Poster Reprint

ASMS 2021
Poster number FP568

Rapid Analysis of mRNA 5’-Capping with High-Resolution LC-MS

Brian Liau, PhD. [1]

[1]Agilent Technologies Inc., Santa Clara, CA

Introduction

Industrial-scale production of mRNA has taken on renewed importance due to the on-going pandemic. Efficient translation of mRNA to protein depends critically on a 5'-terminal dinucleotide modification called capping [1], making it a critical quality attribute that must be characterized and monitored.

We present a rapid LC-MS method for mRNA capping analysis with a total analysis time of only 75 minutes. Sample preparation is accelerated by using thermostable RNase-H to liberate capped 5' terminal oligonucleotides. An optimized LC-MS method then separates liberated oligonucleotides from the sample matrix, simplifying cleanup procedures.

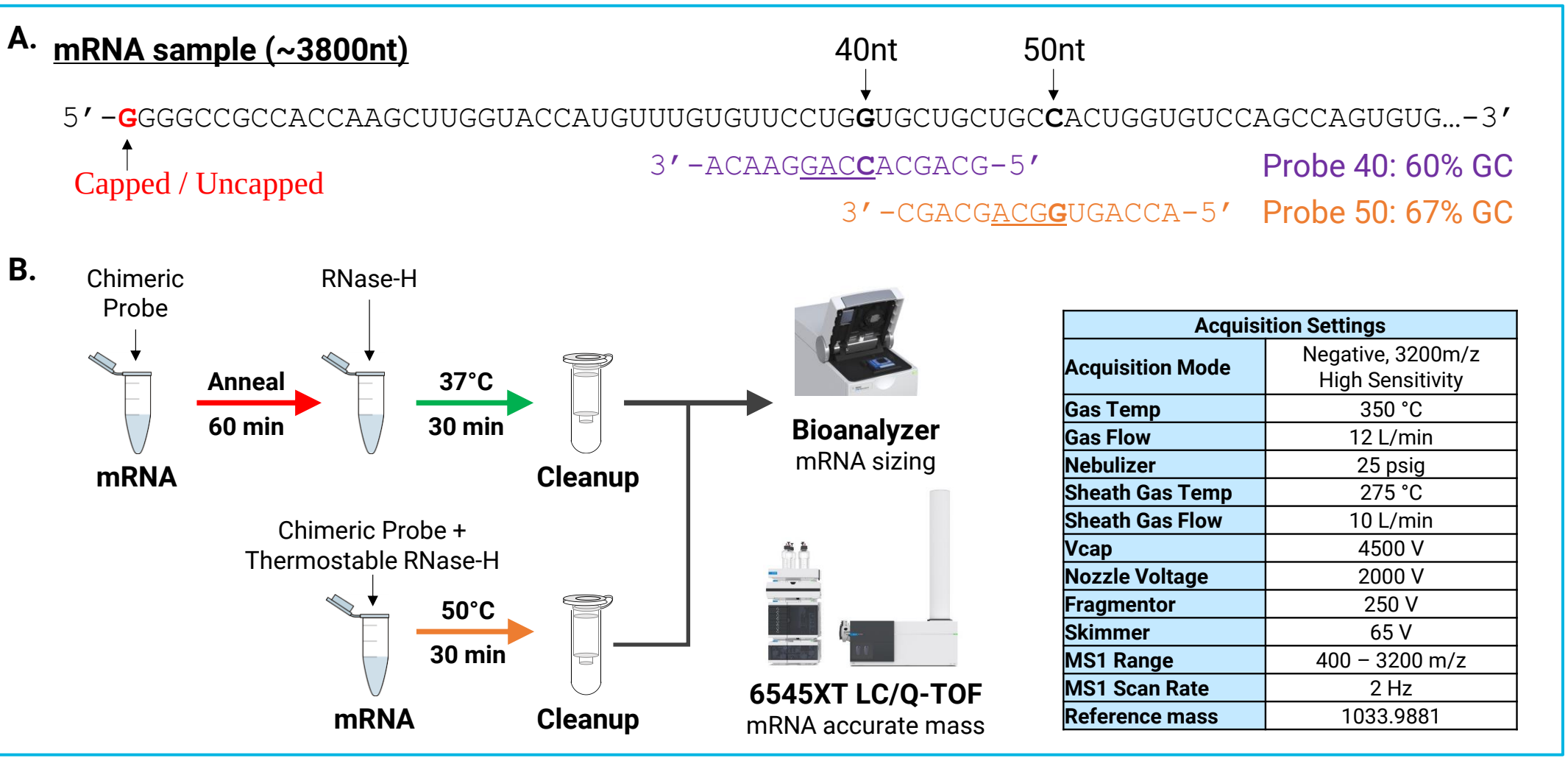
Capping intermediates with different phosphorylation and methylation states were monitored, permitting rational optimization of the capping process.

Experimental

Template DNA approximately 3800nt in length was transcribed to mRNA using T7 polymerase. Co-transcriptional capping with anti-reverse cap analog (ARCA) or enzymatic capping with Vaccinia capping enzyme was performed.

15nt chimeric 2'-O-methyl RNA / DNA probes were designed to direct thermostable RNase-H cleavage for 30-min at 50°C. Oligonucleotides containing the 5' cap were thus liberated, visualized using an Agilent 2100 Bioanalyzer, then analyzed using IP-RP LC in tandem with a 6545XT AdvanceBio LC/Q-TOF. MS data was analyzed with MassHunter BioConfirm 10.0.

Separation was carried out using an AdvanceBio Oligonucleotide column (2.1x50 mm, 2.7µm) using a Water/MeOH mobile phase containing 25mM HFIP and 15mM dibutylamine.



Acquisition Settings	
Acquisition Mode	Negative, 3200m/z High Sensitivity
Gas Temp	350 °C
Gas Flow	12 L/min
Nebulizer	25 psig
Sheath Gas Temp	275 °C
Sheath Gas Flow	10 L/min
Vcap	4500 V
Nozzle Voltage	2000 V
Fragmentor	250 V
Skimmer	65 V
MS1 Range	400 – 3200 m/z
MS1 Scan Rate	2 Hz
Reference mass	1033.9881

Figure 1. (A) Chimeric probes directed RNase-H cleavage at 40nt (purple) and 50nt (orange). Probe nucleotides were composed of DNA (underlined) and 2'-O-methyl RNA. (B) Sample preparation using RNase-H (top scheme) or thermostable RNase-H (bottom scheme). Total analysis time: cleavage (30 min) + cleanup (10 min) + LC-MS (25 min) + data analysis (10 min) = 75 min.

Uncapped		Capping Intermediates		Capped Species	
Name	**Sequence**	**Name**	**Sequence**	**Name**	**Sequence**
Triphosphate	pppGGGGCCG…	Diphosphate	ppGGGGCCG…	ARCA Capped	3'-O-Me-m7GpppGGGGCCG…
		G-Cap	GpppGGGGCCG…	Vaccinia Cap 0	m7GpppGGGGCCG…

Table 1. Uncapped, Capping Intermediate and Capped Species. Red letters indicate 5' terminal nucleotides with phosphates marked by 'p'. These may be elaborated or substituted by enzymatic reactions into capped structures.

Sample Preparation with Thermostable RNase-H

As full-length mRNA is too large for standard LC-MS, site-directed RNase-H cleavage was used to liberate 40 – 50nt capped oligonucleotides for analysis. The typical workflow [2] entails a 1-hour annealing step, which can be avoided by using thermostable RNase-H (Figure 1B). Elevated reaction temperatures permit specific binding of the chimeric probe, thereby yielding a cleaner sample (Figure 2).

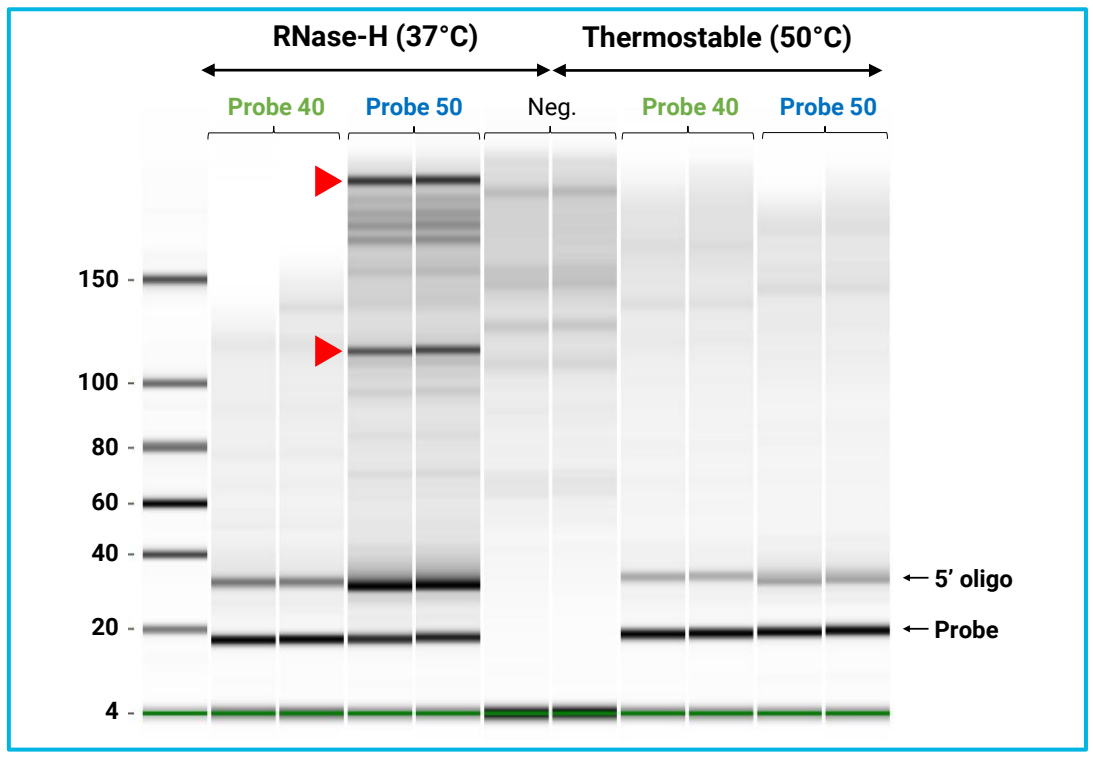


Figure 2. Agilent 2100 Bioanalyzer gel representation showing off-target fragments (red arrows) when Probe 50 directs RNase-H cleavage at 37°C. These are absent when using thermostable RNase-H at 50°C.

LC Separation of 5' Oligos from mRNA Matrix

Uncapped mRNA was digested using Probe 50 + thermostable RNase-H, then analyzed by IP-RP LC-MS. Uncapped 5' oligonucleotides and chimeric probe were well-separated from the remaining mRNA (Figure 3), facilitating data analysis using automated workflows built into BioConfirm software.

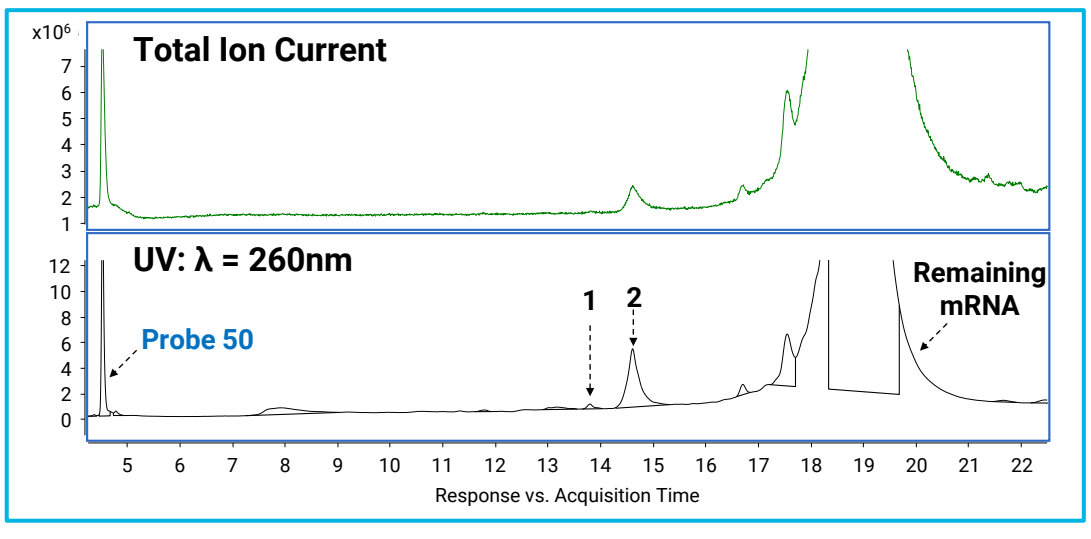


Figure 3. LC separation of uncapped 5' oligonucleotides from chimeric probe and sample matrix. Peak 1: 5' diphosphate, Peak 2: 5' triphosphate oligonucleotides.

Deconvolution of 5' Oligo Mass Spectra

Mass spectra were extracted from the time segments corresponding to peaks 1 and 2 (Figure 4), and deconvoluted masses matched to putative identities shown in Table 1.

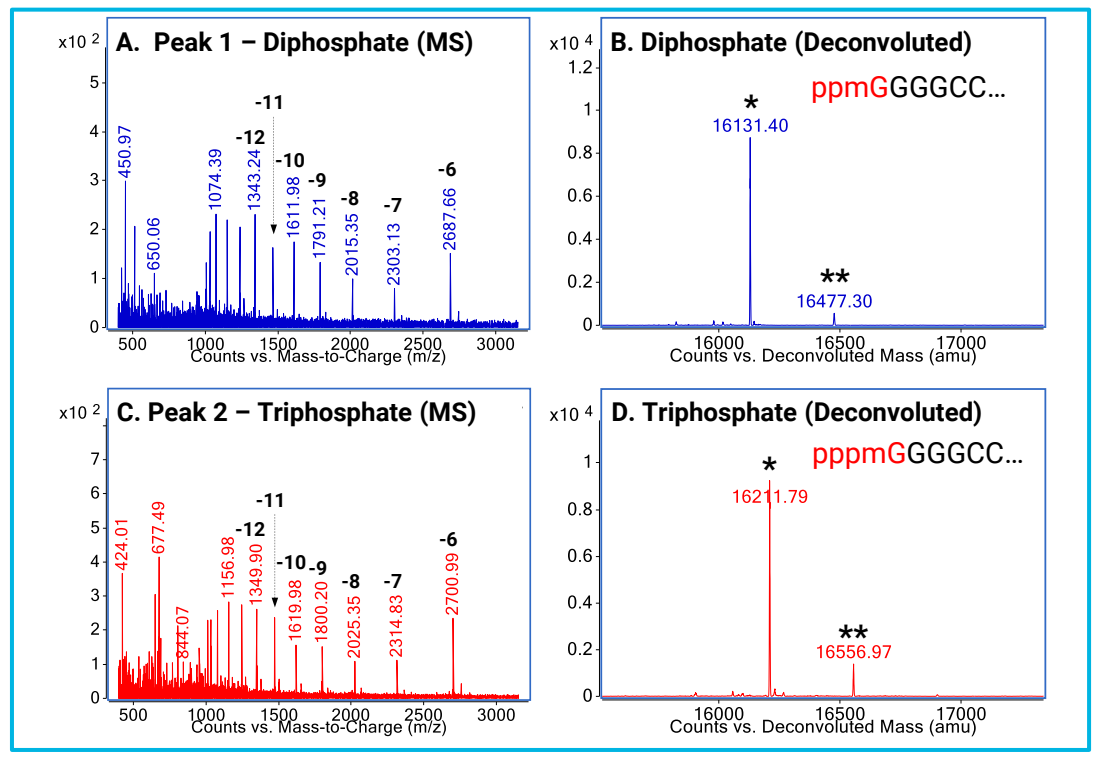


Figure 4. Extracted and deconvoluted mass spectra from Peaks 1 and 2 of Figure 3. Black numbers (A and C) indicate charge states. Mass peaks marked with single asterisks (16131.40 Da and 16211.79 Da) were matched to their putative diphosphate and triphosphate identities (sequences inset) with < 10 ppm mass error. Double asterisks: +G sequence variants (+345.17 Da) due to transcriptional slippage.

LC Separation of ARCA-Capped and Uncapped 5' Oligos

Uncapped mRNA was spiked into ARCA-capped samples at different percentages and used to assess separation, linearity and reproducibility of the method.

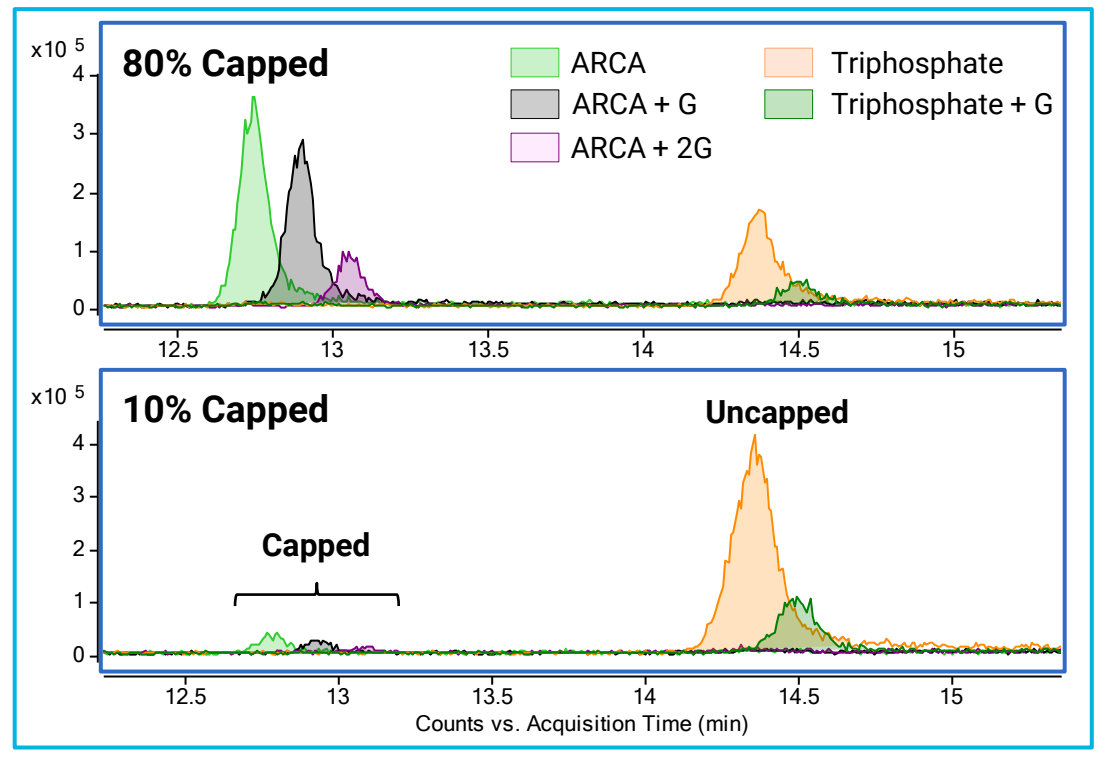


Figure 5. Extracted mass spectra of ARCA-capped and uncapped 5' oligos (all < 10 ppm mass error).

Results and Discussion

Assay Linearity and Inter-Day Reproducibility

Spiked samples ranging from 5 – 80% expected capping ratio were analyzed over three days. Four injections (3µg digested mRNA per injection) were performed per day, and up to three outliers were discarded. N ≥ 9 injections were analyzed for linearity and reproducibility of the observed capping ratio, as well as retention time reproducibility.

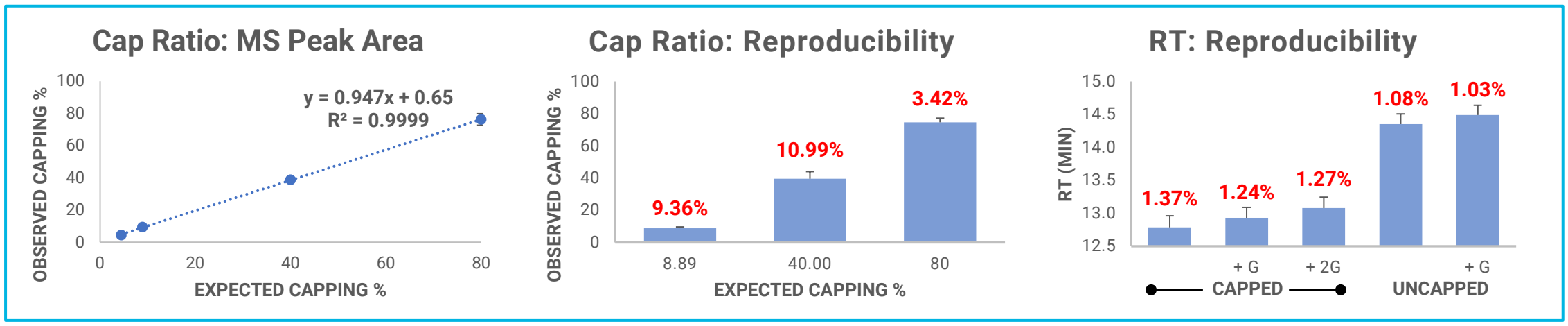


Figure 6. Linearity and reproducibility of capping ratios and retention times. The capping ratio was quantified using MS peak areas. Red numbers indicate relative standard deviation. The LLOQ was estimated as ~8% capped material.

Optimization of Vaccinia Enzyme Capping

Although this reaction has been reported [3] to be almost 100% efficient, it performed only modestly with our sample. Optimizing the ratio of reactants to input mRNA increased capping efficiency from 2.6% to 32.9%.

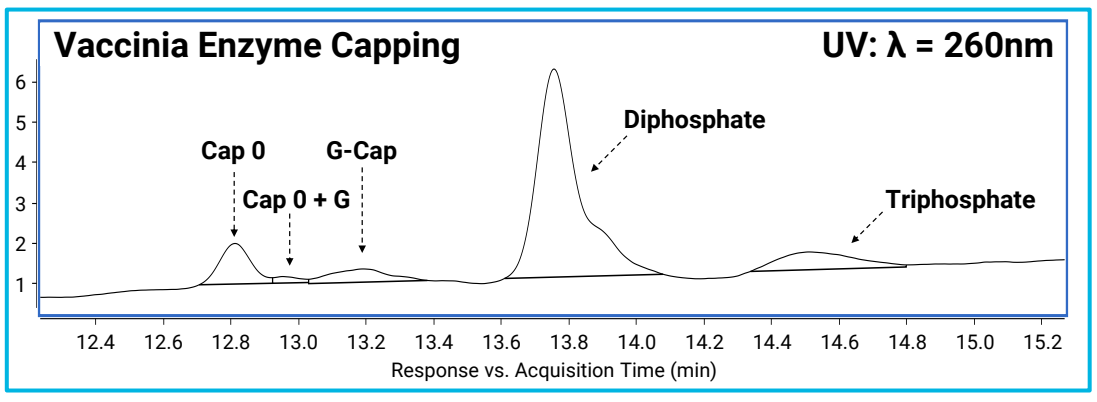


Figure 7. LC-MS of Vaccinia enzyme capped oligonucleotides and capping intermediates.

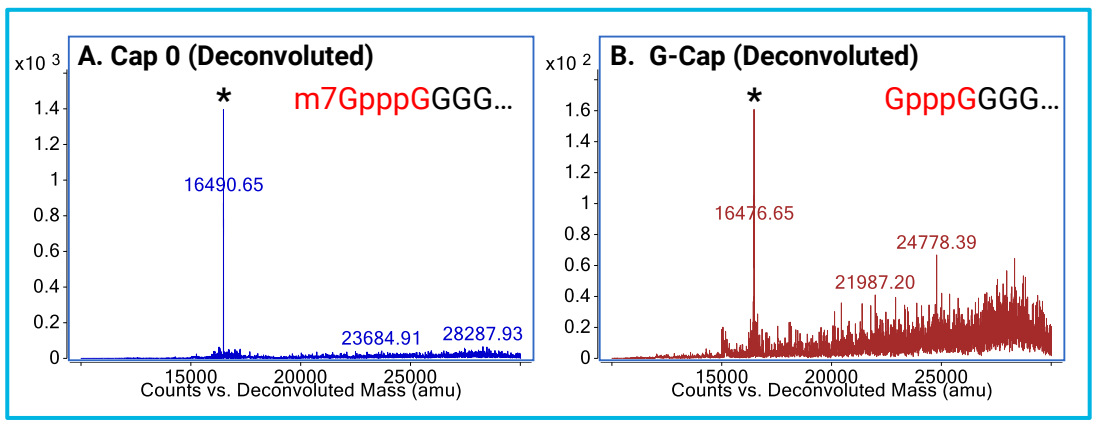


Figure 8. Deconvoluted mass spectra of capped oligonucleotides. Mass peaks marked with single asterisks (16490.60 Da and 16476.65 Da) were matched to their putative identities (sequences inset) with < 5ppm mass error.

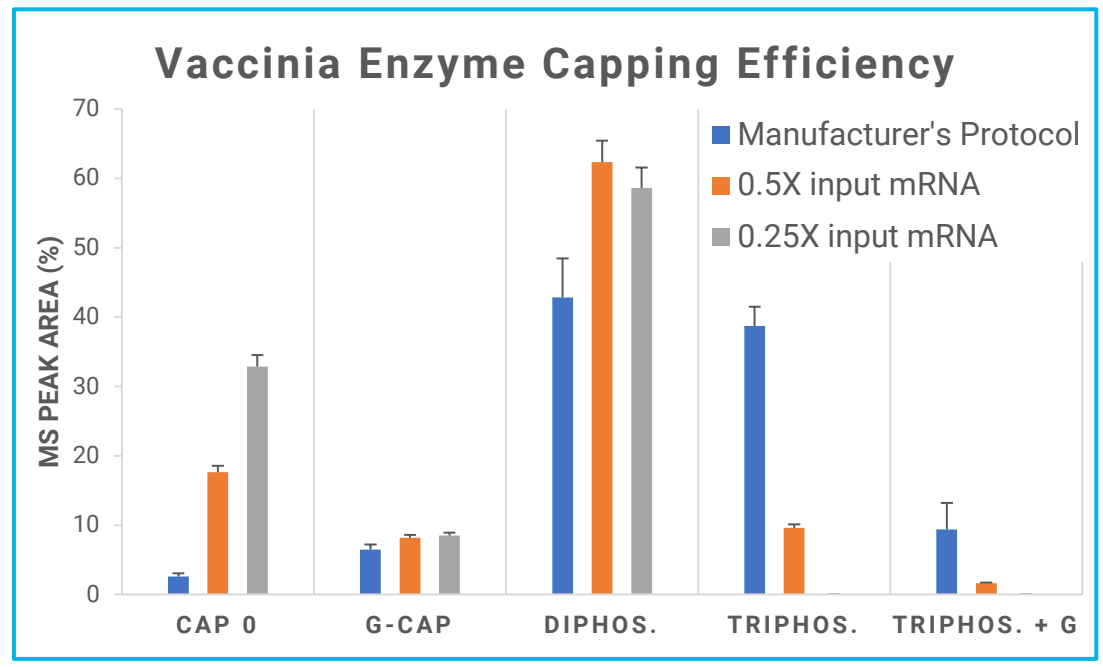


Figure 9. Increasing the ratio of reactants to input mRNA resulted in a decrease in uncapped triphosphate, an increase in diphosphate and G-cap capping intermediates, and an increase in Cap 0 fully capped oligonucleotides.

Conclusions

We have developed a faster LC-MS method for quantifying mRNA 5' capping, and have shown its utility with both ARCA and vaccinia enzyme capping. It can separate capping intermediates and sequence variants, and can be used for rational process optimization.

References

[1]KJ.Hassett et al, Mol. Thera. Nucleic Acids, 2019, 15, 1 – 11.

[2]M.Beverly et al, Anal. Bioanal. Chem, 2016, 408, 5021 – 5030.

[3]AL.Fuchs et al, RNA, 2016, 22, 1454 – 1466.

Download this poster after ASMS at https://explore.agilent.com/asms

DE44480.6067939815

Published in USA, October 20, 2021

Agilent

Trusted Answers