Application Note

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Analyzing Poly(A) Tails of In Vitro Transcribed RNA with the Agilent Fragment Analyzer System

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Abstract

Poly(A) tails are important for the process of mRNA translation. The length of a poly(A) tail has been shown to affect translation efficiency and the rate of mRNA degradation. Therefore, confirming the expected or optimal length of the poly(A) tail is important for research including in vitro transcribed (IVT) RNA. Agilent Fragment Analyzer systems provide efficient, cost-effective, and consistent analysis of nucleic acids. This application note describes the procedures necessary to obtain reliable sizing for poly(A) tails using the Fragment Analyzer.

Introduction

An important component of mRNA is the poly(A) tail. Poly(A) tails are composed of multiple adenine nucleotides added to the 3′ end of mRNA. Research has shown that the length of the poly(A) tails helps minimize mRNA degradation and plays a key role in translation[1]. Poly(A) tails are found in various lengths from 20 nt to over 200 nt[2]. The length of the poly(A) tail contributes to mRNA stability and protein expression[1,2].

Poly(A) tails can be added to in vitro transcribed (IVT) RNA and are critical for IVT RNA used in therapeutics. There are two ways to add poly(A) tails to IVT RNA, either as part of the IVT RNA template or enzymatically, with a recombinant poly(A) polymerase. Poly(A) tails added as part of the IVT RNA template produce poly(A) tails of specific, known lengths. However, tails greater than 30 nt in length are difficult to synthesize since long poly(A) sequences cause polymerases to slip. Poly(A) enzymes can quickly add a poly(A) tail longer than 100 nucleotides. The length of a poly(A) generated with poly(A) enzyme is varied and undefined. Changing the amount of enzyme, ATP, reaction time, or concentration of RNA can alter the length of the final poly(A) tail.

Analyzing the size and quality of a poly(A) tail is a critical part of the IVT RNA workflow since the length of a poly(A) tail affects mRNA stability and influences translation efficiency. There are several techniques for poly(A) quality control (QC), including next-generation sequencing (NGS), chromatography, mass spectrometry (MS), and gel electrophoresis methods[1,2]. However, even with these techniques it is challenging to accurately analyze the length of poly(A) tails. Most of these methods are labor intensive, require large amounts of RNA, are time consuming, or expensive[2]. Additionally, each technique varies in accuracy[2].

An alternative method to accurately assess poly(A) tails is the Agilent 5200 Fragment Analyzer system. In this application note, template and enzymatically added poly(A) tails were analyzed on the Fragment Analyzer system with the Agilent Small RNA kit. This application note demonstrates that the Fragment Analyzer system can quickly, cost-effectively, and reliably analyze the size and quality of poly(A) sequences.

Experimental

The experiments in this study were performed using the Agilent 5200 Fragment Analyzer system and can be replicated on the Agilent 5300 and 5400 Fragment Analyzer systems.

Poly(A) tail samples

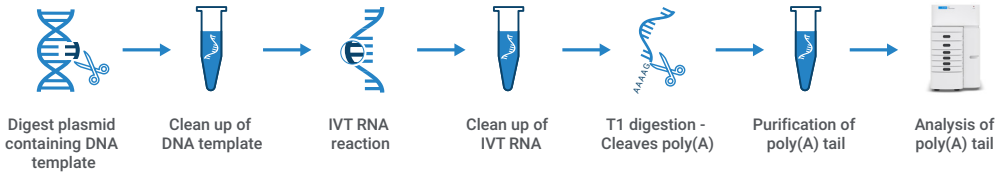
Three poly(A) tail samples were analyzed in this experiment (Table 1). Sample 1, generated from IVT RNA, synthesized from a plasmid borne DNA template, has a 30 nt poly(A) tail. Cleaving the IVT RNA with T1 RNase (described below) at the G nucleotide, just before the poly(A) sequence, generates a 31 nt fragment. Figure 1A illustrates the workflow to generate sample 1.

Sample 2 was generated by enzymatically adding a poly(A) tail to IVT RNA, derived from a 2,055 bp PCR amplified DNA template. Cleaving the IVT RNA with T1 RNase produces a nonuniform poly(A) fragment, expected to be smaller than 200 nt. Figure 1B illustrates the workflow to generate sample 2. Additionally, an RNA oligo control, which consists of a single G followed by a 30 nt poly(A) sequence, was analyzed as a known standard for sample 1.

Table 1. Description of the samples used in this application note.

Sample	Size (nt)	DNA Template	Poly(A) Source
1	31	Plasmid	Template
2	Smear	PCR Amplified	Enzymatic
Control	31	NA	Oligo

A) Sample 1 workflow



B) Sample 2 workflow

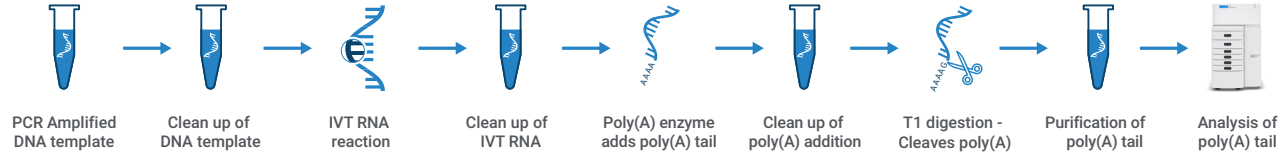


Figure 1. Schematic of the workflows used to generate IVT RNA with poly(A) tails from A) a DNA plasmid template (sample 1) or B) enzymatically added (sample 2).

DNA templates

A pT7CFE1-NHis-GST-CHA vector (Thermo Fisher Scientific p/n 88871) was digested with SpeI (NEB p/n R3133S) to prepare a DNA template with a 30 nt poly(A) sequence for sample 1. For sample 2, a 2,055 bp DNA template lacking a poly(A) sequence was prepared from PCR amplification of Lambda DNA (Thermo Fisher Scientific p/n SD0021), with Phusion High-Fidelity DNA polymerase (Thermo Fisher Scientific p/n F530S) and appropriate primers (IDT). Both the SpeI digested vector and PCR amplified templates were isolated with NucleoSpin Gel and PCR Clean up (Takara Bio p/n 740609.50) following standard protocols[3].

IVT RNA

IVT RNA was generated from the DNA templates using T7 RiboMAX express (Promega p/n P1320) following the manufacturer's instructions[4]. DNA templates were removed using the provided RQ1 RNase-Free DNase, followed by purification with the Zymo RNA Clean & Concentrator-5 kit (p/n R1013) using standard procedures[5].

Poly(A) tail synthesis

Poly(A) tails were added to IVT RNA generated from the 2,055 bp PCR amplified DNA template (sample 2) with *E. coli* Poly(A) polymerase (NEB p/n M0276S) following the manufacturer's instructions[6]. The poly(A) IVT RNA was purified with the Zymo RNA Clean & Concentrator-5 kit and standard procedures[5].

T1 RNase digestion

IVT RNA containing poly(A) sequences were digested with RNase T1 (Thermo Fisher Scientific p/n EN0541), which cleaves RNA at G nucleotides. Each reaction contained 200 U of RNase T1 for each ug of RNA in 1x RNase H reaction buffer (NEB p/n M0297S). The reactions were incubated at 37 °C for 3 hours.

Poly(A) purification and analysis

Immediately following T1 digestion, the poly(A) tails for both sample 1 and sample 2, as well as a 31 nt RNA oligo control were purified using Invitrogen Dynabeads mRNA purification kit (Thermo Fisher Scientific p/n 61-006), following standard protocols[7]. The poly(A) sequences were eluted with 10 mM Tris-HCl at 80 °C for 2 minutes following the kit protocol. The eluates were diluted with nuclease-free water and immediately analyzed on the Agilent 5200 Fragment Analyzer system with the Agilent Small RNA kit (p/n DNF-470-0275).

Results and discussion

The Fragment Analyzer is an automated capillary electrophoresis instrument used for quality control of nucleic acids, including IVT RNA. The Fragment Analyzer generates an electropherogram and digital gel image. Electropherograms can display either a single peak or a smear. A single peak indicates that the sample is uniform in size. A smear is likely the result of several differently sized fragments being displayed together, generating an elongated image and indicating that the sample is not of uniform size. Since fragments and smears yield different separation profiles during electrophoresis, poly(A) tail samples were generated both from a DNA template to yield a poly(A) fragment (sample 1), and with poly(A) enzyme to yield a smear (sample 2). Both samples were analyzed on the 5200 Fragment Analyzer system with the Small RNA kit to demonstrate the difference between the two sample types.

Sample 1, a 31 nt poly(A) tail, produced a single peak (Figure 2A). Sample 1 measured slightly larger than the expected size of 31 nt. Moreover, the sample had little variation between replicates, with approximately 2.0% CV (n=3).

Sample 2, an enzymatically added poly(A) tail, displayed a smear less than 200 nt in length (Figure 2B). A smear analysis was generated using the Agilent Fragment Analyzer ProSize data analysis software. A smear size range was set from 25 to 130 nt to encompass the entire smear. As shown in Figure 2B, the smear is widespread, spanning from approximately 25 to 130 nt, with a larger peak at 59 nt, and a large portion of the smear sloping downwards from the peak towards the right. Triplicate analysis of the sample resulted in an average smear size of 76 nt (n=3).

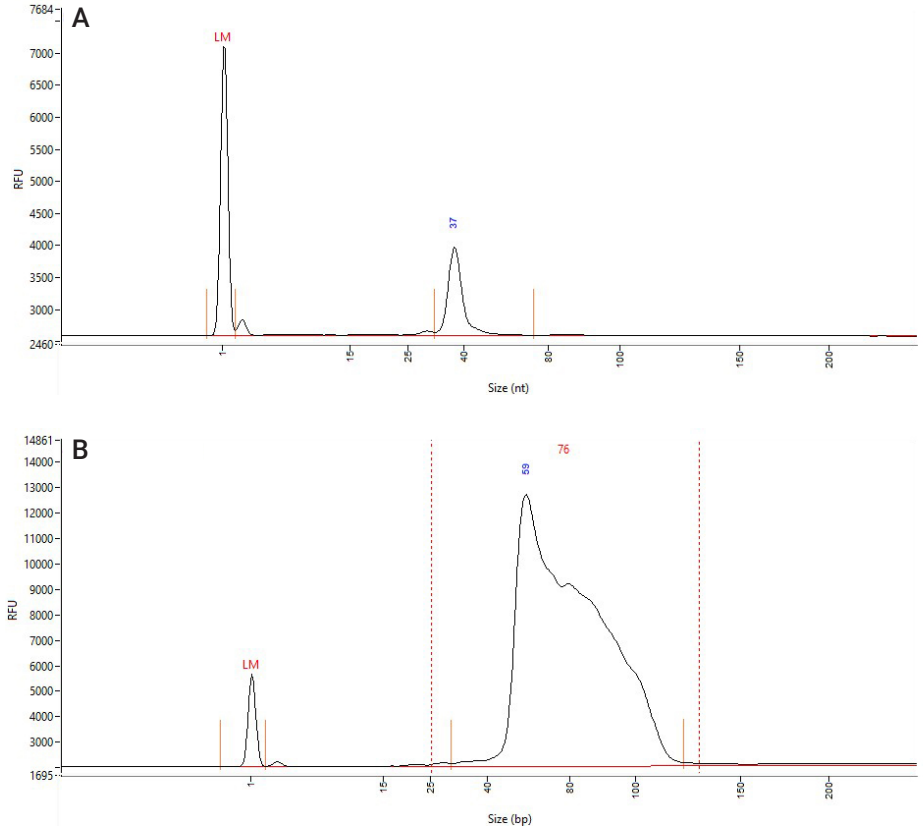


Figure 2. The Agilent Fragment Analyzer system was used to analyze IVT RNA poly(A) tails. Shown are the electropherograms of (A) sample 1, a template derived 31 nt poly(A) tail which is displayed as a fragment, and (B) sample 2, an enzymatically added poly(A) tail which appears as more of a smear. Red dotted lines indicate smear region.

Size analysis using a standard

An established method for determining if the length of a given sample represents the expected size is to compare the sample poly(A) tail length to the observed length of a standard poly(A) tail of a known size. An RNA oligo was designed with a G nucleotide followed by 30 A nucleotides to use as a standard, which the poly(A) fragment (sample 1) could be compared to. The poly(A) fragment displayed an average size of approximately 38 nt and the control displayed an average size of 37 nt (Table 2 and Figure 3), indicating that sample 1 is the same size as the control, as expected.

Table 2. Size comparison of sample 1, a 31 nt poly(A) tail, and a 31 nt oligo standard (n=3).

Sample	Average Size (nt)	%CV
1	37.7	1.25%
Control	37	0.00%

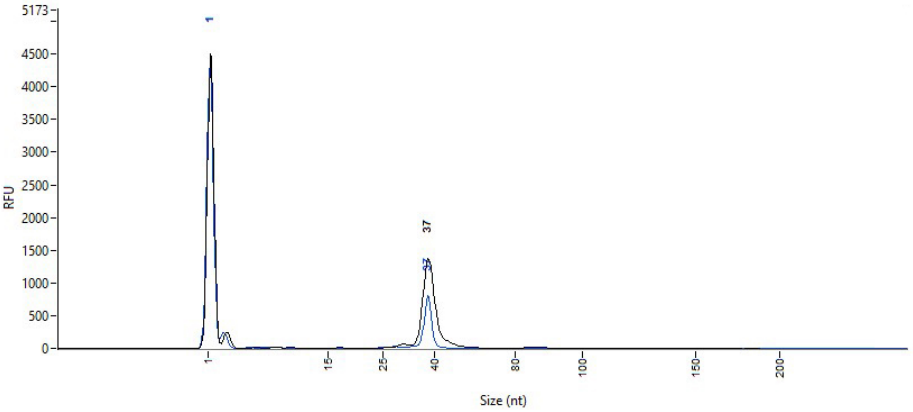


Figure 3. Comparison of representative electropherograms from sample 1, the 31 nt poly(A) tail (black), and a 31 nt poly(A) oligo control (blue) were analyzed on the Agilent Fragment Analyzer system. An overlay of the electropherograms indicates that the samples are the same size (37.7 and 37 nt, respectively) (n=3).

Sample salt concentration

In gel electrophoresis, the accuracy of sizing can be affected by the salt concentration of the samples[8]. The standard protocol for eluting mRNA from the Dynabeads mRNA purification kit uses 10 mM Tris-HCl[7]. To evaluate the effects of salt concentration on the poly(A) tail sizing, the oligo standard was eluted with different concentrations of Tris-HCl: 10 mM, 1 mM, 0.1 mM and no Tris-HCl (nuclease-free water). Each elution was diluted to a ratio of 1 µL of sample to 3 µL of nuclease-free water before analysis on the Fragment Analyzer. Regardless of the elution type, the poly(A) oligo displayed sizes between 35 and 39 nt (Figure 4).

The concentration of Tris-HCl in the elution buffer affected the observed size of the poly(A) fragment (Table 3). The 1 mM and 0.1 mM Tris-HCl samples yielded sizes of 35 and 36 nt, respectively. The 10 mM Tris-HCl and nuclease-free water samples yielded sizes of 37 and 38 nt, respectively. These results indicate that minimal amounts of Tris-HCl are required to achieve the most accurate sizing when analyzing poly(A) tails on the Fragment Analyzer.

Table 3. Aliquots of the control sample were purified with 10 mM, 1 mM, and 0.1 mM Tris-HCl elution buffer, and nuclease-free water. The samples were analyzed on the Agilent 5200 Fragment Analyzer system with the Small RNA kit. The 1 mM Tris-HCl elution displayed a size within 15% of the expected size.

	10 mM Tris-HCl	1 mM Tris-HCl	0.1 mM Tris-HCl	Nuclease-Free Water
Size (nt)	37	35	36	38
%CV	0.0	0.01	0.0	0.01
%Error	19.4%	13.8%	16.1%	23.7%

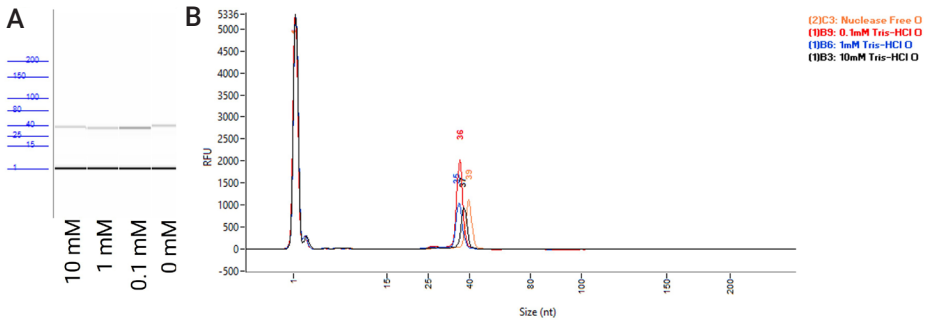


Figure 4. Analysis of Tris-HCl elution buffer dilution series. The oligo standard was eluted in various dilutions of Tris-HCl (10 mM (black), 1 mM (blue), 0.1 mM (red)) and nuclease-free water (orange) and analyzed with the Agilent Fragment Analyzer systems. A) The digital gel image of the representative samples for each dilution and B) the electropherogram overlay of the representative samples of each dilution. Examination of the results indicates that the samples eluted with the 1 mM and 0.1 mM Tris-HCl display more accurate sizing than those eluted with 10 mM Tris-HCl or nuclease-free water.

Conclusions

Analyzing the length of poly(A) tails is an important part of IVT RNA workflows where efficient gene expression is required. Typical methods of poly(A) analysis, such as qPCR and MS, are time consuming and expensive. The Fragment Analyzer system with the Small RNA kit is a cost-effective, efficient, and reliable way to determine the length of poly(A) sequences. This application note demonstrates that the Agilent Fragment Analyzer systems can accurately and reliably separate and analyze poly(A) tails from IVT RNA fragments.

Reference

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