Application Note

Genomics

Agilent

Trusted Answers

# Analysis of *in Vitro* Transcribed Cas9 mRNA Pre- and Postpolyadenylation with the Agilent 5200 Fragment Analyzer System


**Authors**

Kyle Luttgeharm,
Chava Pocernich, and
Kit-Sum Wong
Agilent Technologies, Inc.



## Abstract

DNA-free CRISPR gene editing has become a popular way to control for off-target effects during CRISPR transfection. However, the size of the Cas9 protein does not always allow for transfection of Cas9/sgRNA ribonucleoprotein complexes. A way to overcome this method of DNA-free CRISPR gene editing is to transfect both *in vitro* transcribed sgRNA and Cas9 mRNA. Polyadenylation (poly(A)) of the Cas9 mRNA allows for a longer translation time by protecting and increasing the stability of the Cas9 transcript. The Agilent 5200 Fragment Analyzer system coupled with the Agilent HS RNA kit provides high resolution separation and consistent sizing of the Cas9 mRNA with or without polyadenylation.

## Introduction

CRISPR gene editing can be accomplished by transformation of DNA plasmids encoding both Cas9 and sgRNA. However, the constitutive presence of these plasmids and the transcripts can result in high levels of off-target gene editing. Many researchers are turning to DNA-free CRISPR gene editing systems to achieve their desired gene editing effect. DNA-free CRISPR gene editing encompasses two different gene editing options that bypass the need for transfection of DNA plasmids into cells. The first system uses ribonucleoprotein (RNP) complexes composed of an sgRNA transcribed *in vitro* complexed with Cas9. These RNP complexes are transfected into cells enabling gene editing. Degradation of the RNP complexes prevents further gene editing events, limiting the number of off-target effects. While the RNP system has proven to be efficient, the large size of Cas9 can prevent its effective transfection into certain cell types. The second DNA-free CRISPR gene editing system overcomes size-restricted protein transfection by transfecting both *in vitro* transcribed sgRNA and Cas9 mRNA. This system relies on the translation of the Cas9 mRNA *in vivo*, thus bypassing size restricted protein transfection. Polyadenylation (poly(A)) of the Cas9 mRNA prior to transfection is required to prevent Cas9 mRNA degradation before *in vivo* translation can occur. In order to influence how long the Cas9 mRNA is present *in vivo*, different lengths of poly(A) tails can be added to the Cas9 mRNA. The length of the poly(A) tail acts as a timer; greater polyadenylation results in a longer lasting transcript compared to a shorter poly(A) tail. The Agilent 5200 Fragment Analyzer system with the Agilent HS RNA kit (15 nt) can easily size the Cas9 mRNA and determine the approximate length of the poly(A) tail.

## Experimental

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

### *In vitro* transcription of Cas9 mRNA

The 4,246 nt pT7-Cas9 plasmid (OriGene, #GE100014) was linearized using MssI (Thermo Fisher, #FD1344) according to manufacturer instructions. The linearized plasmid was purified using the Monarch PCR and DNA cleanup kit (NEB, #T1030) according to manufacturer instructions. Following purification, the Cas9 mRNA was transcribed using the mMESSAGE mMACHINE T7 ULTRA transcription kit (Thermo Fisher, #AM1345) according to manufacturer instructions with a 2-hour incubation time at 37 °C. TURBO DNase was added to stop transcription. Prior to polyadenylation, a 1 µL aliquot was removed and diluted into 40 µL of nuclease free water. The polyadenylation reaction was started with the addition of the E-PAP enzyme and incubated for 50 minutes at 37 °C. Every 10 minutes, a 1 µL aliquot was removed from the reaction and diluted into 40 µL nuclease-free water for analysis.

### Analysis of Cas9 mRNA

Qubit 2.0 was used for quantification of the Cas9 mRNA transcripts. Approximately 1 ng/µL of sample was heat denatured at 70 °C for 2 minutes and analyzed on the Agilent 5200 Fragment Analyzer system using the Agilent HS RNA kit (15 nt) (p/n DNF-472) with the DNF-472M33 (mRNA) separation method and analyzed with ProSize data analysis software. The run time was extended by 10 minutes on the 5200 Fragment Analyzer system to ensure a complete separation.

## Results & Discussion

### Separation on the 5200 Fragment Analyzer system

Cas9 mRNAs with varying reaction times of polyadenylation were analyzed on the 5200 Fragment Analyzer system with the HS RNA kit (15 nt). The length of the poly(A) tail became longer with increased polyadenylation reaction times, as noted by the increase in size of the Cas9 mRNA (Figure 1). An extended run time may be required for complete separation of the Cas9 mRNA with the longer poly(A) tails. Separation of the Cas9 mRNA without polyadenylation exhibited a large peak at 4,132 nt, without additional secondary

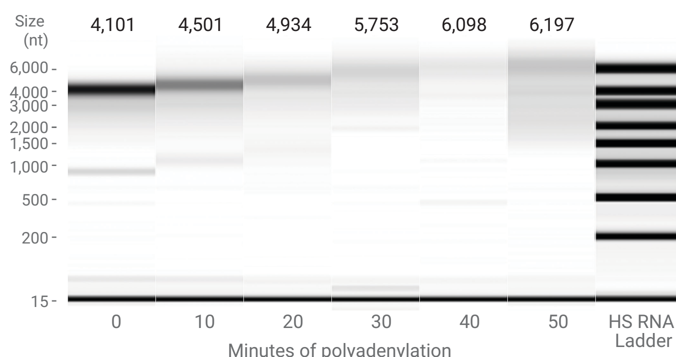


**Figure 1.** A digital gel image of Cas9 mRNA with polyadenylation reaction time= 0, 10, 20, 30, 40, and 50 minutes separated on the Agilent 5200 Fragment Analyzer system with the Agilent HS RNA kit (15 nt) after heat denaturation.

structure peaks observed (Figure 2A). In addition to the heat denaturation, the presence of denaturing formamide in the diluent marker of the Agilent HS RNA kit (15 nt) aids in limiting secondary structure formation. After 50 minutes of polyadenylation, Cas9 mRNA displayed a smear representing many different lengths of poly(A) tails (Figure 2B) with a major peak at 6,197 nt representing the longer poly(A) tail. Secondary structure formation is probable due to the presence of poly(A) tails. ProSize automatically reported the size of the largest peak.

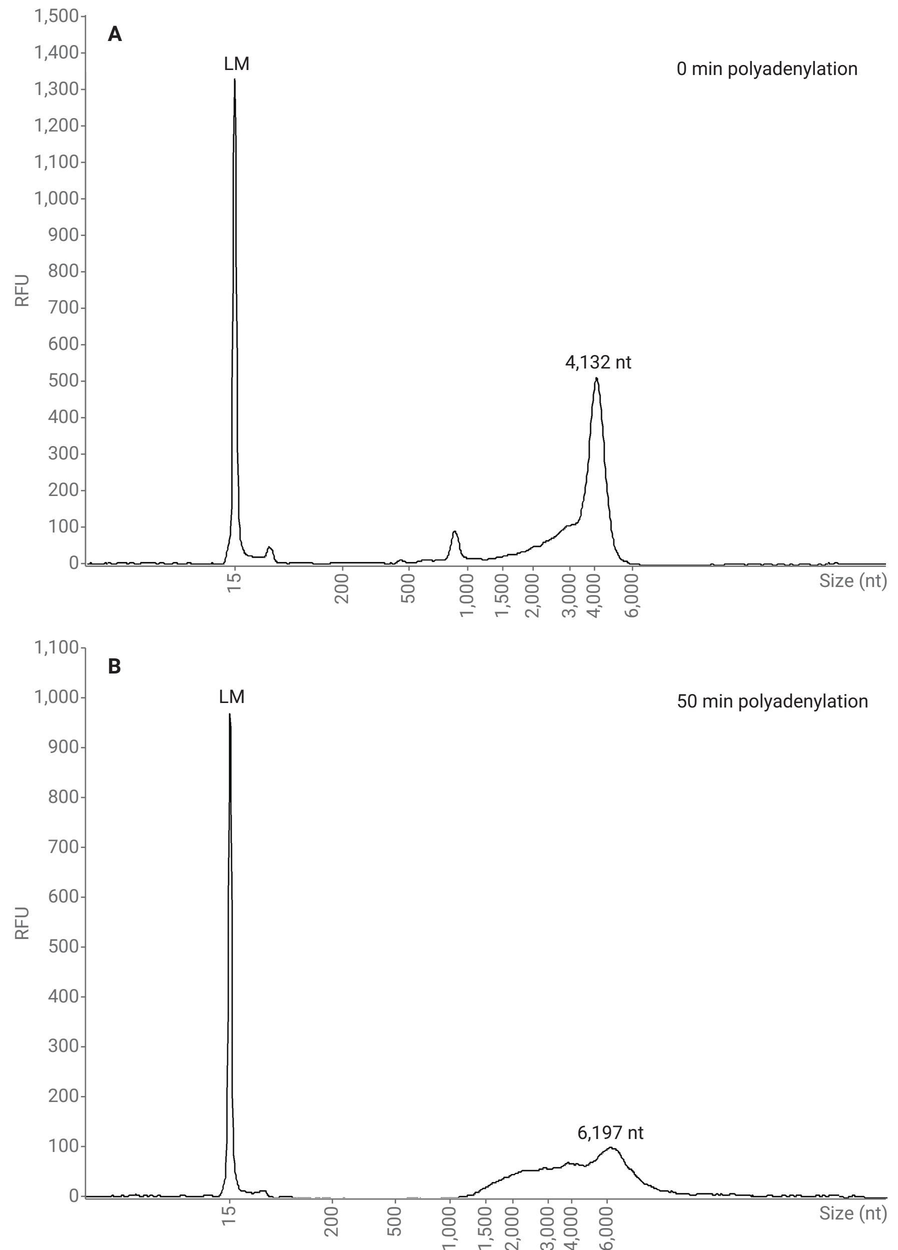


**Figure 2.** Heat denatured Cas9 mRNA (known size 4,246 nt) separated on the Agilent 5200 Fragment Analyzer system with the Agilent HS RNA kit (15 nt): (A) 0 minutes polyadenylation (B) 50 minutes polyadenylation. LM = lower marker.

The 5200 Fragment Analyzer system ladder is essential in providing accurate sizing. The Agilent HS RNA Ladder (DNF-386-U015) has eight fragments ranging from 200 to 6,000 nt with excellent baseline resolution between each fragment, allowing for accurate sizing through 6,000 nt (Figure 3). The 5200 Fragment Analyzer system sized the Cas9 mRNA with no polyadenylation extremely accurately at 4,132 nt with a percent error of 3 %. Sizing of the Cas9 mRNA with polyadenylation increased proportionally with an increase in polyadenylation time, with an average size of 6,197 nt after 50 minutes. The 5200 Fragment Analyzer system can accurately size RNA fragments larger than 4,000 nt due to the excellent resolution and the fragment sizes of the HS RNA Ladder.

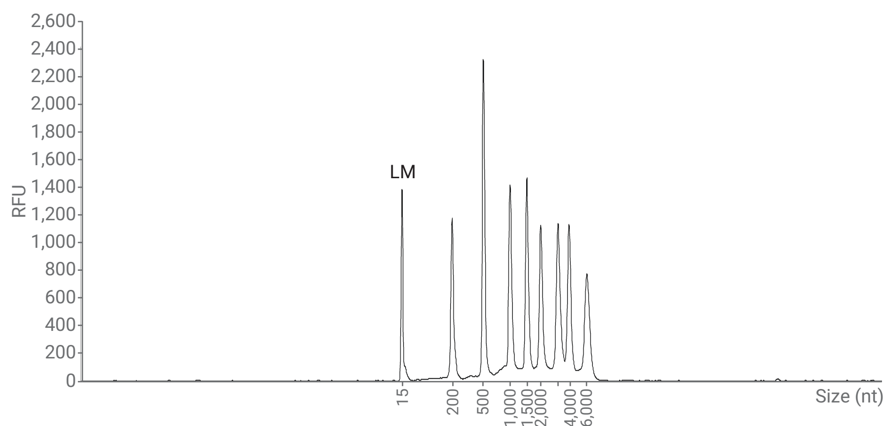


**Figure 3.** The Agilent HS RNA Ladder separated on the Agilent 5200 Fragment Analyzer system with the Agilent HS RNA kit (15 nt). LM = lower marker.

## Conclusions

The 5200 Fragment Analyzer system allowed for easy analysis of the Cas9 mRNA transcript and Cas9 mRNA post-polyadenylation by providing excellent resolution and reliable sizing. Knowledge of the approximate length of the poly(A) tail helps to determine the length of time *in vivo* Cas9 transcription will occur and aids in the efficiency of gene editing.

**www.agilent.com**

**For Research Use Only. Not for use in diagnostic procedures.**

This information is subject to change without notice.

© Agilent Technologies, Inc. 2019

Printed in the USA, February 14, 2019
5994-0583EN

Agilent
Trusted Answers