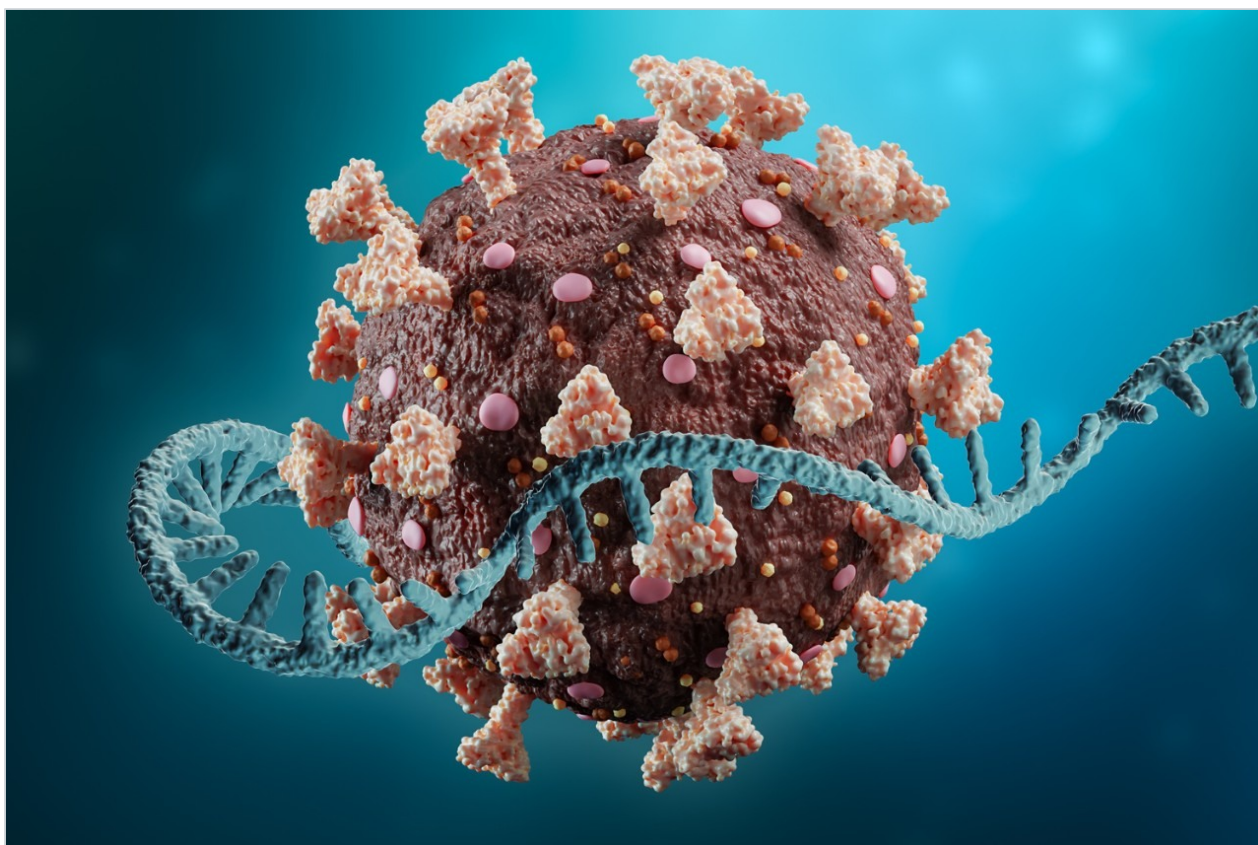


Rapid Analysis of Synthetic mRNA Cap Structure Using Ion-Pairing RPLC with the BioAccord LC-MS System

Jennifer M. Nguyen, Siu-Hong Chan, Bijoyita Roy, Martin Gilar, Brett Robb, Weibin Chen, Matthew A. Lauber

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Abstract

Due to the crucial need to fight the SARS-CoV-2 pandemic, the development of mRNA vaccines progressed rapidly throughout 2020. This has led to the emergency use authorization of two highly efficacious mRNA vaccines.¹ These vaccines contain synthetic mRNA encapsulated in a lipid nanoparticle for delivery, and they use the recipient's body to conduct *in vivo* translation of a stabilized version of the viral spike protein and elicit the immune response.

The mRNA in these vaccines is produced by an enzymatic process known as *in vitro* transcription (IVT). Much like cellular mRNAs, synthetic mRNAs also need to be modified on their 5' ends to include a 7-methyl guanosine nucleotide in a 5'-5'-linkage that is referred to as a 5' cap. The vaccine mRNA is also modified to include a chain of adenosines on the 3' end, called the poly(A) tail (typically ~120 nucleotides). These modifications are critical features of mRNA structure, playing important roles in recognition of the mRNA by cellular factors, stability of the synthetic mRNA, and translational efficiency of the synthetic mRNA molecule.² As such, determining the nature of the 5' cap and the measurement of the poly(A) tail length by LC-MS analysis is vital to understand the product quality and ensure the safety and efficacy of these new vaccines.³

In this application note, we developed a rapid and sensitive LC-MS method applicable to synthetic mRNA capping analysis. Previously, a LC-MS method has been reported to analyze pre-defined 5' fragments of synthetic mRNA.⁴ Here, we demonstrate that ACQUITY Premier Columns and their MaxPeak High Performance Surfaces (HPS), previously shown to enhance oligonucleotide recovery, can also improve the performance of mRNA fragment analysis. This, in combination with an easy-to-use, compliance-ready BioAccord System, provides a fit-for-purpose platform for the rapid analysis of 5' capping of IVT mRNA preparations. A less than 5-minute method was established, and linearity was demonstrated to detecting product-related impurities down to less than 0.1% of the target 5' capped fragment.

Benefits

- Fast, quantitative analysis of synthetic mRNA 5' analysis
- Minimal to no column conditioning required for ACQUITY Premier Columns with MaxPeak High Performance Surfaces
- Linearity, reproducibility, and robustness suitable in both development and QC testing
- High quality MS spectra produced with DIPEA (diisopropylethylamine) mobile phase

Introduction

The success of the Pfizer-BioNTech and Moderna SARS-CoV-2 vaccines has brought a surge of interest to mRNA molecules while also placing new demands on analytics to better support the development and manufacturing of a new modality. Among critical quality attributes (CQAs) such as percentage of full-length mRNA, untranslated regions (UTRs), 3' poly(A) tail length, the sequence, structure, and chemical modifications of the mRNA,⁵ the presence of a proper 5' cap structure is important to ensuring maximal gene expression, to evading innate recognition mechanisms against foreign RNA, and to increasing resistance towards exonuclease degradation.⁶

In eukaryotes, the modified 5' end of the mRNA is an N7-methyl-guanosine connected to the 5' nucleotide by a 5' to 5' triphosphate group. Such a modified structure is known as the cap. In lower eukaryotes such as yeast, the first nucleotide of the capped mRNA does not have any 2'-O modifications, and the cap is known as Cap-0. In higher eukaryotes such as humans, the first nucleotide of the capped mRNA contains a methyl group at the 2'-O position. This mammalian cap structure is known as Cap-1.⁶

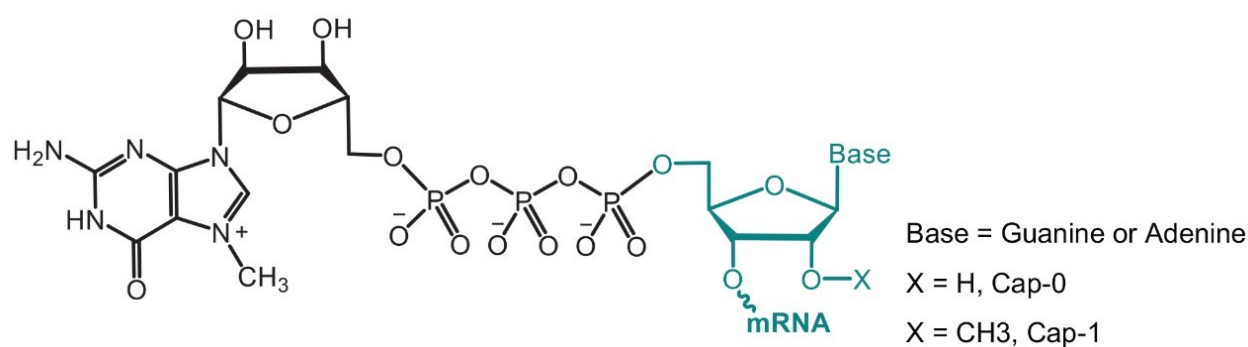


Figure 1. mRNA cap structure.

Since proper cap structure plays a critical role in mRNA translation and innate immune response, effective manufacturing of capped RNA is required. The Cap-1 structure can be added to the synthetic mRNA after IVT enzymatically using vaccinia mRNA capping enzyme (VCE) (Reactions 1.1, 1.2 and 1.3, Figure 2) in concert with vaccinia cap 2'-O-methyltransferase (Reaction 2, Figure 2), or during IVT by using an appropriate cap analog. Verifying the extent of RNA capping, however, is not trivial. RNA capping is the addition of a single nucleotide to an RNA that is often kilobases in length. For example, the BioNTech/Pfizer BNT162b2 is 4,284 nt-long. To date, there is no effective analytical method available to detect and quantify such a small change in physical property of an intact RNA molecule. Beverly and co-workers from the Novartis Institutes of Biomedical Research reported a liquid chromatography coupled to mass spectrometry (LC-MS) method that can be used to identify 5' cap structures and quantify the capping efficiency of synthetic mRNA.⁴ The method employs biotinylated probes to direct RNase H to cleave off a pre-defined fragment from the 5' end of the synthetic mRNA. After enrichment via streptavidin beads, the 5' cleavage fragments can be analyzed by LC-MS to examine the extent of 5' capping.

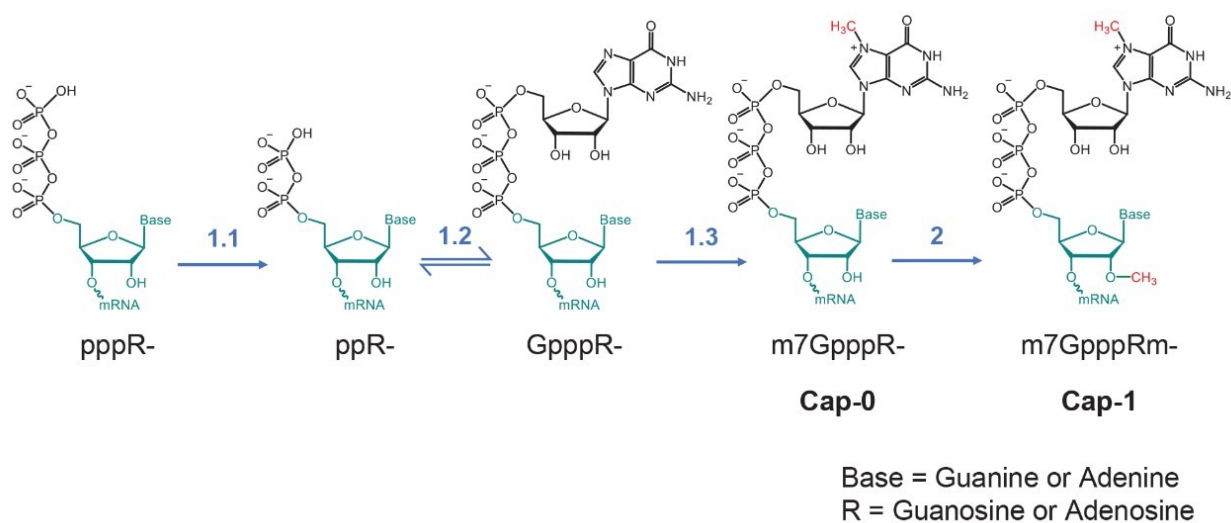


Figure 2. Enzymatic capping of RNA.

In this application note, we demonstrate the advantages of LC-MS to evaluate the 5' capping efficiency of synthetic mRNA using an easy-to-use, compliant-ready BioAccord LC-MS System and ACQUITY Premier Oligonucleotide BEH C₁₈ Columns, which feature MaxPeak High Performance Surfaces Technology. This hybrid organic/inorganic surface technology has been shown to minimize adsorptive losses by blocking interactions to metal surfaces.^{7, 8} Here, the performance of the MaxPeak Premier Technology versus conventional columns greatly improves recovery of the mRNA 5' fragments from the first injection. This, coupled with the BioAccord LC-MS System, can facilitate the analysis of 5' cap structure of synthetic mRNA through MS quantitation.

Experimental

Sample Preparation

In this application note, we demonstrate the advantages of LC-MS to evaluate the 5' capping efficiency of synthetic mRNA using an easy-to-use, compliant-ready BioAccord LC-MS System and ACQUITY Premier Oligonucleotide BEH C₁₈ Columns, which feature MaxPeak High Performance Surfaces Technology. This hybrid organic/inorganic surface technology has been shown to minimize adsorptive losses by blocking interactions to metal surfaces.^{7, 8} Here, the performance of the MaxPeak Premier Technology versus conventional columns greatly improves recovery of the mRNA 5' fragments from the first injection. This, coupled with the BioAccord LC-MS System, can facilitate the analysis of 5' cap structure of synthetic mRNA through MS quantitation.



25 nt = GUAGAACUUCGUCGAGUACGCUCAA

Figure 3. RNA oligonucleotides used in this study.

An equimolar solution of Cap-1 and the targeting oligo at 2.5 pmol/ μ L concentrations was prepared in water. A 1:10, 1:100, and 1:1000 dilution series of the four precursor oligonucleotides versus Cap-1 at a constant 2.5 pmol/ μ L concentration was prepared in water.

Data Treatment and Analysis:

LC Conditions

LC system:	BioAccord LC-MS
Detection:	UV detection at 260 nm
Vials:	Polypropylene vials (p/n: 186002639)
Column(s):	ACQUITY Premier Oligonucleotide BEH C ₁₈ , 130 Å, 1.7 μ m, 2.1 x 50 mm (p/n: 186009484); ACQUITY UPLC Oligonucleotide BEH C ₁₈ , 130 Å, 1.7 μ m, 2.1 x 50 mm (p/n: 186003949)

Column temp.:	60 °C
Sample temp.:	4 °C
Injection volume:	5.0 μ L (sample)
Flow rate:	0.3 mL/min
Mobile phase A:	1.0% hexafluoroisopropanol (HFIP), 0.1% N,N-diisopropylethylamine (DIPEA) in water
Mobile phase B:	0.75% HFIP, 0.0375% DIPEA in 65:35 acetonitrile:water
Gradient:	5–25% B in 5, 10, or 20 min

MS Conditions

MS system:	BioAccord LC-MS
Ionization mode:	Full scan, ESI negative
Acquisition range:	400–5000 <i>m/z</i>
Capillary voltage:	0.8 kV
Cone voltage:	40 V
Desolvation temp.:	400 °C

Data Management

Chromatography and MS software:	waters_connect with UNIFI v1.9.12
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Results and Discussion

Initial Column Performance

Before developing a LC-MS method for 5' capping efficiency, we evaluated the chromatographic recovery of the mRNA fragments from a LC-MS analysis. As described in literature and prior application notes, we have demonstrated that oligonucleotides as well as phosphorylated compounds can adsorb to metal surfaces such as stainless steel. This unwanted effect can be mitigated with the use of MaxPeak Premier Columns.⁹⁻¹¹ For RNA 5' cap analysis, we investigated the LC-UV-MS separations of the Cap-1 species, consisting of the m⁷GpppGm group on the 5' end, using either a conventional ACQUITY UPLC Oligonucleotide BEH C₁₈ Column or an ACQUITY Premier Oligonucleotide BEH C₁₈ Column packed with the same lot of stationary phase. It was confirmed that the ACQUITY Premier Column with its MaxPeak High Performance Surfaces confers benefits like improved recoveries and out-of-the-box performance without the need for conditioning or passivation.

For oligonucleotide LC-MS analysis, ion-pairing reversed-phase (IP-RP) separations with amine mobile phase additives is the preferred mode of chromatography given their resolving power and amenability to MS. However, amines with acidic counter ions, such as acetate or bicarbonate salts, produce too much ion suppression at the concentrations required for effective separations, though reducing the concentration of IP reagent can help. Since its introduction in 1997 by Apffel and co-authors, hexafluoroisopropanol (HFIP) has been preferred for IP-RP separations that are hyphenated to MS technology.¹² HFIP is a weak acid that can help buffer amine containing mobile phases without significantly impairing ionization efficiency. Additionally, it has been theorized that alkylamines are more likely to adsorb onto the stationary phase to act as a better IP system in the presence of HFIP and that it positively affects the desolvation of oligonucleotides in ESI-MS.¹³ Here, we used the amine N,N-diisopropylethylamine (DIPEA) buffered with HFIP, as it has been shown to greatly increase MS signal intensity and peak shape versus triethylamine.¹⁴

Recovery of the Cap-1 fragment as based on peak area from the first injections were evaluated for the conventional ACQUITY UPLC Column versus the ACQUITY Premier Column. Representative UV chromatograms resulting from the first injection on the ACQUITY Premier Column and from the first to fifth injection of the conventional ACQUITY UPLC Column are shown in Figure 4. No peak representing the Cap-1 species was seen upon the initial use of the conventional column (Figure 4). In contrast, the ACQUITY Premier Column gave a high intensity peak on its first injection and reproducible peak areas from subsequent injections. Even after successive injections of Cap-1 on the conventional column, by injection 5, the peak area of the Cap-1 fragment was still only 37% of that of the ACQUITY Premier Column.

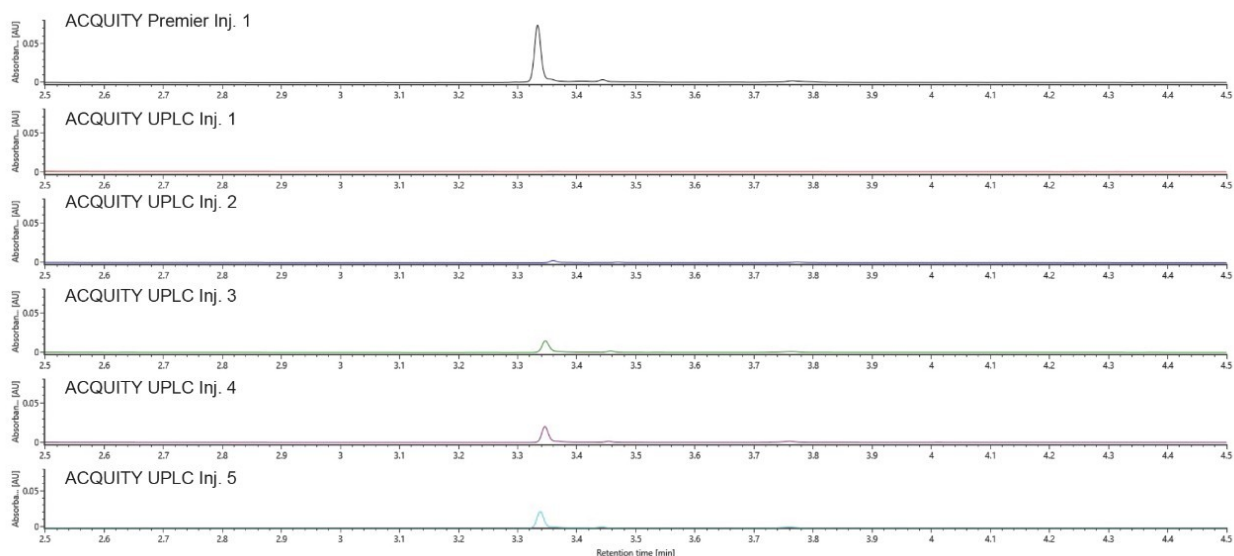


Figure 4. UV chromatograms of the first injection of Cap-1 fragment obtained using an ACQUITY Premier Oligonucleotide BEH C₁₈, 1.7 μm, 2.1 x 50 mm Column and from the first to fifth injections of an ACQUITY UPLC Oligonucleotide BEH C₁₈, 1.7 μm, 2.1 x 50 mm Column. Separations were performed with a BioAccord LC-MS System using a flow rate of 0.3 mL/min, column temperature of 60 °C, DIPEA-HFIP modified mobile phases, and 2.5 pmol mass loads.

Development of a Fast LC-MS Method

We sought to develop a LC-MS method for 5' cap quantification and mass confirmation on the BioAccord System using the ACQUITY Premier Columns. As an easy-to-use LC-optical-MS platform, the BioAccord System is designed for comprehensive analysis of biotherapeutics with reduced complexity in MS instrument operation.¹⁵⁻¹⁷ With the BioAccord LC-MS System, robust and efficient LC-optical-MS methods can be developed for oligonucleotide analysis, reducing the cost and time for gene-based therapy development without compromising product quality. While quantitation of the 5' cap fragments via UV is often favored for some analysts without access to MS instrumentation, we chose to optimize the method of quantitation based on MS signals using the BioAccord, because of the benefits to shorten run times while also taking advantage of accurate MS detection.

Using this strategy, we developed a fast LC-MS method using a short 50 mm ACQUITY Premier Column. Our aim was to rapidly elute the species and use the high-quality mass spectra for mass confirmation and relative quantitation. In Figure 5, it is demonstrated that a method of less than 5 minutes can be employed with little or no loss in recovery of the Cap-1 species. Moreover, Figure 5A suggests even a shorter run time of less than 5 minutes could be achieved; however, the lower abundant species would have become less resolved from the main peak when a run time is shorter, which could interfere with the interpretation of the mass spectra.

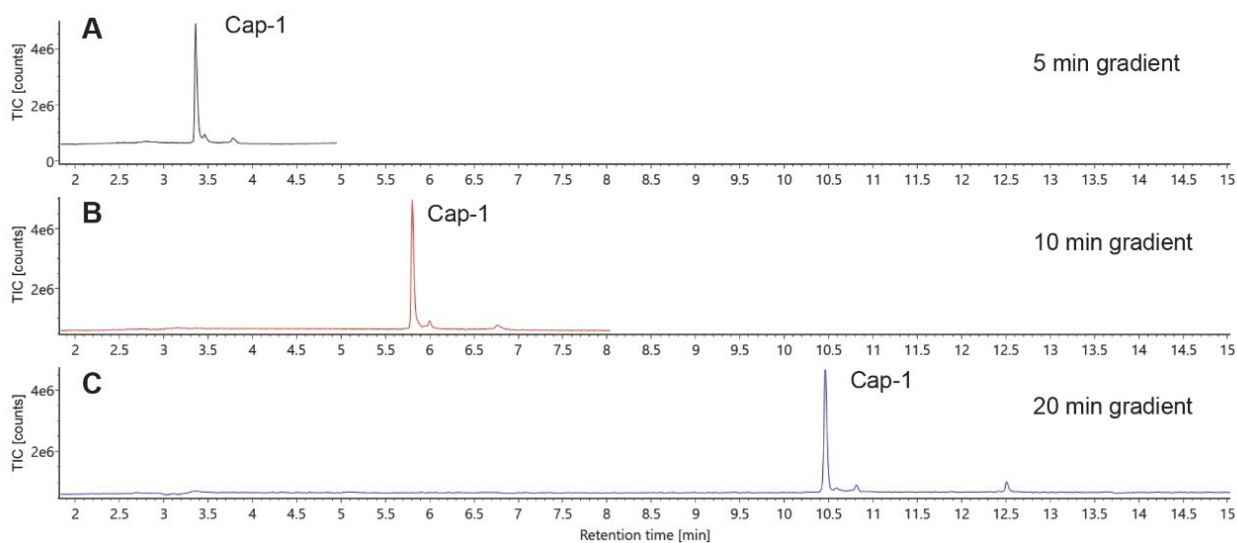


Figure 5. TIC chromatograms of Cap-1 fragment obtained using an ACQUITY Premier Oligonucleotide BEH C₁₈, 1.7 μ m, 2.1 x 50 mm Column. Separations were performed with a BioAccord LC-MS System using a flow rate of 0.3 mL/min, column temperature of 60 °C, DIPEA-HFIP modified mobile phases, 2.5 pmol mass loads, and a gradient from 5–25% B in (A) 5, (B) 10, or (C) 20 min.

Relative Quantitation of 5' Cap Fragment Variants for the Evaluation of 5' Capping Efficiency

As previously mentioned, the percentage of Cap-1 mRNA molecules generated from IVT is an important CQA and must be determined accurately to understand and predict the efficacy of the synthetic mRNA preparation. To demonstrate the capability of the BioAccord LC-MS System for this measurement, we created a series of solutions, in which the ratios between the four precursor species to Cap-1 is 1:10, 1:100, and 1:1000, respectively, while the concentration of Cap-1 remained constant in all solutions. The charge state profiles as obtained from the solution series are displayed in Figure 6.

In Figure 6, the most abundant charge state for Cap-1 was the $[M-10H]^{10-}$ charge state. When running samples containing each individual mRNA fragment (data not shown), the 10 times deprotonated ion was also the most abundant for each fragment, and these charge states can be seen visually in the 1:10 dilution sample. Therefore, we chose this charge state for data analysis and quantification using extracted ion chromatograms (XICs).

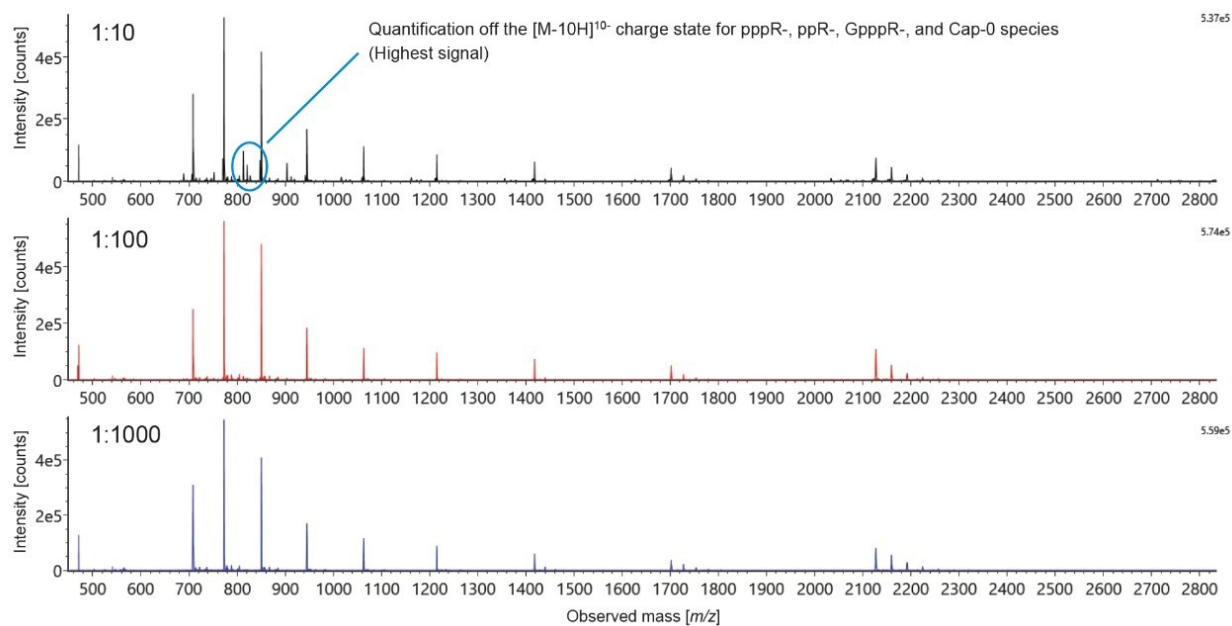


Figure 6. Mass spectra of Cap-1 fragment and its product related impurity fragments (pppG, ppG, GpppG, and m7GpppG, or Cap-0) obtained using an ACQUITY Premier Oligonucleotide BEH C₁₈, 1.7 μm, 2.1 x 50 mm Column. Separations were performed with a BioAccord LC-MS System using a flow rate of 0.3 mL/min, column temperature of 60 °C, DIPEA-HFIP modified mobile phases, and a dilution series of 1:10, 1:100, and 1:1000 versus a 12.5 pmol/μL concentration of Cap-1.

Representative XICs for the [M-10H]¹⁰⁻ charge state of Cap-0 are shown in Figure 7 for each dilution solution. For each dilution, the isotopic distribution for the charge state can be clearly observed, and XICs were taken from the entire isotope mass at a mass error of 10 ppm. Even at a 1:1000 dilution, corresponding to a mass load on column of only 12.5 fmol of the product related impurities, the XIC of the Cap-0 species could be readily generated and integrated.

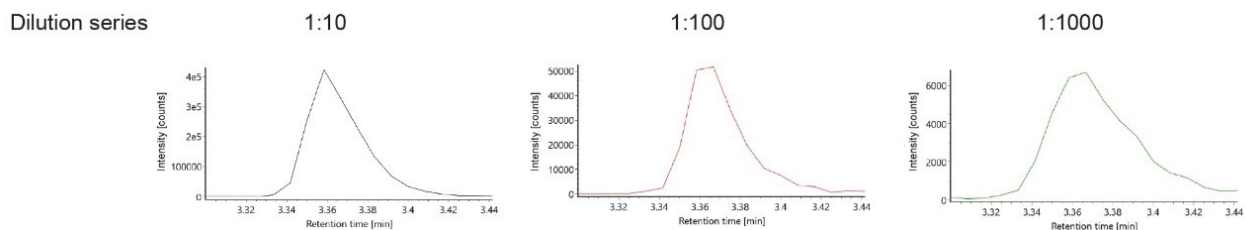


Figure 7. XICs as obtained from the $[M-10H]^{10-}$ charge state of the Cap-0 fragment using an ACQUITY Premier Oligonucleotide BEH C_{18} , $1.7 \mu m$, 2.1×50 mm Column. Separations were performed with a BioAccord LC-MS System using a flow rate of 0.3 mL/min, column temperature of 60 °C, DIPEA-HFIP modified mobile phases, and a dilution series of 1:10, 1:100, and 1:1000 versus a 12.5 pmol/L concentration of Cap-1.

From the XICs of the dilution series, calibration curves could be generated and are displayed in Figure 8 for each of the precursor fragments. Here, the linear regression for each chart produces an R squared value of 1.000, indicating the capability of this rapid LC-MS method for evaluating the extent of 5' capping.

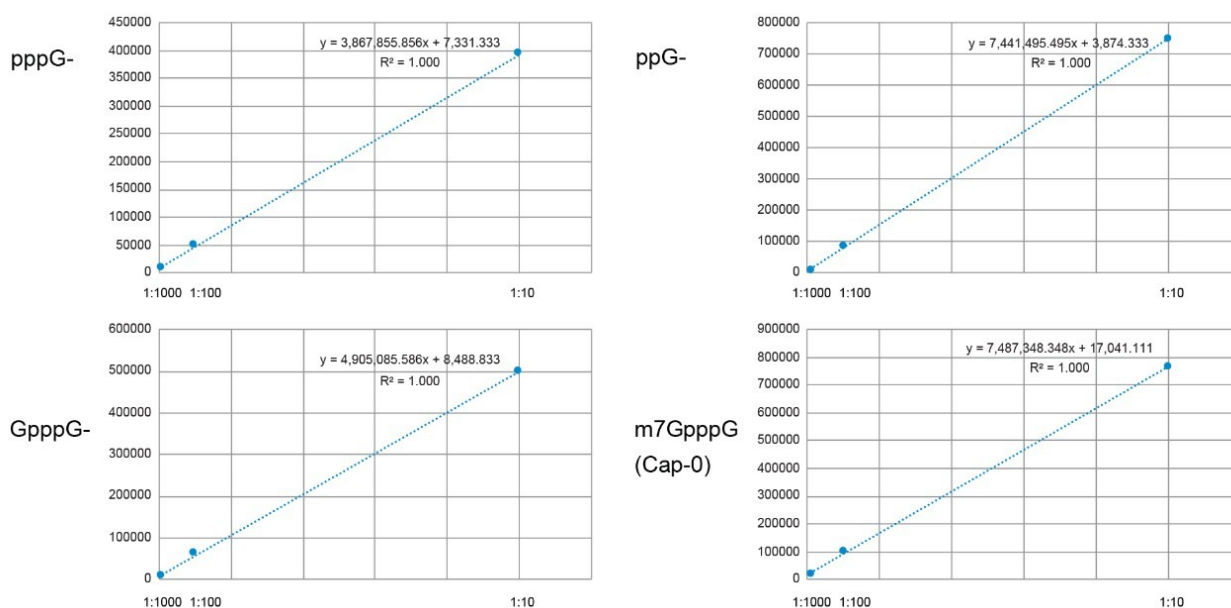


Figure 8. Calibration curves generated from a 1:10, 1:100, and 1:1000 dilution series of Cap-1 at 12.5 pmol/L versus its product-related impurity fragments (pppG, ppG, GpppG, and m7GpppG, or Cap-0).

Separation of the RNase H Probe (Targeting Oligo) and RNA 5' Fragments

An important consideration when analyzing RNase H-based mRNA fragment analysis, is the LC separation of the RNase H probe, or targeting oligo, from the analyte species. In this example, the RNase H probe is a 3'

desthiobiotin affinity tag-modified RNA:DNA chimera approximately the same length as the cleaved 5' fragment. The probe is designed to be a complement of the target 5' fragment such that the two hybridize in solution to form a double-stranded RNA-DNA duplex structure. After RNase H cleavage at a pre-defined site, the probe and the 5' fragment remain associated as a duplex in solution and thus, it is necessary to resolve the species of interest from the RNase H oligo probe that is expected to be present in the sample mix.

To validate the LC separation of the RNase H probe and the analyte fragments, an RNase H probe that could be used to generate the 25-nt RNA oligonucleotide from a longer RNA was designed and synthesized. Figure 9 shows that the rapid LC-MS method can separate the Cap-1 RNA oligonucleotide and the RNase H probe in an equimolar solution of the two species.

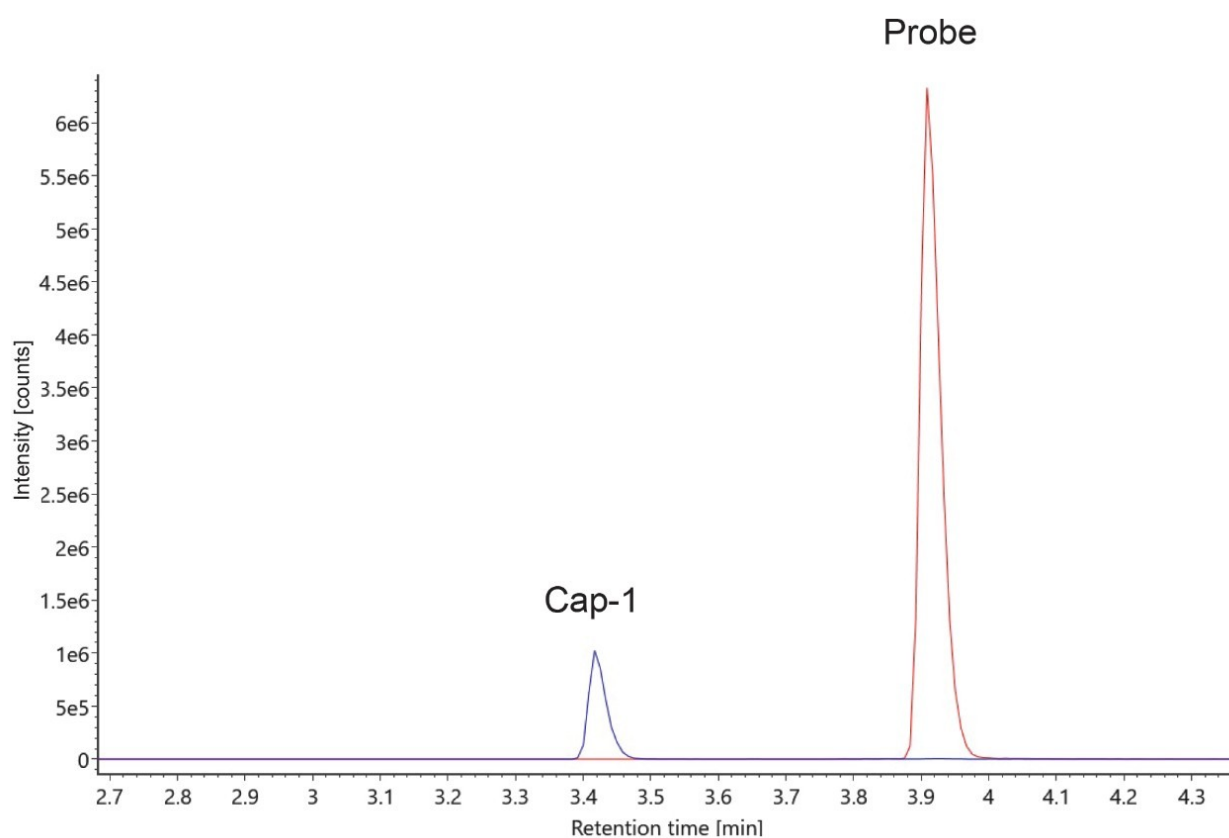


Figure 9. Overlaid XICs of Cap-1 and a representative probe from an equimolar solution using an ACQUITY Premier Oligonucleotide BEH C₁₈, 1.7 μ m, 2.1 x 50 mm Column. Separations were performed with a BioAccord LC-MS System using a flow rate of 0.3 mL/min, column temperature of 60 °C, DIPEA-HFIP modified mobile phases, and 12.5 pmol mass loads.

Conclusion

The value of mRNA as a vaccine modality is now proven by the high effectiveness of the mRNA-based vaccines against COVID-19 that have now been administered to over 25% of the world population. Analytical techniques to ensure their proper design, development, and reproducible manufacture are important. In this application note, we demonstrate that a rapid LC-MS method applicable to evaluating the extent of 5' capping of synthetic mRNA, an important CQA for synthetic mRNAs, using an ACQUITY Premier Oligonucleotide BEH C₁₈ Column couple with a BioAccord LC-MS System. The ACQUITY Premier Column with MaxPeak High Performance Surfaces Technology could provide considerable improvements in RNA recovery upon first injection. Paired with the BioAccord LC-MS, this combination of technologies allows for accurate quantitation, even at low limits of detection, such that it is possible to validate the manufacturing of Cap-1 mRNA molecules, and potential presence of product precursor related impurities. Moreover, these results highlight the potential of MS-based quantitation for high throughput assays, which could help accelerate the development of mRNA modalities.

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Siu-Hong Chan, Bijoyita Roy, Brett Robb (New England Biolabs Inc., Ipswich, MA, USA).

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