Application note | 001108

Biopharma

Characterization of mRNA 5’ capping products using an LC-HRAM-MS/MS analytical platform and Thermo Scientific BioPharma Finder software solution

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Keywords

Orbitrap Exploris 240 MS, BioPharma Finder software, mRNA, 5' cap, IVT, IP-RP, HRAM

Goal

To develop a sensitive and robust LC-HRAM-MS/MS method for identification, sequence confirmation, and relative quantitation of the 5’ cap in in vitro transcription (IVT) mRNA products

Application benefits

- The capabilities of high-resolution accurate mass (HRAM) and data-dependent tandem mass spectrometry (ddMS^2) enable confident identification, mapping, and relative quantitation of mRNA 5’ capping oligonucleotide digestion products.
- Thermo Scientific™ BioPharma Finder™ 5.0 software offers a streamlined workflow for identification and characterization of mRNA 5’ digestion products.

Introduction

Production of non-natural mRNA transcripts by IVT for vaccines and therapeutics development and manufacturing[1] requires the addition of the modified cap structure post purification.[2] In eukaryotic systems, non-mitochondrial mRNA are capped at the 5’ end by an inverted 7-methylguanosine (m7G) joined to the transcript by a 5’ to 5’ triphosphate linkage, m7G(5’)ppp(5’)N, referred to as Cap(0).[3–5] Once added to the transcript, the first nucleotide in the strand, (N), is then methylated at the 2’ hydroxyl position to give the Cap(1) structure, m7G(5’)ppp(5’)Nm.[6–9]

Capping and polyadenylation help the transcript to resist degradation by the innate exonucleases in the cell.[10] Besides blocking 5' to 3' exonucleases,[11] the 5' cap facilitates exportation from the nucleus to the cytoplasm[12,13] and aids the translation initiation process by acting as a determinate for ribosomal docking complexes.[14,15] Therefore, as one of the mRNA Critical Quality Attributes (CQAs), the 5' capping needs to be fully characterized during product and process development.

The enzyme RNase H will cleave the RNA strand in an RNA:DNA double-stranded hybrid at the Watson-Crick RNA:DNA base pair.[16] This enzyme specificity can be exploited to measure capping efficiency. The method[17] involves designing a biotinylated molecular probe ~25 nt in length, complementary to the 5' end of the transcript, and containing several DNA nucleotides in tandem. The probe is annealed to the 5' end of the mRNA, complexed to magnetic beads, then subjected to an RNase H digestion. The cleaved products are separated by a magnet, washed, eluted from bead, dried, then analyzed by LC-HRAM-MS/MS.

Here we show the applicability of the Thermo Scientific™ Orbitrap Exploris™ 240 mass spectrometer coupled to the Thermo Scientific™ Vanquish™ Horizon UHPLC system, and utilizing BioPharma Finder 5.0 software for the accurate identification and characterization of mRNA 5' capping digestion products.

Experimental

Reagents, consumables, and lab equipment

- Fisher Chemical™ Optima™ LC/MS Grade Water (P/N W6500)
- Thermo Scientific™ UHPLC-MS Methanol (P/N A456-1)
- Thermo Scientific™ Ethanol, 99.5% (P/N 615100020)
- Thermo Scientific™ RNase H with reaction buffer (P/N 18021014)
- Invitrogen™ Dynabeads™ MyOne™ Streptavidin C1 (P/N 65001)
- Invitrogen™ LiCl Precipitation Solution (7.5 M) (P/N AM9480)
- Invitrogen™ NaCl (5 M), RNase-free (P/N AM9759)
- Invitrogen™ Nuclease-Free Water (not DEPC-Treated) (P/N 9938)
- Thermo Scientific™ $MgCl_2$ (1 M) (P/N AM9530G)
- Invitrogen™ UltraPure™ 1 M Tris-HCl Buffer, pH 7.5 (P/N 15567027)
- Thermo Scientific™ MagJET Separation Rack, 12 × 1.5 mL tube (P/N FERMR02)
- Thermo Scientific™ F1-ClipTip™ Variable Volume Single Channel Pipettes (P/N 4641210N)
- Thermo Scientific™ SureSTART™ Polypropylene Vials (P/N 6ESV9-04PPT)
- Thermo Scientific™ SureSTART™ 9 mm Screw Caps (P/N 6ASC9ST1)
- Invitrogen™ Nonstick, RNase-free Microfuge Tubes, 1.5 mL (P/N AM12450)
- Invitrogen™ Nonstick, RNase-free Microfuge Tubes, 2 mL (P/N AM12475)
- Thermo Scientific™ Savant™ SpeedVac™ Medium Capacity Vacuum Concentrators (P/N SPD131DDA)
- Thermo Scientific™ NanoDrop™ One/One^C Microvolume UV-Vis Spectrophotometer (P/N ND-ONEC-W)

Sample preparation

RNA 5' pyrophosphohydrolase (RppH) digestion (decapped reference standard)

To a low binding Eppendorf tube was added 100 pmol mRNA, 10 µL of 10× reaction buffer and 2 µL of RppH enzyme. The solution was brought up to 100 µL with Nuclease-Free water and allowed to digest for 1 hr in a thermal mixer set to 37 °C and 300 RPM. At the end of the hour, the tube was removed, and the decapped mRNA was precipitated by the addition of 50 µL of 7.5 M LiCl solution. The tube was placed in the -20 °C freezer for 30 minutes, then centrifuged at 12,000 RPM for 15 minutes. The tube was removed from the centrifuge and the supernatant was discarded. The pellet was then washed with 100 µL of cold 75% EtOH, centrifuged again, and then the supernatant was discarded. The pellet was allowed to dry at RT for ~10 minutes.

RNase H digestion (capped mRNA sample)

To a low binding RNase/DNase free 1.5 mL Eppendorf tube was added 300 pmol of 5' capped mRNA. The sample was brought up to 100 µL using Nuclease-Free water then precipitated as before with LiCl. An annealing buffer solution of 10 mM TRIS-HCl, pH 7.5 and 100 mM NaCl was prepared. To the decapped pellet was added 100 µL of annealing buffer, the mRNA was resolubilized, then transferred to the tube containing the capped mRNA pellet and mixed by pipetting. This creates a 1:4 ratio of decapped to capped mRNA in ~100 µL. To the tubes were then added 10 µL (1,000 pmol) of a biotinylated chimeric 25-mer containing 5 DNA bases. The tube was then heated to 95 °C for 5 minutes in a heat block, which was then powered off and allowed to come to room temperature.

To this tube, 50 U of RNase H was added and the mixture was allowed to digest for three hours in a thermal shaker at 37 °C and 300 RPM. The solution was then added to 100 µL of Dynabeads™ MyOne™ Streptavidin C1 magnetic beads, previously prepared per manufacturer's specifications. RNase digest and magnetic beads were mixed by gently pipetting, then the tube was gently mixed for 30 minutes. The tube was removed, and the beads were isolated with a magnetic rack. The supernatant was saved for poly(A) tail characterization. Beads were then washed a total of 3× per manufacturer's recommendation using the annealing buffer. Annealing buffer was used over the 1× B&W buffer recommended for the beads to decrease the residual Na^+ content. The B&W buffer is at a 1 M concentration, where the annealing buffer is 100 mM. Note: A less concentrated wash could be used here for the final rinse if the concentration does not interfere with the Tm of the annealed probe.

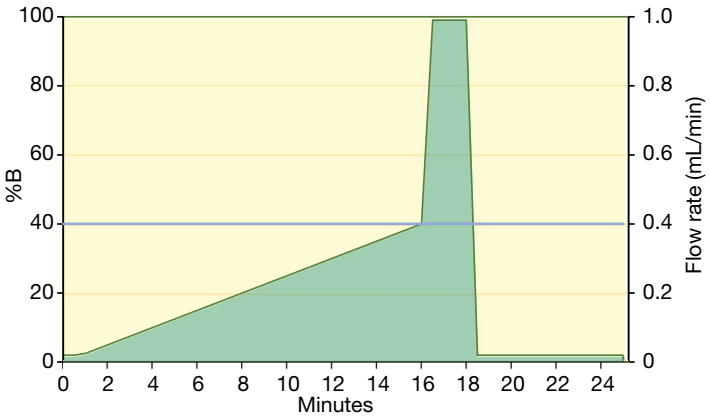
RNase H digestion products were eluted off the beads by resuspending in 100 µL 75% LC-MS grade EtOH:water solution. The tube was placed in an 80 °C heat block for ~3 min, removed to the magnetic rack and magnetic beads allowed to collect. Clear supernatant was removed to a clean low binding Eppendorf tube. The elution was repeated with a second 100 µL elution and combined. The tubes were then placed in a SpeedVac vacuum concentrator and taken to dryness under no heat. Samples were resuspended in MPA and injected.

Ion-pair reversed-phase liquid chromatography (IP-RP LC)

For all experiments, the Thermo Scientific™ Vanquish™ Horizon UHPLC system was used, consisting of:

- Thermo Scientific™ Vanquish™ Binary Pump H (P/N VH-P10-A-02)
- Thermo Scientific™ Vanquish™ Split Sampler HT (P/N VH-A10-A-02)
- Thermo Scientific™ Vanquish™ Column Compartment H (P/N VH-C10-A-02)
- Thermo Scientific™ Viper™ MS Connection Kit Vanquish™ LC systems (P/N 6720.0405)
- Mobile phase A 50 mM HFIP, 15 mM triethylamine in H_2O
- Mobile phase B 50 mM HFIP, 15 mM triethylamine in MeOH

Oligonucleotide separations were performed with a Thermo Scientific™ DNAPac™ RP column (4 µm, 2.1 × 100 mm, (P/N 088924). The autosampler was held at 5 °C while the column was maintained at 70 °C with the column oven thermostat mode set to Still Air. The LC gradient used in this study is shown in Figure 1.



Time (min)	%B
0	2
0.5	2
16	40
16.5	99
18	99
18.5	2
25	2

Figure 1. LC-MS grade water:methanol gradient used in this experiment. Both mobile phase A (water) and B (methanol) contain the modifiers hexafluorisopropanol (HFIP) and triethylamine (TEA).

Mass spectrometry

The Thermo Scientific Orbitrap Exploris 240 mass spectrometer (P/N BRE725535) was operated with Thermo Scientific™ Chromeleon™ Chromatography Data System software version 7.3.1 and controlled by the Thermo Scientific™ Orbitrap Exploris™ Series 4.0 SP1 instrument control software. Instrument calibration was performed using Thermo Scientific™ Pierce™ FlexMix™ calibration solution. Data acquisition was performed in negative ion mode. The $ddMS^2$ method was built in the method editor using the standard MS and MS^n templates provided with the Orbitrap Exploris instrument control software then modified accordingly. Table 1 lists the scan parameters of the MS and MS/MS methods. Global settings were default for the flow rate (400 µL min^{-1}) used in the experiment.

Table 1. MS and MS/MS settings for 5' cap acquisition

Master scan	
Full Scan	**Value**
Orbitrap resolution	120,000
Scan Range *(m/z)*	550–2,500
RF lens (%)	70
AGC target	Custom
Normalized AGC target (%)	100
Maximum injection time mode	Custom
Maximum injection time (ms)	50
Microscans	1
Data type	Profile
Polarity	Negative
Filters	
Intensity	**Value**
Intensity threshold	5.00E+03

Dynamic exclusion	Value
Dynamic exclusion mode	Custom
Exclude after n times	1
Exclusion duration (s)	3
Mass tolerance	ppm
Low	10
High	10
Exclude isotopes	TRUE
Perform dependent scan…	FALSE
Data dependent	**Value**
Data dependent mode	Number of scans
Number of dependent scans	5

Scan	
ddMS²	**Value**
Multiplex ions	FALSE
Isolation window *(m/z)*	1.6
Isolation offset	Off
Collision energy type	Absolute
HCD collision energy (V)	20
Orbitrap resolution	30,000
Scan range mode	Define *m/z* range
Scan range *(m/z)*	600–1,500
AGC target	Standard
Maximum injection time mode	Auto
Microscans	1
Data type	Profile

Software

- Chromeleon 7.3.1 CDS software (P/N CHROMELEON7)
- Thermo Scientific™ FreeStyle™ 1.8.2 application
- Thermo Scientific BioPharma Finder 5.0 software (P/N OPTON-30988)

mRNA data analysis in BioPharma Finder software

To map the identified digestion products from the RNase H digestion of in vitro produced mRNA, a reference sequence must be created. This is done from the BioPharma Finder software home screen using the Sequence Manager option where the oligonucleotide sequence is added and modified. Under *Sequence Information,* the oligonucleotide name is created and the **Category** for processing is chosen. Here we choose the option “Sequencing” from the drop down. Once all sequences are added, the *Sequence Information* panel is populated with the monoisotopic and average mass, as well as the formula for each sequence (Figure 2). Under *Manual Input Sequence* the chain name is given, and the sequence is copied (Figure 3). For our analysis we created three sequences, a truncated 5’ end 40 nucleotides in length containing a cap, a second 40-mer containing only a 5’ phosphate on the first nucleotide past the cap, and the sequence of the biotinylated probe.

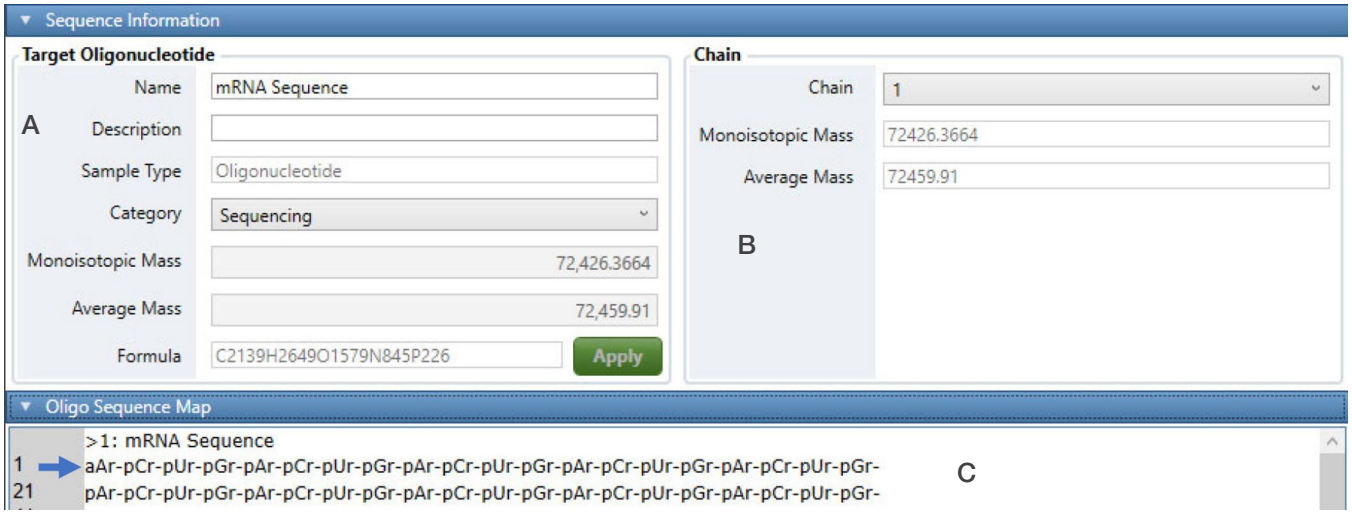


Figure 2. Sequence Manager panel. (A) Creation of named sequences and selection of processing category. (B) Monoisotopic and average mass calculation for each created sequence. (C) Sequence Map showing each created oligonucleotide.

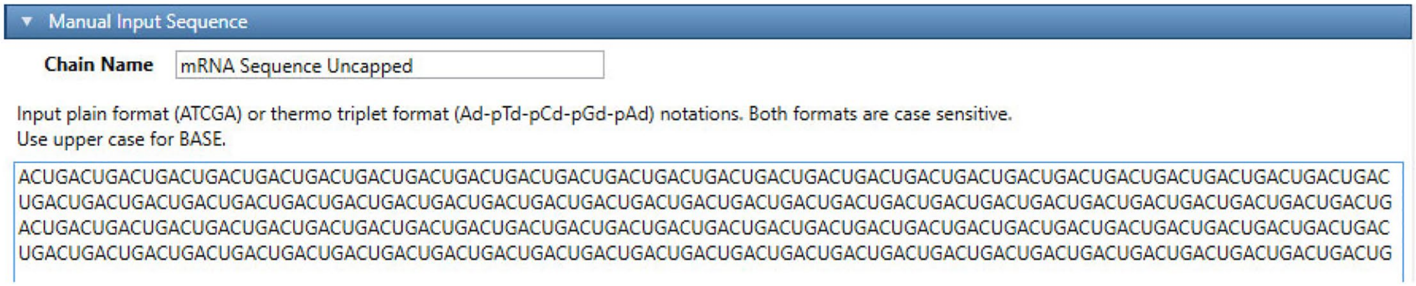


Figure 3. Representative mRNA sequence entered in the Manual Input Sequence panel in Sequence Manager. Up to 10 sequences can be created for a single experiment, each one differentiated by its "Chain Name". Mass and formula values are populated in the Target Oligonucleotide section.

After the sequence has been created, a 5' cap structure is created under the *Building Block and Variable Modification Editor.* From the Subunit dropdown, **5' terminal** is chosen, it is named, and its formula is added (Figure 4). This automatically populates the Monoisotopic and Average mass boxes. Modifications for 5', 3', nucleobase and linker are all created under this panel.

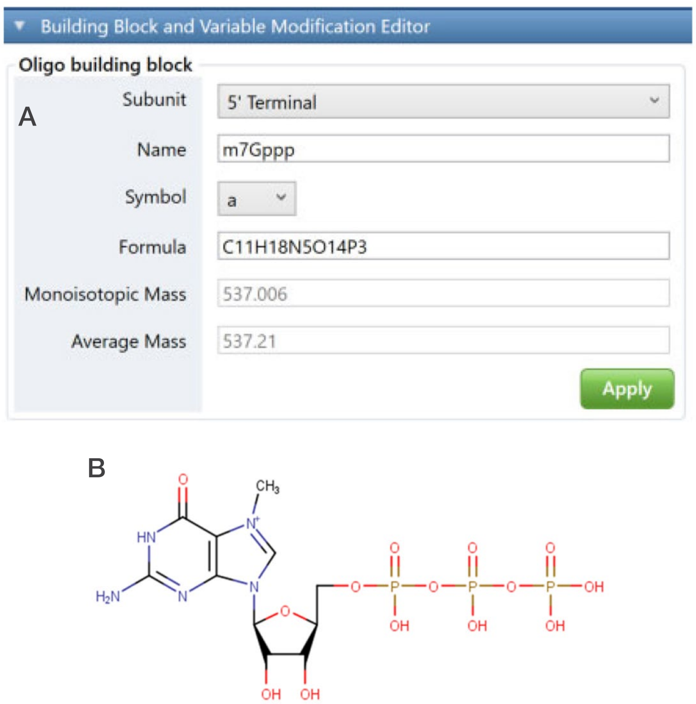


Figure 4. Building Block and Variable Modification Editor (A) is used to create the 5' cap structure and added as a molecular formula that can be generated through a chemical drawing (B). For a 5' capping characterization, the modification is added to the 5' Terminal option under the Subunit dropdown.

After creation of the 5' terminal modification, the *Edit Sequence* panel is accessed (Figure 5). It is from this panel that any nucleotide in the sequence can be modified, using either the default modifications in the dropdown menus, or the user-created modifications generated in the *Building Block and Variable Modification Editor.* Any change to the sequence in the edit sequence panel is reflected in the *Oligo Sequence Map.* For example, addition of the m7G cap structure generated in the *Building Block* editor to the 5' terminal of our mRNA sequence is reflected as an addition of a lowercase (a) to the 5' end of the mRNA sequence (Figure 2, blue arrow). The lowercase (a) is a user-defined symbol chosen when the modification is created.

Once the sequence(s) are created and saved, the sequence manager is closed, and the Oligonucleotide Analysis option is chosen from the home screen. The processing analysis is named, and the data file is chosen. Next the oligonucleotide sequence that was just created is chosen along with Basic Default Method. Depending on chromatographic and digestion conditions, parameters such as signal to noise, peak width, etc. are defined as well as nuclease. For this method, a "Nonspecific" nuclease is chosen with no 3' phosphate. The method is then saved under a new name and the data processed.

The identification and mapping of mRNA 5' cap oligonucleotides were performed in the Oligonucleotide Analysis workflow within BioPharma Finder software as previously described.[18] Here the Basic Default method was chosen with minor modifications. The main identification parameters include the following: Under Component Detection, "Find All Masses in the Run" with a S/N of 200 and an MS noise level of 20K. Under Identification, Search by Full MS Only was set to "No", with Use MS/MS = Use All MS/MS. Because we are only looking for the 5' end, we used a maximum oligonucleotide mass of 10K, slightly more than the mass of the probe, with Mass Accuracy = 5 ppm, and Minimum Confidence = 0.80. Most importantly, under *Select Ribonuclease,* the RNase option "Nonspecific" was chosen with a custom specificity of A, G, or T. Finally, the Phosphate Location was set to "None" with High Specificity.

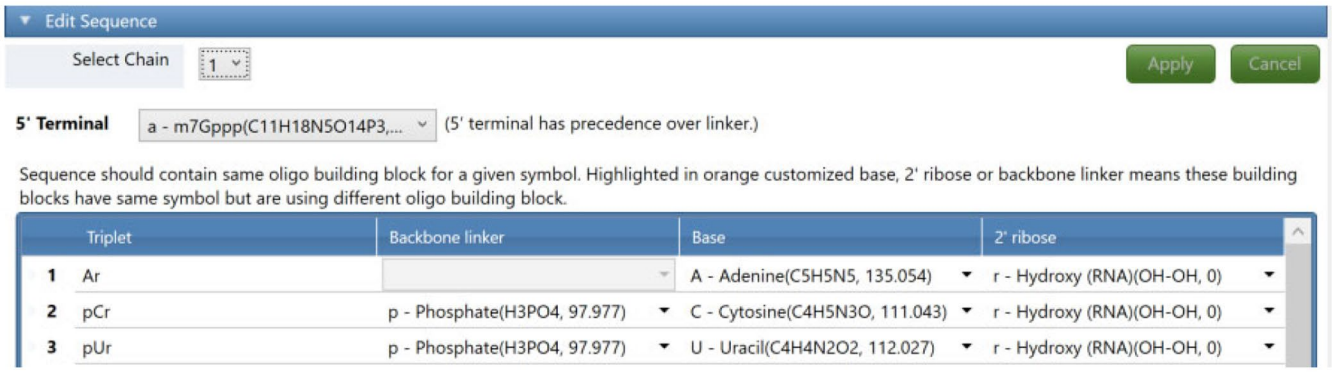


Figure 5. *Edit Sequence* panel. Each individual nucleotide in the individual sequences can be customized. For the 5' capping, the 5' terminal dropdown menu is accessed and the cap structure that we created under building blocks is added to the sequence. Addition of the 5' terminal cap, once applied, is then added as a separate character in the oligo sequence map (blue arrow, Figure 2).

Results and discussion

UHPLC-HRAM MS analysis of mRNA RNase H digestion products

Characterization of the mRNA 5' cap is accomplished through the application of the enzyme RNase H.[17] RNase H is a naturally occurring enzyme that has a role in DNA repair,[19] functioning as an endonuclease to cleave the RNA strand in a DNA:RNA duplex. The enzyme's specificity for chimeric duplexes is exploited for 5' cap characterization. This is accomplished by designing a biotinylated probe that contains a minimum of 5 DNA nucleotides in its sequence and is complementary to the 5' end of the mRNA. When annealed to the mRNA, a DNA:RNA duplex is created, resulting in a cleavage motif for RNase H.

In this study, we performed the IP-RP LC-MS/MS analysis using an RNase H digestion on a commercially available capped mRNA. To enhance the contrast between capped and decapped detection while highlighting the accuracy and robustness of the relative quantification, an aliquot of the mRNA was subjected to a 1 hr pyrophosphohydrolase digestion to remove the m7G, leaving only a single phosphorylation at the first nucleotide of the mRNA transcript. This aliquot was then mixed with three aliquots of capped mRNA and subjected to the digestion protocol. Figure 6 shows extracted ion chromatograms for both capped and decapped oligonucleotides from the RNase H digest. Co-elution was observed for the two products and was not unexpected given the difference between the two digestion

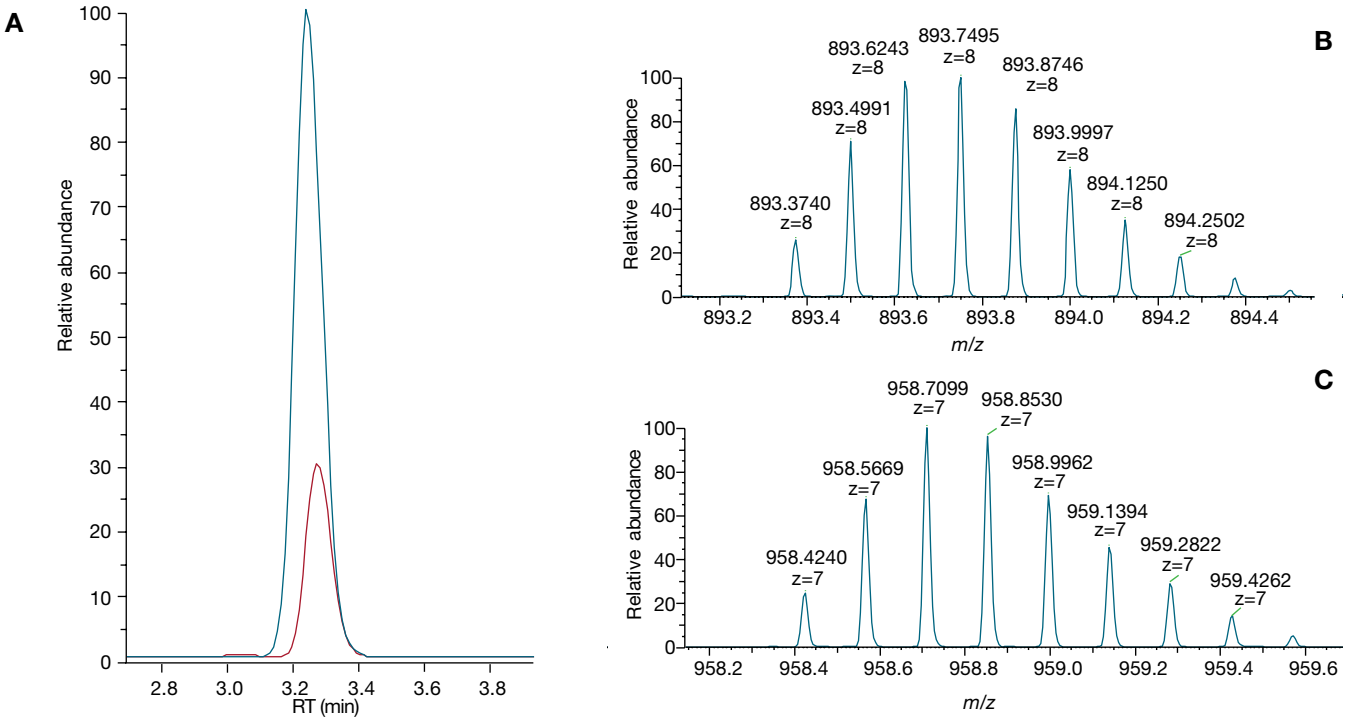


Figure 6. LC-MS data from RNase H digestion. (A) Overlay of extracted ion chromatograms for both capped and decapped oligonucleotides. Ratio of decapped (red trace) to capped (blue trace) was determined to be within 4% of the theoretical value. (B) Isotopic peaks for the most abundant charge state (8th) in the cropped oligonucleotide detected in the sample. (C) Isotopic peak envelope for the most abundant charge state (7th) in the decapped digestion products detected in the sample. The top five peaks were averaged to generate the extracted ion chromatograms used to measure abundance. Chromatogram and spectra were generated in Freestyle software 1.8.2.

products is a single GDP. Extracted ion chromatograms were generated using a mass range from the top 5 isotopic peaks of the most abundant charge state (8th and 7th, respectively) in both capped (893.4994–893.9995 Da) and decapped (958.5666–959.1395 Da) at a mass tolerance of 5 ppm.

Using the same strategy, the top three charge state areas from both capped and decapped were averaged and compared (Figure 7). Comparison of capped to decapped peak area returned a ratio of 24%, within 4% variance from the 4:1 molar ratio of starting material.

Due to the nature of the magnetic bead purification, residual metal contamination was observed at greater than 30% for the capped species (Figure 7). Sodium adduction reduces sensitivity in detection through suppression of the negative charge sites along the oligonucleotide. For these experiments, we reduced the recommended amount of sodium in the wash buffer (1 M) prior to elution from the beads yet kept the concentration high enough (100 mM) to not interfere with the Tm of the probe.

Challenges with manual interpretation of RNase H digestions can arise due to the nature of the experiment. Probe design, annealing conditions, and digestion times are critical for successful digestion. Equally important is analysis of the data post digestion. Historically, LC-MS/MS oligonucleotide data was interpreted manually using online calculators or academic software. Oligonucleotide sequences were generated in silico and the calculated masses were searched for in the raw data files, a time consuming and tedious process that can result in annotation errors. For example, failure sequences during probe synthesis are carried over into the final product used in digestion. These truncated oligonucleotides will complicate manual data interpretation. By adding the probe sequence into our workflow, BioPharma Finder software will report detection of these contaminates. Figure 8 shows the BioPharma Finder software mapping result of a 5' cap RNase H digestion. Red and green highlights at 3.24 min are the capped and decapped 5' ends of the mRNA, the large peak at 6.12, highlighted in blue, is the intact molecular probe used in the experiment. All other blue highlighted areas are detected oligonucleotides matching failure sequences. The peak at 4.73 was identified as the biotinylated first nucleotide in the probe. All detected masses are tabulated with sequence assignments annotated, listing modification, monoisotopic mass, peak area, etc. (Table 2).

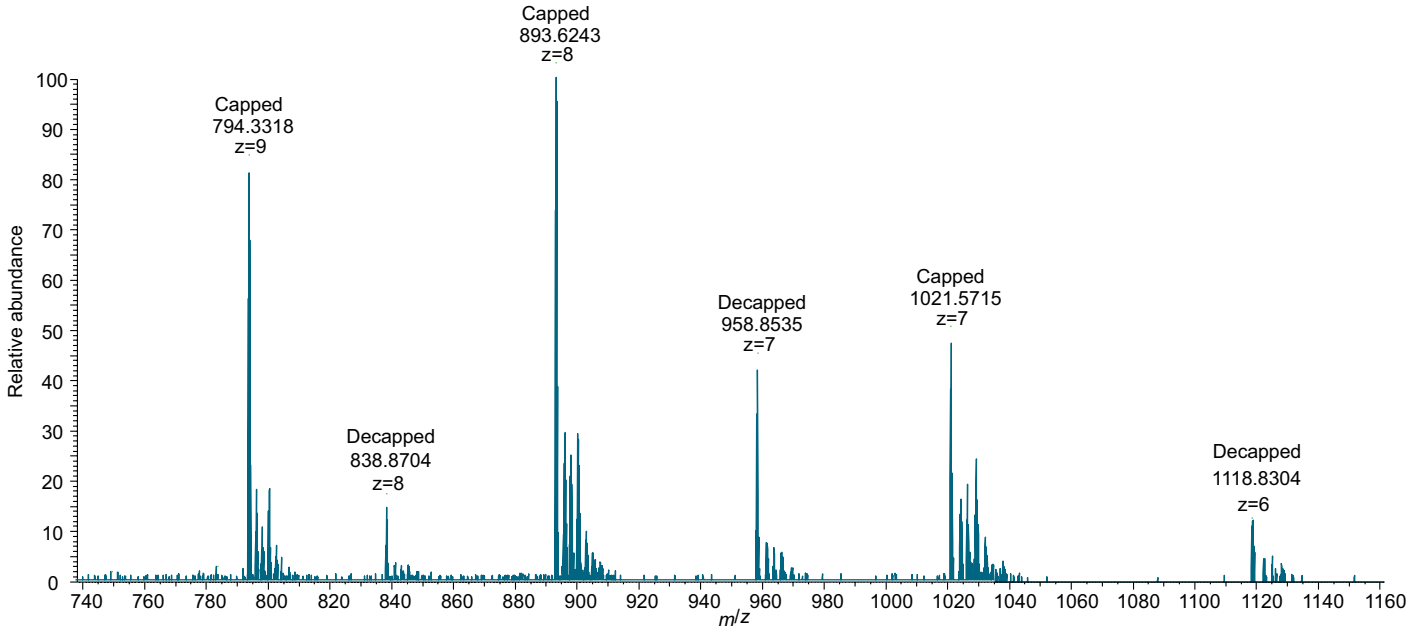


Figure 7. Mass spectrum of top three charge states from capped and decapped 5' oligonucleotides generated through an RNase H enzymatic digestion. Spectra were generated in Freestyle software 1.8.2.

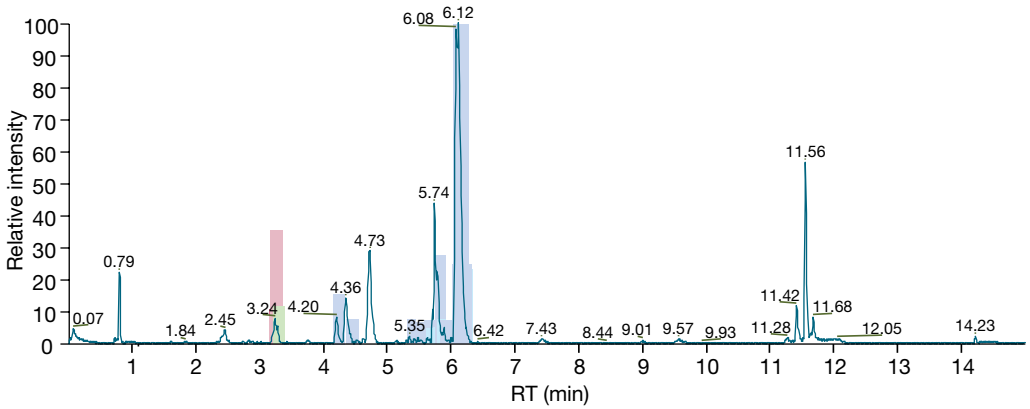


Figure 8. BioPharma Finder software 5.0 chromatogram of RNase H digestion products. Blue highlighted peaks are mapped failure sequences of the probe, as well as the probe itself (6.12 min).

Table 2. BioPharma Finder software results table for identified features in the processed data file

Identification	Oligo Sequence	Mod	Site	Δ ppm	Conf. Score	ID Type	RT	M/Z	Charge St.	Mono Mass Exp.	Theor. Mass	Oligo	MS Area
3:A1-U25 = 8452.478m	Am-pCm-pUm-pCm-pT...	(3' Biotin-TEG)	(U25)	0.46	100.0	MS2	6.10	767.672	-11	8452.4814	8452.4776	Probe	1,437,068,160.00
1:A1-A20 = 7155.0585m	Am-pGr-pGr-pAr-pAr-p...	(m7Gppp)	(A1)	-0.88	100.0	MS2	3.24	893.749	-8	7155.0522	7155.0585	Capped	69,959,344.00
3:A1-U25 = 8452.478m(Na+)	Am-pCm-pUm-pCm-pT...	Na+, (3' Biotin-TEG)	(U25)	-1.10	100.0	MS2	6.11	769.669	-11	8474.4502	8474.4595	Probe	54,028,096.00
3:A1-U25 = 8452.478m(Na+)	Am-pCm-pUm-pCm-pT...	Na+, (3' Biotin-TEG)	(U25)	-4.21	100.0	MS2	6.09	846.736	-10	8474.4238	8474.4595	Probe	32,876,560.00
3:U10-U25 = 5646.0240m	pUm-pUm-pCm-pUm-p...	(3' Biotin-TEG)	(U25)	-1.58	100.0	MS2	5.79	704.995	-8	5646.0151	5646.0240	Probe	19,019,854.00
1:A1-A20 = 7155.0585m(Na+)	Am-pGr-pGr-pAr-pAr-p...	Na+, (m7Gppp)	(A1)	-1.49	99.9	MS2	3.26	896.496	-8	7177.0298	7177.0405	Capped	19,015,378.00
3:A1-U25 = 8452.478m	Am-pCm-pUm-pCm-pT...	(3' Biotin-TEG)	(U25)	-0.70	100.0	MS2	6.07	1690.288	-5	8452.4717	8452.4776	Probe	17,176,314.00
2:A1-A20 = 6716.0294m	Am-pGr-pGr-pAr-pAr-p...	(Phosphate)	(A1)	-1.77	100.0	MS2	3.26	958.710	-7	6716.0176	6716.0294	Uncapped	11,341,699.00

Relative quantification is easily determined from annotated peak areas. MS/MS fragment identification of the highest charge state in the capped oligonucleotide is shown in Figure 9. In addition to identification by full-scan analysis and the mass deconvolution of multiple charge states, fragmentation identification provides the confirmation of sequence order as well as information pertaining to potential modifications and the modification site within the sequence. MS/MS fragmentation identification and annotation are automatically generated and color coded by the data analysis software.

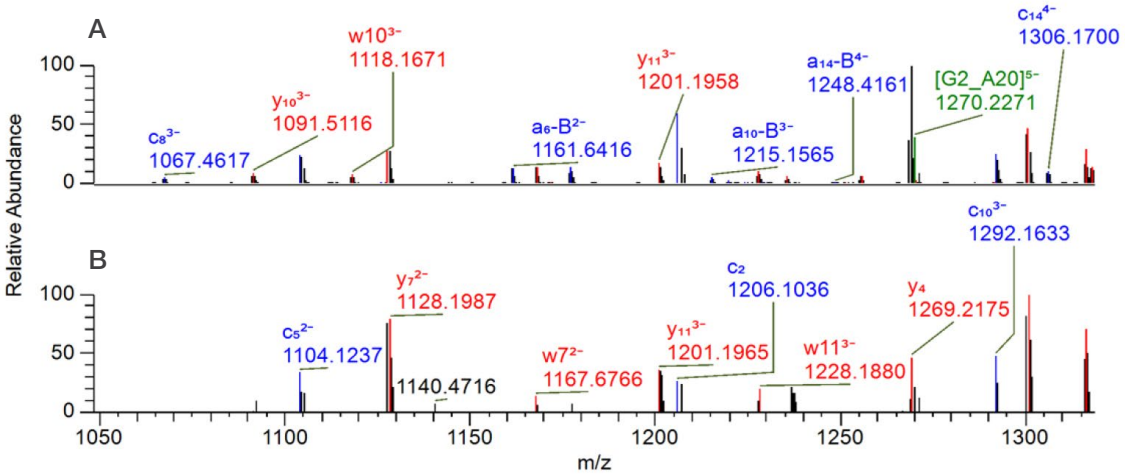


Figure 9. Zoomed view of tandem mass spectra of an RNase H digestion product. (A) Theoretical fragmentation pattern. (B) Actual fragmentation. Color-coded annotated MS/MS spectrum is automatically generated for each oligonucleotide identification. Matched spectra lend confidence to the identification.

MS/MS fragmentation identification is also evaluated using the fragment coverage map tool, which provides a visual representation of all assigned MS/MS fragmentation and is color coded by intensity for each digestion fragment (Figure 10). Visualization of the fragmentation ladder provides a quick means of optimization of fragmentation energies during method development. For this experiment, activation energy was optimized using the molecular probe where multiple injections of increasing energy were acquired and processed using BioPharma Finder software as previously reported.[18] Mapped sequences are visualized and reported based on recovered peak area. For the capping analysis, the mapped 5' capped end of both capped and decapped mapped digestion products are visually represented in Figure 11. Mapped products are represented based on parameters set in the processing method.

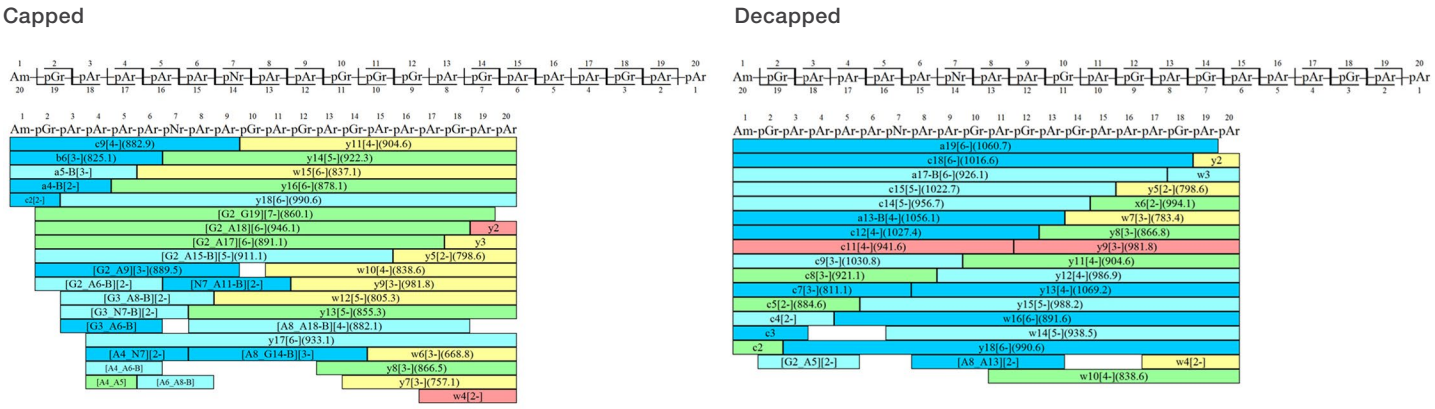


Figure 10. MS/MS fragmentation identification maps for both capped and decapped oligonucleotides produced by RNase H enzymatic digestion

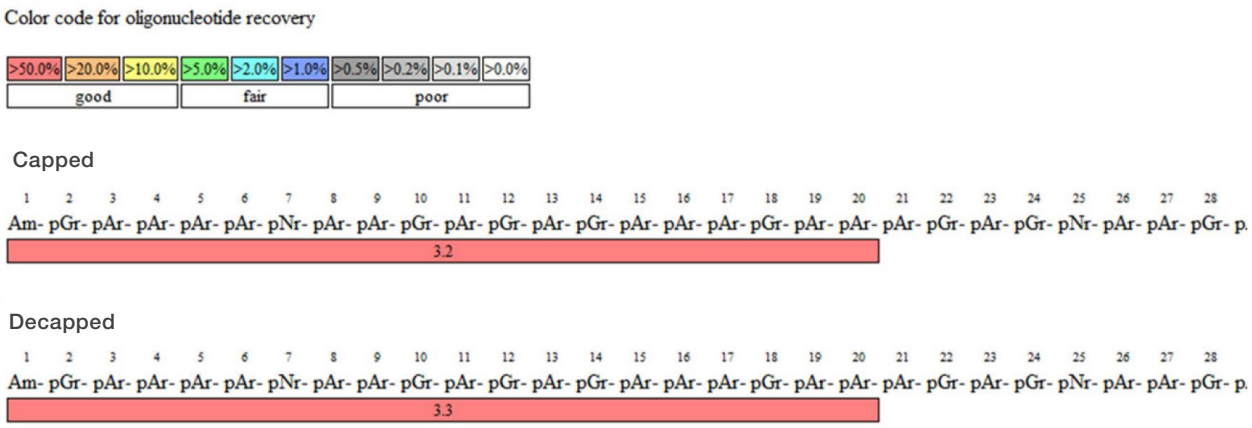


Figure 11. BioPharma Finder software mapping results of 5' end of a commercially available mRNA

Conclusion

The Orbitrap Exploris 240 mass spectrometer, coupled to the Vanquish Horizon UHPLC system, utilizing the DNAPac RP column for separation, and BioPharma Finder software for data processing yields a robust and powerful analytical platform for detection, characterization, and relative quantitation of mRNA 5' cap oligonucleotides.

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