Application Note

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Benefits of Quality Control in the IVT RNA Workflow Using the Agilent 5200 Fragment Analyzer System

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Abstract

Quality control (QC) analysis is an essential part of the *in vitro* transcription (IVT) RNA workflow. Utilizing QC checkpoints at critical junctures in the IVT RNA process eliminates unsuitable samples, saves time and money, and maximizes resources. The Agilent 5200 Fragment Analyzer system provides sensitive and reliable quality control analysis of RNA with the Agilent RNA kit (15 nt) and Agilent HS RNA kit (15 nt). Analysis of DNA with various DNA kits. QC during IVT allows for detection of poor PCR amplification, poor transcription, DNA template contamination of RNA, degraded RNA, ensuring downstream applications are starting with high-quality RNA.

Introduction

The importance of quality control (QC) in laboratory processes is often overlooked but should be considered an essential part of every procedure. Parallel capillary electrophoresis solutions from Agilent provide the benefit of extremely sensitive, reliable, and high throughput QC for *in vitro* transcription (IVT) RNA applications. These solutions help to deliver a consistent and reproducible RNA product. RNA is prone to degradation due to temperature and ever-present RNases. Hence, there are two critical QC checkpoints in the IVT RNA workflow to ensure the production of quality RNA: after amplification and after transcription (Figure 1). The Agilent 5200 Fragment Analyzer system aids in determining if the PCR amplification, transcription, and product cleanup procedures were successful. Detection of degraded, contaminated, or otherwise unsuitable RNA allows researchers to rework or remove these samples early in the process. Analyzing IVT RNA starting material for downstream applications confirms it meets or exceeds quality requirements and ensures that the experiment is starting off in the right direction.

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.

PCR product was analyzed on the Agilent 5200 Fragment Analyzer system with the Agilent dsDNA 930 Reagent kit (75-20000 bp) (p/n DNF-930). Various lengths of IVT RNA were diluted with nuclease-free water and analyzed on the Agilent 5200 Fragment Analyzer system with the Agilent RNA kit (15 nt) (p/n DNF-471), with the separation time extended by 5 minutes. mRNA separated from ribosomal RNA was analyzed on the Agilent 5200 Fragment Analyzer system with the Agilent HS RNA kit (15 nt) (p/n DNF-472) and the HS mRNA 15 nt method.

RNA analysis kit selection

The Agilent RNA kit and Agilent HS RNA kit were designed for analysis of RNA fragments and smears ranging from 200 to 6,000 nt in size. The Agilent RNA kit is intended for a higher concentration of total RNA samples between 5 to 500 ng/µL RNA. In addition, the kit works well for RNA fragments such as IVT RNA or IVT mRNA between approximately 10 to 20 ng/µL up to 100 ng/µL. In contrast, the Agilent HS RNA kit is aimed at lower concentration total RNA and mRNA depleted ribosomal RNA samples between 50 and 5,000 pg/µL. Both kits provide reliable sizing and quantification of IVT RNA, including sgRNA.[1]

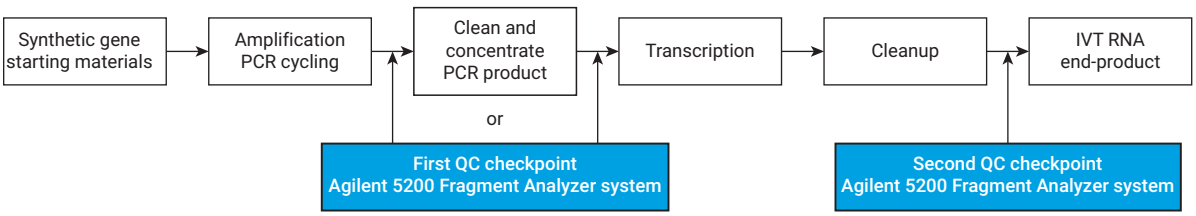

Figure 1. The Agilent 5200 Fragment Analyzer system provides dependable quality control analysis throughout the IVT process.

Sizing IVT RNA

The Agilent RNA kit is the recommended kit for analysis of IVT RNA and IVT mRNA due to the high concentration of a single fragment. Various concentrations (100, 50, 10, and 1 ng/µL) of 1,800 and 6,000 nt IVT RNA were analyzed on the Agilent 5200 Fragment Analyzer system with the Agilent RNA kit (Figure 2). The 1,800 and 6,000 nt RNA fragments had an average size of 1,836 and 6,503 nt, respectively, with low % CV and % error indicating consistent data analysis and accurate sizing throughout the dilution series (Table 1). Concentration measurements at 50 and 100 ng/µL for both the 1,800 and 6,000 nt RNA fragments reported excellent accuracy below 18 % error, within the specifications of the RNA kit.

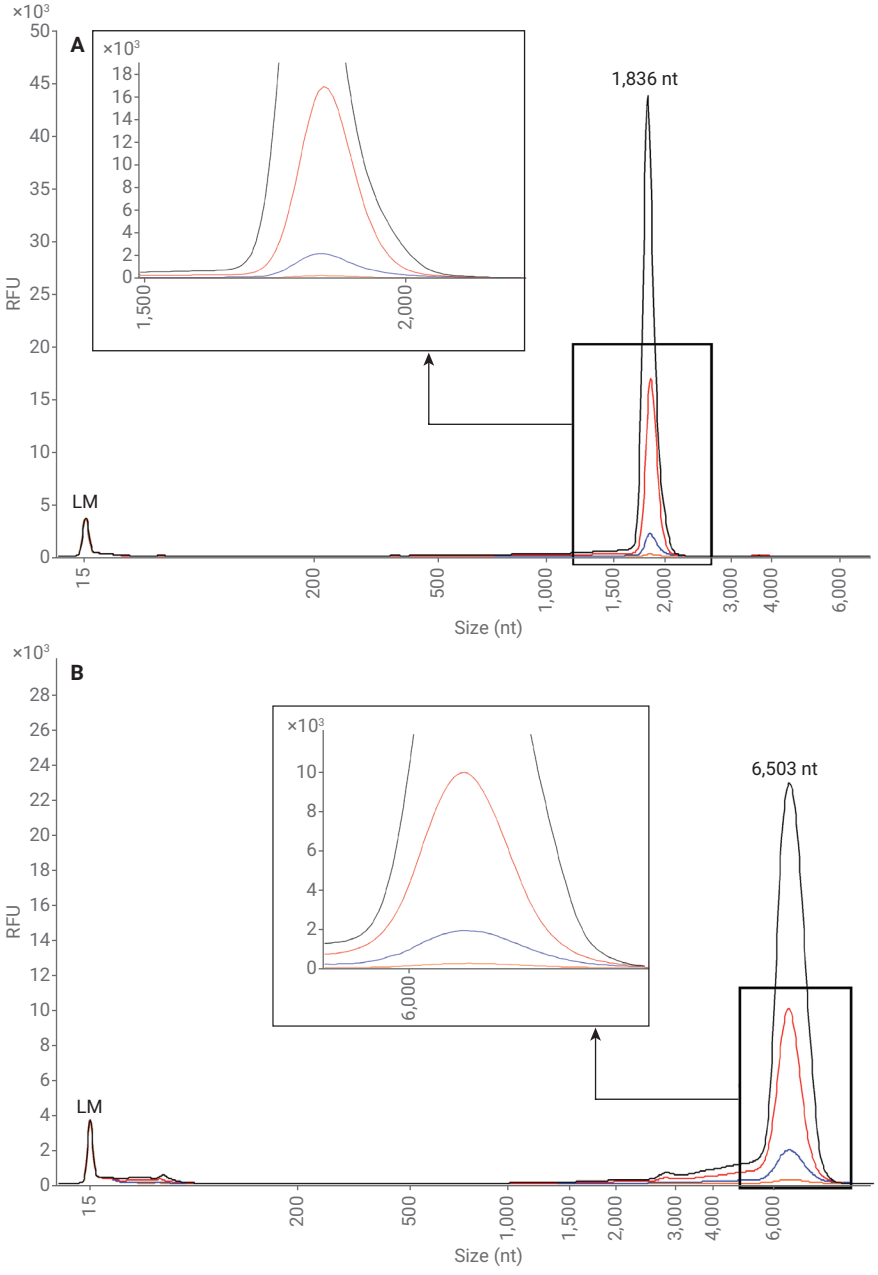

Figure 2. Overlay of the IVT RNA fragment analyzed on the Agilent 5200 Fragment Analyzer system with the Agilent RNA kit (15 nt) over a dilution series of 100, 50, 10, and 1 ng/µL. Run time was extended to 5 minutes. (A) 1,800 nt RNA fragment. (B) 6,000 nt RNA fragment. LM = lower marker.

Analysis of ribosomal depleted mRNA

mRNA pulled from ribosomal RNA was analyzed on the Agilent 5200 Fragment Analyzer system with the Agilent HS RNA kit – HS mRNA 15 nt. mthds file (Figure 3). The HS mRNA method file in the Agilent HS RNA kit was designed for analysis of ribosomal depleted mRNA. This method produces low concentration mRNA that separates as a smear. The HS mRNA method utilizes a longer sample injection time to accommodate the low concentration mRNA and is not recommended for highly concentrated synthetic mRNA or IVT mRNA. In addition, ProSize data analysis software automatically reports the percent of ribosomal RNA contamination in each sample with the HS mRNA method.

Quality control

There are several key challenges associated with IVT RNA workflows such as overall time, yield, RNA degradation, and incomplete enzyme reactions. Many IVT reactions take 2 to 3 hours to produce maximum RNA yields, with low yields leading to repeated IVT reactions. The Agilent 5200 Fragment Analyzer system offers reliable and sensitive quantification and sizing of nucleic acids to help optimize reaction conditions and determine input volumes needed for successful amplification and transcription yields.

Table 1. Sizing of 1,800 and 6,000 nt IVT RNA throughout a dilution series (100, 50, 10, and 1 ng/µL), *n=3.

Concentration (ng/µL)	Sizing throughout dilution series					
	1,800 nt Fragment ~Theoretical Size 1,800 nt			6,000 nt Fragment ~Theoretical Size 6,039 nt		
	Average* (nt)	%CV	% Error	Average* (nt)	%CV	% Error
100	1,819	0.5 %	1.1 %	6,503	0.4 %	7.7%
50	1,838	0.6 %	2.1 %	6,503	0.2 %	7.7%
10	1,838	0.1 %	2.1 %	6,503	0.4 %	7.7%
1	1,847	0.5 %	2.6 %	6,503	0.4 %	7.7%
Entire Range	1,836	0.7 %	2.0 %	6,503	0.3 %	7.7%

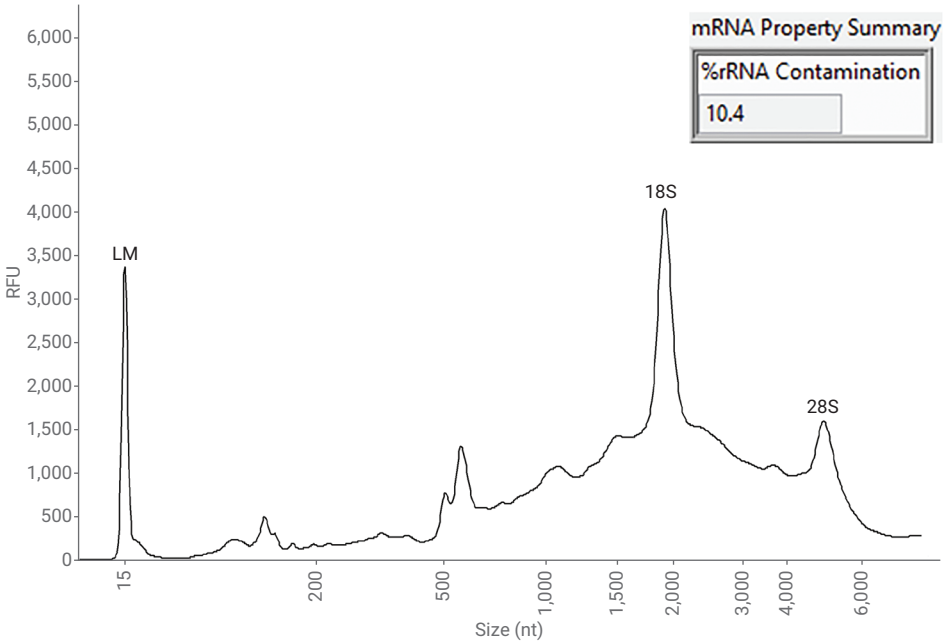

Figure 3. Ribosomal depleted rat kidney mRNA was analyzed on the Agilent 5200 Fragment Analyzer system with the Agilent HS RNA kit (15 nt) and the HS mRNA method. The mRNA still contained some 18S and 28S ribosomal RNA. LM = lower marker.

First QC checkpoint

The first quality control check for IVT is recommended after amplification and can be performed either before or after PCR clean-up. The Agilent 5200 Fragment Analyzer system provides essential information including the concentration of the PCR product, which is required for transcription and the size of the fragment, which reveals if the product of interest was amplified. In addition, the quality or purity of the amplification is assessed through visualization of the electropherogram. The purity of the amplification product will help the user to determine if further cleanup is necessary. Successful PCR reactions produce a single fragment, while a poor PCR reaction generates multiple unwanted peaks (Figure 4). Salt concentrations during PCR can reach 100 to 200 mM chloride levels, which may inhibit transcription. Noisy or spiky baselines seen on the electropherograms are indications of high salt levels. Salt levels over 50 mM are easily detected[2]. PCR cleanup or dilution of samples – or both – can help eliminate high salt concentration concerns.

Second QC checkpoint

The second recommended QC checkpoint occurs after transcription. Cleanup of the transcription product involves the use of DNase to eliminate the DNA template, leaving a purified RNA product. The Agilent 5200 Fragment Analyzer system can detect the presence of a DNA template in RNA. The DNA template is distinguishable from RNA due to the size difference. In instances where DNase is not utilized, or the reaction is incomplete, the DNA template will be visible in the electropherogram.

For example purposes only, IVT RNA not treated with DNase was separated on the Agilent 5200 Fragment Analyzer system with the Agilent RNA kit (Figure 5). An expected single RNA product was seen at 1,831 nt along with a much larger DNA template. Running the separation an extra five minutes is recommended to ensure visualization of any possible DNA template. Successful transcription and cleanup will produce a single RNA peak, while problematic transcription results in several smaller unwanted peaks (Figure 6).

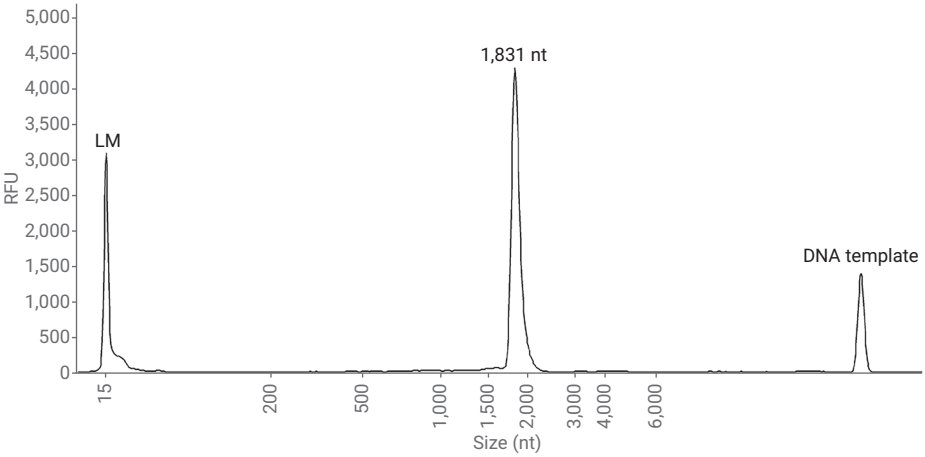

Figure 5. IVT RNA analyzed on the Agilent 5200 Fragment Analyzer system with the Agilent RNA kit (15 nt). The DNA template is detected when the sample is not treated with DNase. LM = lower marker.

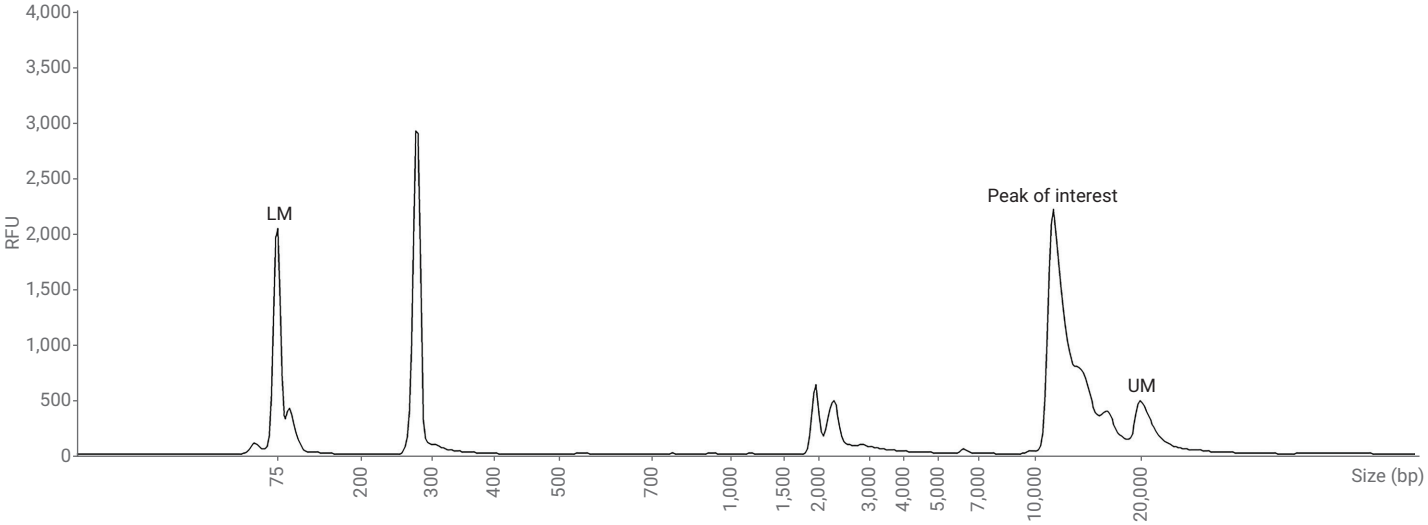

Figure 4. PCR product analyzed on the Agilent 5200 Fragment Analyzer system with the Agilent dsDNA 930 Reagent kit (75-20000 bp). Multiple unwanted peaks indicate a poor PCR reaction. LM = lower marker; UM = upper marker.

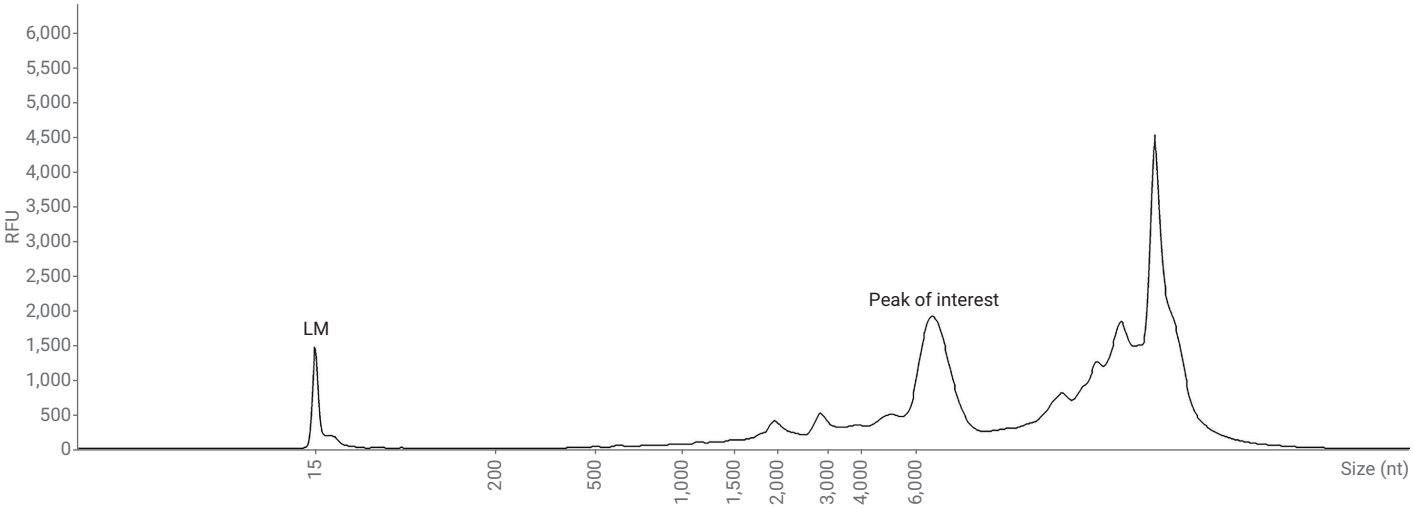

Figure 6. IVT RNA analyzed on the Agilent 5200 Fragment Analyzer system with the Agilent RNA kit (15 nt). Low-quality transcription results in multiple peaks. LM = lower marker.

Conclusions

Quality control analysis is an important part of IVT RNA workflow, which is utilized in multiple downstream applications. Beginning with quality RNA assists in starting the applications off in the right direction. QC after PCR ensures that the DNA of interest is amplified and free of contamination. The second quality control step ensures successful transcription of the RNA of interest and detects the presence of RNA degradation or DNA template contamination, or both. The Agilent 5200 Fragment Analyzer system provides sensitive and reliable quality control analysis of the IVT RNA workflow.

References

1. Luttgeharm, K.; Pocernich, C. Single-Guide RNA Quality Assessment by the Agilent 5200 Fragment Analyzer System, *Agilent Technologies Application Note*, publication number 5994-0523EN, **2017**.
2. Pocernich, C.; Deist, B.; Pike, W.; Warzak, D. Best Sizing Practices with the Agilent 5200 Fragment Analyzer System. *Agilent Technologies Application Note*, publication number 5994-0585EN, **2018**.

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