New Methods for Determining Key Quality Attributes for mRNA by Enzymatic Digestion Followed by LC-MS/MS

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Introduction

mRNA is probably the most rapidly growing therapeutic modality with numerous drug candidates in the pipeline globally. With this success came a high demand for robust analytical methods able to determine the quality of the mRNA product. The manufacturing process of mRNA molecules consists of *In vitro* transcription reactions in which the desired final product is over 1000 nucleotides in length, has a 5' Cap, and a Poly(A) tail. Both the 5' Cap and the Poly(A) tail help to stabilize the RNA molecule and improve its translation. Contaminants such as degradation products, incomplete capping, and shorter than expected Poly(A) tails can still be present in the final product and need to be quantified to assess mRNA purity. Additionally, the length of the mRNA molecule can make it challenging to analyze. Here, we show a reliable workflow for the characterization and relative quantitation of mRNA therapeutics by using enzymatic digestion followed by High-Resolution Mass Spectrometry (HRMS) coupled to liquid chromatography. This workflow can serve as a base for researchers to establish their own mRNA purity assessment analytical methods.

Materials and Methods

Reagents and Chemicals

RNA oligonucleotides were purchased from Integrated DNA Technologies (Coralville, IA, USA). All other chemicals were obtained from Sigma-Aldrich® Company (St. Louis, MO, USA).

LC Conditions – QC Oligonucleotides (RNA)

Column: Biozen™ 2.6 µm Oligo
Dimensions: 50 x 2.1 mm
Part No.: 00B-4790-AN
Mobile Phase: A: 95 mM Hexafluoro-2-propanol, 5.7 mM N, N-Diisopropylethylamine in Water
B: 7.1 mM Hexafluoro-2-propanol, 2.1 mM N, N-Diisopropylethylamine in 80 % Methanol

Gradient: Time (min)	%B
0	3
15	35
15.6	80
20	80
20.5	3
25	3

Flow Rate: 0.3 mL/min
Injection Volume: 5 µL
Temperature: 70 °C
Instrument: Vanquish™ UHPLC
Detection: HRMS
Detector: Q Exactive™ Plus

LC Conditions – mRNA

Column: Biozen 2.6 µm Oligo
Dimensions: 50 x 2.1 mm
Part No.: 00B-4790-AN
Mobile Phase: A: 95 mM Hexafluoro-2-propanol, 5.7 mM N, N-Diisopropylethylamine in Water
B: 7.1 mM Hexafluoro-2-propanol, 2.1 mM N, N-Diisopropylethylamine in 80 % Methanol

Gradient: Time (min)	%B
0	3
45	35
45.6	80
50	80
50.5	3
60	3

Flow Rate: 0.3 mL/min
Injection Volume: 5 µL
Temperature: 70 °C
Instrument: Vanquish UHPLC
Detection: HRMS
Detector: Q Exactive Plus

HRMS Conditions

Ionization Mode: Negative (HESI Source)

Scan Type: MS1
Resolution: 140,000
AGC Target: 1e6 ms
Maximum IT: 200 ms
Scan Range: 450 to 3500 m/z

Scan Type: MS2
Resolution: 35,000
AGC Target: 1e5 ms
Maximum IT: 50 ms
Loop Count: 8

Sample Preparation

Oligonucleotide (RNA) standards were resuspended in water at a concentration of 100 µM each followed by mixing each of them at a 10 µM final concentration in water. mRNA of 1250 nucleotides long was enzymatically digested using Rnase H followed by injection into a Biozen Oligo column. The flow path to the column was diverted to waste during the first 2 min of the start of the run to avoid salts from entering the MS source. Bioinformatic analysis for both, oligonucleotide standards and mRNA, were done using BioPharma Finder® software and Protein Metrics Oligo software.

Results

Figure 1. Oligonucleotide (RNA) Ladder QC Standards to Assess LC and Column Performance.

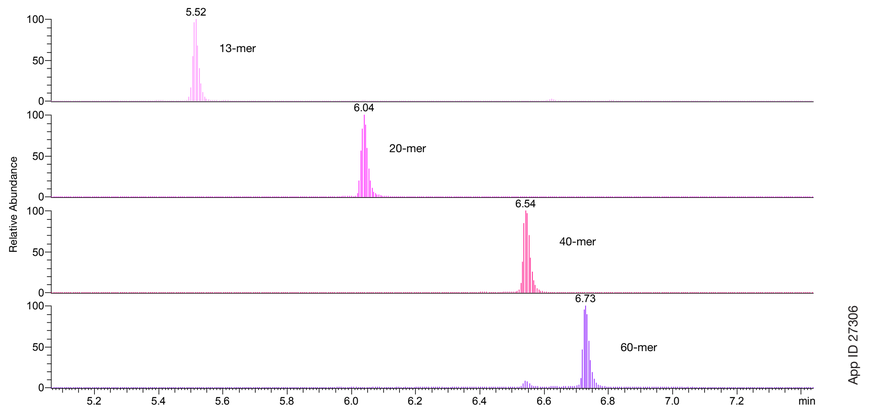


Figure 2. MS2 Fragmentation of 20-mer Oligonucleotide (RNA) QC Standard to Assess MS Performance.

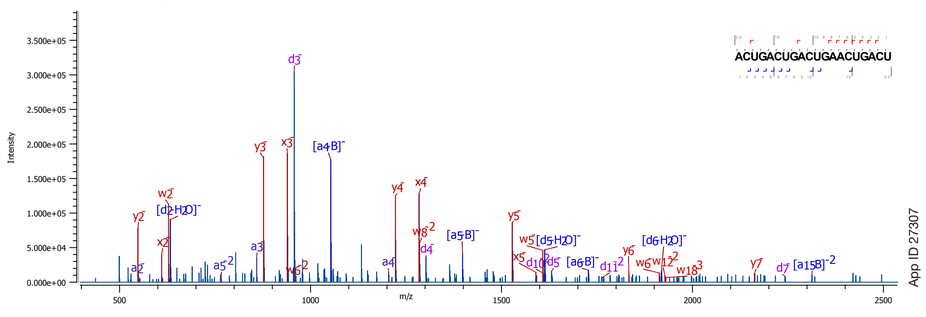


Figure 3. Total Ion Chromatogram of RNase H Digested 1250 Long mRNA.

Table 1. Total Number of Peaks Detected and mRNA Sequence Coverage.

Sample	Number of MS Peaks	Sequence Coverage
mRNA	674	88%

Results

Figure 4. Biozen Oligo Core-shell Particles for mRNA Cap Purity Analysis.

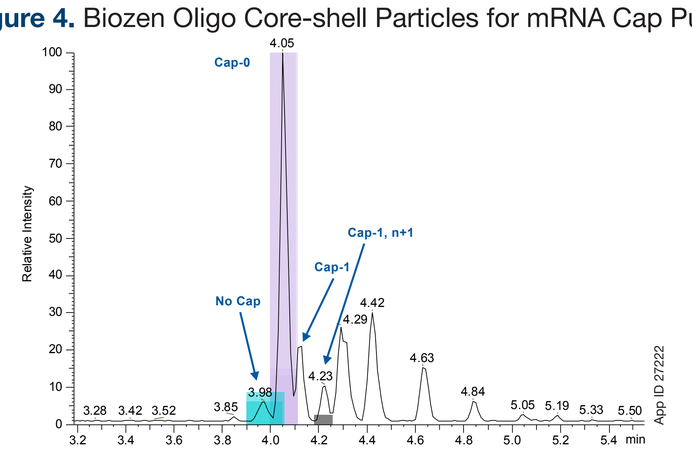


Figure 5. Example of Deconvoluted Charged Envelope for Cap-1.

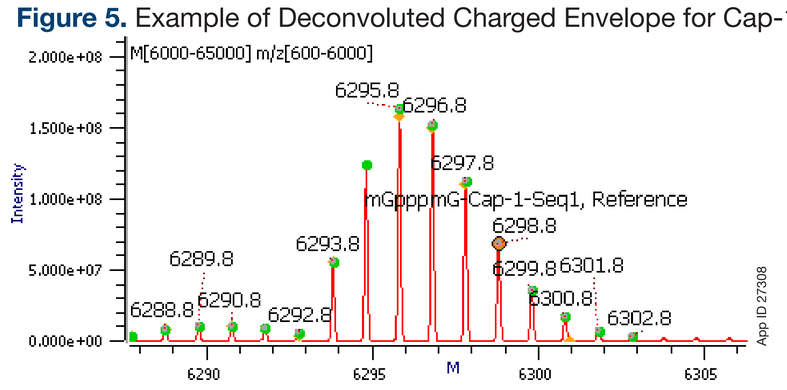


Table 2. 5' Cap Type Relative Abundance (% Area) and Mass Errors.

Type	Delta (ppm)	% Area
No Cap	3.27	9.02
Cap-0	-0.75	79.86
Cap-1	-3.45	9.65
Cap-1,n+1	-1.04	1.47

Results

Figure 6. Biozen Oligo Column Provides Single Nucleotide Resolution of Poly(A) Tail Degradation: XIC Shows Single Nucleotide Resolution.

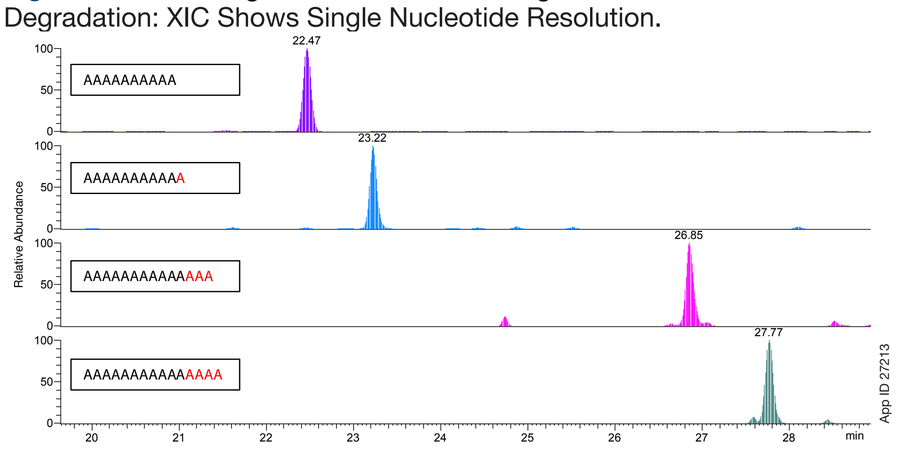


Figure 7. Biozen Oligo Column Provides Single Nucleotide Resolution of Poly(A) Tail Degradation Fragments.

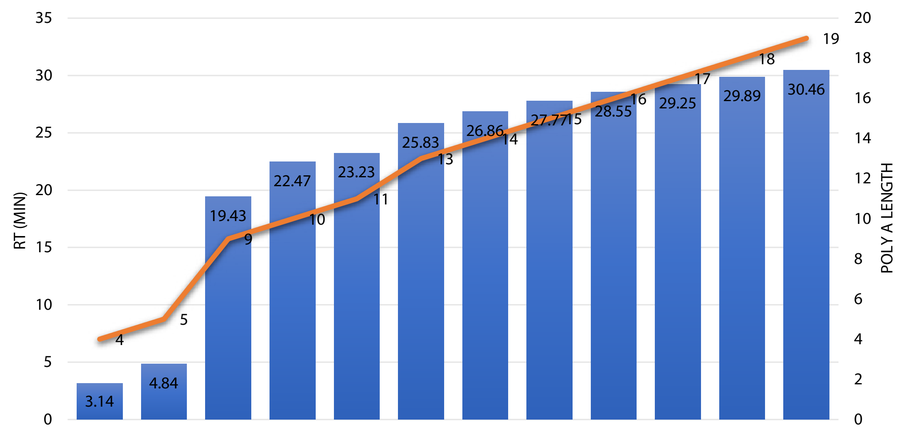
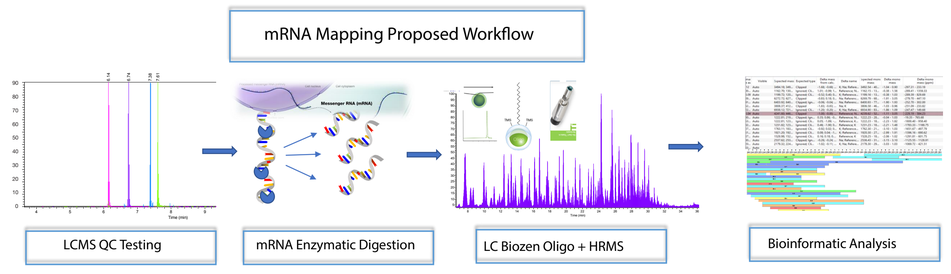


Figure 8. Proposed mRNA Mapping Workflow: From QC Testing to Data Analysis.



Discussion

In order to preserve mRNA from 5' nuclease degradation, a variety of CAPping molecules have been created to preserve the biological stability of the mRNA sequence. Analytical characterization of the diversity and integrity of the 5' Cap is essential for molecule stability and administration safety. Similarly, the Poly(A) tail of a messenger RNA (mRNA) is a repeat sequence of adenosine (A) that is an emergingly popular modality among vaccine and gene therapy developers. Grown at the 3' end, the increasing length of this tail provides additional stability to the "core" sequence that invokes the generation of a protein and subsequent immune response from the host. Given the inherent RNA stability challenges as well as current delivery challenges, the ability to understand the degradation of the Poly(A) tail and also the mRNA's Cap purity is an essential tool to improving delivery of therapeutics.

Conclusion

mRNA enzymatic digestion coupled to High Resolution Mass Spectrometry (HRMS) and Liquid Chromatography using Biozen Oligo columns provides resolution and relative quantitation of mRNA cap as well as single nucleotide resolution of Poly(A) tail fragments that can help assess mRNA purity and synthesis quality. Also, adding a QC method using RNA oligonucleotide standard mix of various sizes helps to ensure that the LC-MS system is functioning properly prior to injection of a digested RNA sample.

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