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Note d'application

LC-MS Analysis of Two mRNA Critical Quality Attributes: 5′-Capping Efficiency and Average Poly(A) Tail Length and Heterogeneity Using the Xevo™ MRT Mass Spectrometer

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Main

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Abstract

An optimized LC-MS and informatics workflow was developed to analyze two critical mRNA quality attributes: Capping Efficiency and Average Poly(A) Tail Length and Heterogeneity. The workflow, enabled by the Xevo™ MRT Mass Spectrometer and three waters_connect™ Apps - SYNTHETIC Library, INTACT Mass and MAP Sequence, allowed rapid and accurate identification and relative quantification of 5′-Cap structures and Poly(A)

Tail variants from mRNA digests.

Benefits

- A single LC-MS assay was developed on the Xevo MRT Mass Spectrometer to measure the Capping Efficiency and the Average Poly(A) Tail Length & Heterogeneity of an mRNA molecule (Fluc mRNA, ~ 1,900 nt)
- waters_connect application software - SYNTHETIC Library, INTACT Mass and MAP Sequence) serve as powerful data analysis tools that streamline the LC-MS data processing of digested mRNA, significantly reducing manual data analysis for mRNA CQA analysis.

Waters Solutions

- Xevo MRT MS (multi-reflecting time-of-flight) Mass Spectrometer
- ACQUITY™ Premier UPLC™ System
- ACQUITY Premier Oligonucleotide BEH C18 Column
- waters_connect Informatics
- INTACT Mass App v1.9
- SYNTHETIC Library App v2.0
- MAP Sequence App v2.0

Keywords

oligonucleotides / oligo sequencing / RNA / mRNA / RNA digestion enzymes / Xevo MRT MS / waters_connect / INTACT Mass / SYNTHETIC Library / MAP Sequence

Introduction

The recent development and regulatory approval of several COVID mRNA-based vaccines, and emerging opportunities for mRNA-based therapeutics, have brought the mRNA molecules to the forefront of the biopharma industry[1-3]. The resulting need for rapid product development has necessitated development of analytics for the

precise characterization of several mRNA critical quality attributes (CQAs), including 5′-Capping Efficiency, Average 3′-Poly(A) Tail Length and Heterogeneity and Sequence Integrity.

Several iterative workflows have previously been developed at Waters Corporation for the direct assessment of the Capping Efficiency and Average Poly(A) Tail Length CQAs, starting with the BioAccord and Xevo G3 Q-Tof mass spectrometry systems in combination with the INTACT Mass waters_connect App, and progressively refined to improve performance and expand capabilities. However, these earlier workflows require two separate LC-MS analysis methods for each of the CQAs[4-6].

A single LC-MS assay simultaneously quantified two pivotal CQAs – 5′-Capping Efficiency and Average Poly(A) Tail Length/Heterogeneity of Fluc mRNA – accelerating method development, reducing runtime and lowering costs. This assay improved the mass accuracy for both CQAs, taking advantage of the exquisite sensitivity, mass resolution and mass accuracy of the Xevo MRT mass spectrometer.

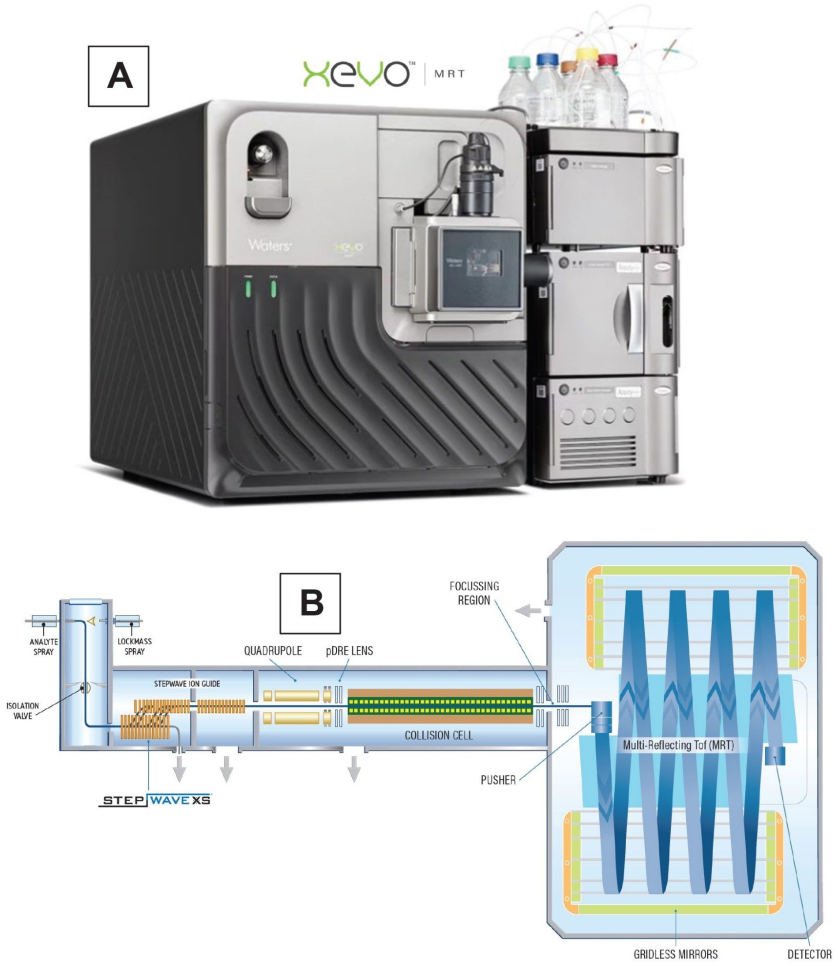


Figure 1. (A) Xevo MRT QTOFMass Spectrometer with the ACQUITY™ Premier System; (B) Schematic diagram of the Xevo MRT QTOF Mass Spectrometer.

Experimental

Reagents and Sample Preparation

Normal-dipropylamine (DPA, 99.0% purity, catalog number D214752-500ML) was purchased from Millipore Sigma (St Louis, MO) and 1,1,1,3,3,3-hexafluoro-2-propanol (IonHance HFIP, P/N 186010781 <

https://www.waters.com/nextgen/global/shop/standards--reagents/186010781-ionhance-hfip-10-ml.html>) was obtained from Waters (Milford, MA). Methanol (LC-MS grade, catalog number 34966-1L) and acetonitrile (LC-MS grade, catalog number 34967-6XL) were obtained from Honeywell (Charlotte, NC). 5M RNase-free ammonium acetate (catalog no AM9071-500ML) was purchased from Thermo Fisher (Waltham, MA). HPLC grade Type I deionized (DI) water was purified using a Milli-Q system (Millipore, Bedford, MA). Mobile phases were prepared fresh daily. Ultrapure nuclease-free water (catalog number J71786.AE) for mRNA digestions was purchased from Thermo Fisher Scientific (Waltham, MA).

An mRNA construct based on the firefly luciferase (Fluc) sequence was custom-made via IVT (in vitro transcription) synthesis by GenScript (GS, Piscataway, NJ). The GS Fluc mRNA molecule was synthesized with a Cap-1 structure, followed by 1813 nucleotides and a Poly(A) Tail sequence.

Fungus-derived animal free purified ribonuclease T1 (catalogue no IFGRNASET1AFLY500KU) was ordered from Innovative Research (Novi, MI) and the lyophilized enzyme was dissolved in 5 mL of 100 mM ammonium bicarbonate (catalogue no 5.33005-50G, Millipore Sigma) to prepare a solution containing 100 units/µL. For mRNA digestion with RNase T1, 20 µL of Fluc mRNA (1.4mg/mL) were mixed with 10 µL of nuclease-free water in an Eppendorf PCR vial and denatured for 2 minutes at 90ºC. 10 µL of RNase T1 enzyme (1,000 units) were added to the vial and the digestion was allowed to proceed at 37ºC for 15 minutes. The digestion mixture was transferred to a Quan Recovery MaxPeak 300 µL vial for LC-MS analysis. The digest was analyzed immediately by LC-MS using 5 µL injections.

The hRNAse4 enzyme (catalogue no M1284S, 2500 units) was purchased from New England Biolabs (Ipswich, MA). 20 µL of Fluc mRNA were denatured following the same procedure described for RNase T1 digestion, then 10 µL (500 units) of hRNase4 were added and the mRNA sample was digested for 90 minutes at 37ºC.

All UPLC-MS^E datasets (data independent analysis - DIA) were acquired with waters_connect (v4.1.0.17) and subsequently processed using the waters_connect SYNTHETIC Library App (v 2.0) INTACT Mass App (v 1.9) and the MAP Sequence App (v2.0).

LC-MS System:

System:	Xevo MRT (multi-reflecting time-of-flight) Mass Spectrometer coupled with ACQUITY Premier UPLC (Binary) System

LC Conditions:

Column:	ACQUITY Premier Oligonucleotide BEH™ C_{18} Column 130 Å, 1.7 µm, 2.1 x 150 mm (p/n: 186009486)
Column temperature:	60 °C
Flow rate:	300 µL/min
Mobile phases:	Solvent A: 10 mM DPA (n-dipropylamine), 40 mM HFIP (1,1,1,3,3,3-hexafluoroisopropanol) in DI water, pH 8.6 Solvent B: 10 DPA, 40 mM HFIP in 50% methanol

Gradient Table

Time (min)	Flow rate (mL/min)	Solvent A composition (%)	Solvent B composition (%)	Curve profile
0.00	0.3	100	0	Initial
1.00	0.3	100	0	6
91.00	0.3	50	50	6
91.20	0.3	15	85	6
94.20	0.3	15	85	6
95.00	0.3	100	0	6
105.00	0.3	100	0	6

Sample temperature:	8 °C
Sample vials:	QuanRecovery MaxPeak HPS Vials (p/n:

	186009186)
Injection volume:	5 µL

Wash solvents:

Purge solvent:	10% methanol in DI water
Sample manager wash solvent:	50% MeOH
Seal wash:	10% methanol in DI water

MS Conditions:

MS system:	Xevo MRT QTOF Mass Spectrometer
Ionization mode:	ESI(-)
Acquisition mode:	MS^E
Acquisition rate:	2 Hz
Capillary voltage:	1.5 kV
Cone voltage:	40 V
Source offset:	10 V
Source temperature:	120 ºC
Desolvation temperature:	550 ºC

Cone gas flow:	0 L/h
Desolvation gas flow:	1000 L/hr
TOF mass range:	50 – 4000 (MS^E acquisition)
Low energy CE:	6 V
High energy CE ramp:	15 – 35 V
Lock-mass:	50 pg/µL Leu Enk in 0.1% formic acid, 50% CAN
Data acquisition:	waters_connect
Data processing:	INTACT Mass App Synthetic Library App MAP Sequence App

Results and Discussion

CQA1: Capping Efficiency Measurement Using the MAP Sequence App

Several examples of mRNA Capping Efficiency assays have previously been reported in application notes published by Waters[4–6]. The mRNA cap is added to the 5′ end of an mRNA molecule and typically consists of a fully matured 7N-methyl guanosine linked to the mRNA through a 5′–5′ triphosphate ester bond (m7Gppp). The SYNTHETIC Library App was first utilized to predict the 5′-end uncapped digestion product generated from hRNase4 digestion of Fluc mRNA, corresponding to a 7-mer nucleotide with the sequence GGG AAA U cP. For the GenScript (GS) Fluc mRNA, the intended Cap-1 structure incorporated at the 5′ end includes a 2′-O-methylated adenosine residue attached to the mRNA, resulting in the expected Cap-1 structure denoted as m7GpppAm. Interestingly, analysis using the MAP Sequence App (sequencing software), also identified a second Cap-1 structure with the sequence m7GpppGm from the hRNase4 digested mRNA sample. These two Cap-1

structures were defined as individual nucleotides in the MAP Sequence App, added to the default Cap structure (m7Gppp). Additionally, the second nucleotide in each Cap-1 sequence (Am or Gm) was custom-defined in the SYNTHETIC Library App, as shown by the two library entries presented in Figure 2.

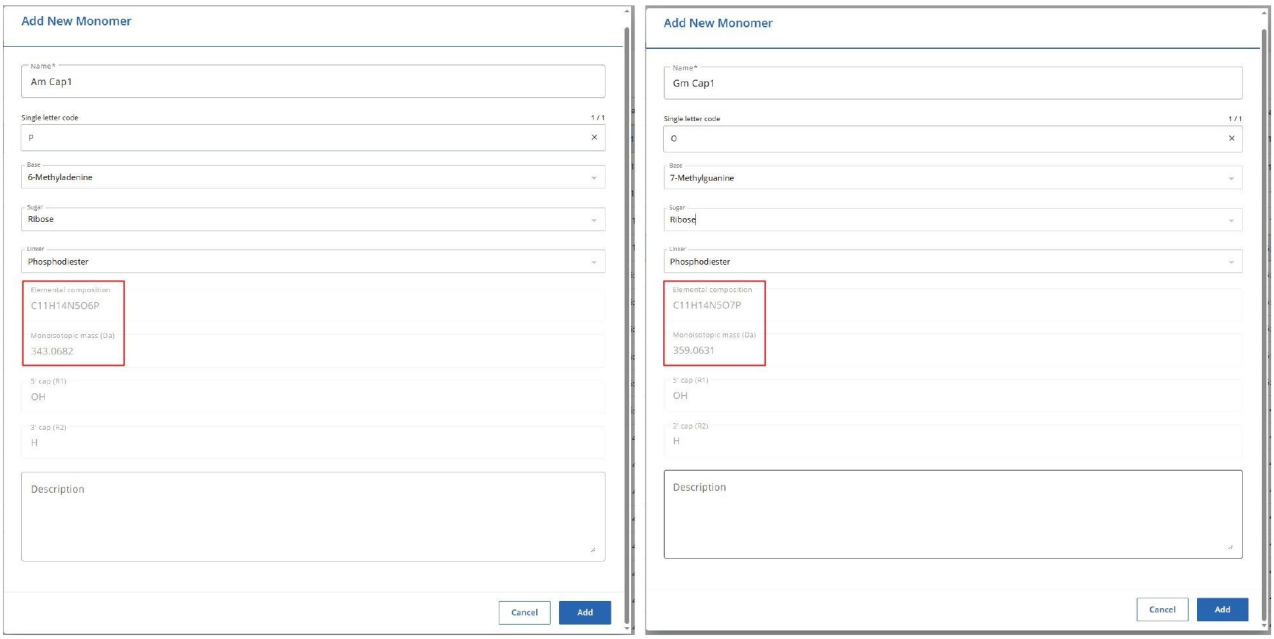


Figure 2. SYNTHETIC Library entries showing two single nucleotide components of the Cap-1 structures (Am and Gm) detected in the Fluc mRNA digest. Each of these nucleotides is an individual component of the two 9-mer digestion products m7GpppAm GGG AAA U cP and m7GpppGm GGG AAA U cP, produced from the 5′-end of Fluc mRNA, upon hRNase4 digestion.

Both 5′-capped Fluc mRNA digestion products were identified in the hRNase4 digest, as demonstrated by the extracted ion chromatograms (XICs) presented in Figure 3. These Cap-1-modified 9-mer digestion products, corresponding to the sequences m7GpppAm GGG AAA U cP and m7GpppGm GGG AAA U cP, were successfully separated chromatographically. Their identities were further confirmed by the isotopic distributions shown in the two spectra from Figure 4, recorded for the most abundant detected charge state, $[M-2H]^{2-}$. High mass resolution (~100,000) achieved on the Xevo MRT mass spectrometer enabled measurement of the corresponding monoisotopic peaks with high mass accuracy, as illustrated by the MAP Sequence App screenshot displayed in figure 4A. The uncapped 7-mer digestion product, GGG AAA U cP (m/z = 1163.13, −2), was not detected in the hRNase4 digest.

Furthermore, data analysis using the MAP Sequence App[7] did not reveal any Cap-related impurities, including truncations of Cap-0, Cap-1, or Cap-2 structures, indicating a capping efficiency of 100% for GS Fluc mRNA. This outcome is consistent with the post-IVT dephosphorylation treatment applied to the GS Fluc mRNA, which enzymatically degrades uncapped mRNA transcripts into single nucleotides.

For Fluc mRNA, RNase T1 is unsuitable for evaluating capping efficiency because the 5′-end sequence contains three guanosine residues, which serve as cleavage sites for this enzyme. As a result, RNase T1 digestion produces very short oligonucleotides for the 5′-end of Fluc mRNA. Under the IP-RP LC-MS assay conditions employed, sufficient chromatographic retention cannot be achieved for such short species (mono-, di-, or trinucleotides), preventing detection of the uncapped RNase T1 digestion products.

Quantitative analysis based on the integrated peak areas of the two Cap-1 variants (see the peak areas reported in Figure 3), indicated that 62.7% of the Fluc mRNA population contains the intended m7GpppAm Cap-1 structure, whereas 37.3% carries an alternative m7GpppGm Cap-1 structure. This observation was unexpected, since conventional 5′ mRNA cap structures generally contain either a 2′-O-methylated adenosine (Am) or a 2′-O-methylated guanosine (Gm) as the second Cap-1 nucleotide, but not a mixture of both within the same mRNA preparation. The utility of a single LC-MS assay developed here for measuring the mRNA Capping Efficiency was clearly illustrated in the case-study presented above.

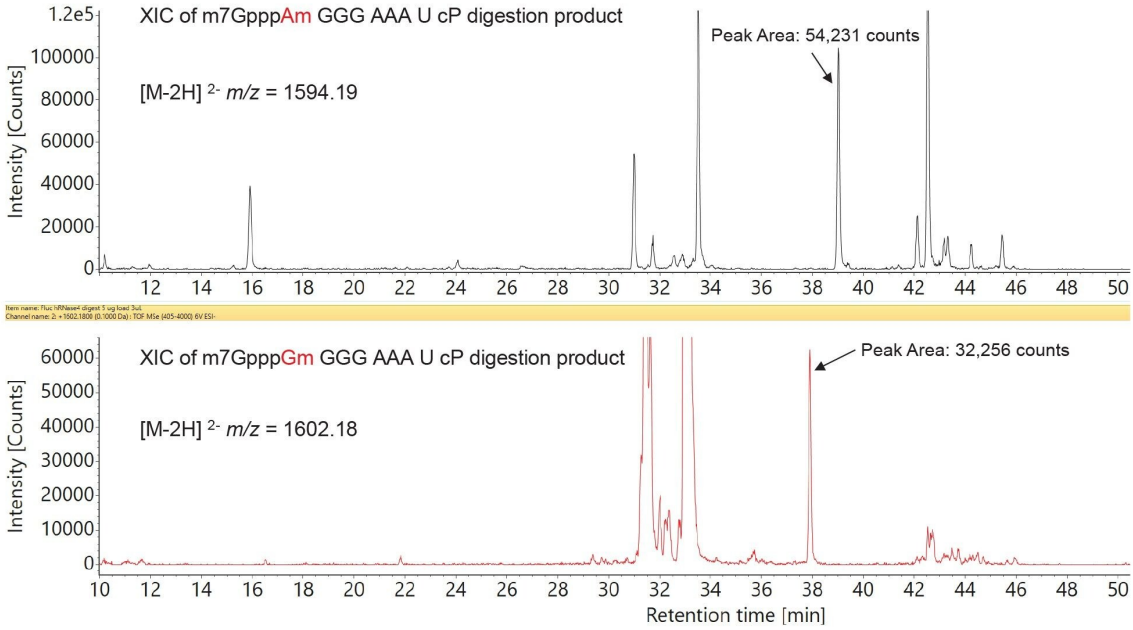
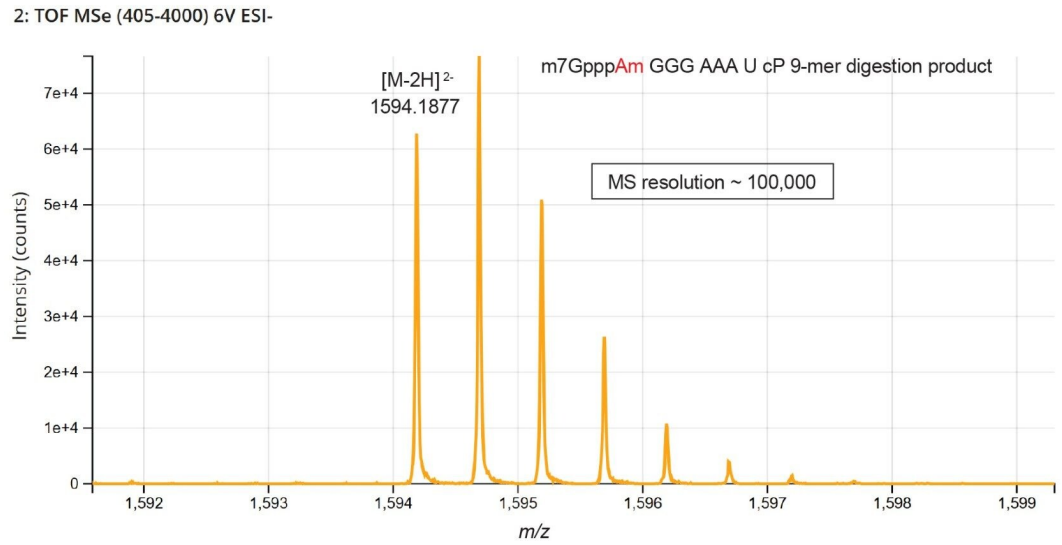


Figure 3. Extracted ion chromatograms corresponding to the doubly charged digestion products containing two Cap-1 structures, m7GpppAm (panel A) and m7GpppGm (panel B), attached to the same 7-mer hRNase4 digestion product with the sequence GGG AAA U cP.

4A

Digestion product	Sequence	Length	Expected mass (Da)	Observed mass (Da)	Response	Mass error (ppm)	Observed RT (min)	Results
m7Gppp(0)Am Cap1(1)G2:U8	GGGAAAU-cP	8	3190.3891	3190.3913	71,471	0.70	35.55	Found



4B

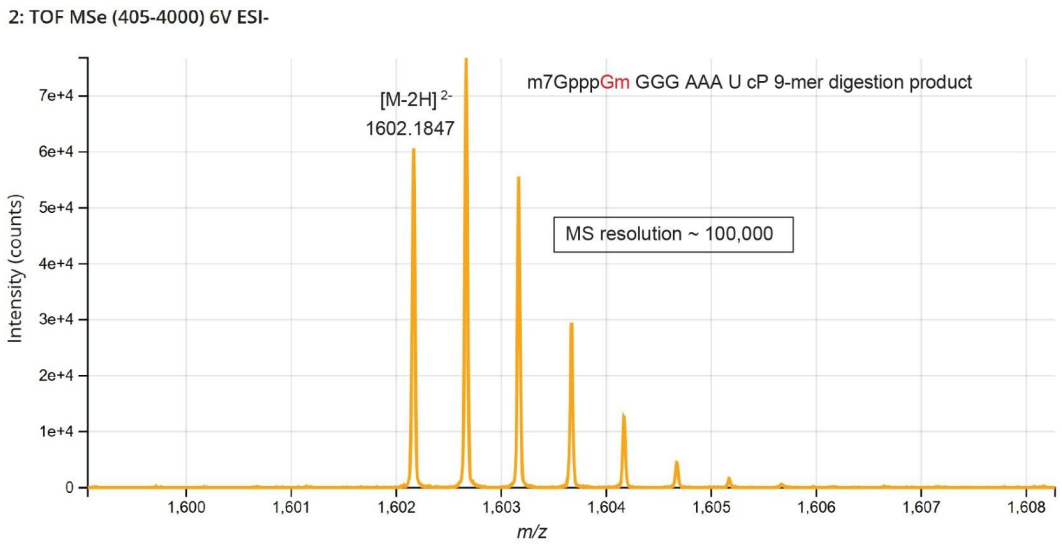


Figure 4. MAP Sequence isotopic distributions of the doubly charged precursors corresponding to two 9-mer Cap-1 digestion products generated following hRNase4 digestion: m7GpppAm GGG AAA U cP (panel A) and m7GpppGm GGG AAA U cP (panel B). Both these Cap-1 structures were detected in the hRNAse4 digest, but the free digestion product (GGG AAA U cP, m/z= 1163.13, -2) was not detected (data not shown), therefore the calculated capping efficiency is 100%.

CQA2: Measurement of the Average Poly(A) Tail Length and Heterogeneity Using the INTACT Mass App

Compared with the Capping Efficiency assays, LC-MS characterization of the various Poly(A) Tail variants presents significantly greater analytical challenges. Because the Poly(A) Tail consists of a substantially longer nucleotide sequence, ionization in negative ESI-MS is significantly less efficient than for the much shorter Cap-related oligonucleotides. Poly(A) Tail oligonucleotides also display great heterogeneity and lower post-digestion stability, making them highly susceptible to enzymatic degradation. Furthermore, the biological mechanisms governing Poly(A) Tail synthesis across different species are not yet fully understood, which complicates accurate prediction of all sequence variants that may arise during the IVT process.[8-11]

A previous report[12] described an isolation approach for the Poly(A) Tail oligonucleotide from a complex mRNA RNase T1 digest using (dT)25 magnetic beads to enrich this fraction prior to LC-MS analysis. In our study, consistent with several other reports,[13,14] direct IP-RP chromatographic separation of the complex RNase T1 digestion mixture (Figure 5) yielded a well-resolved and abundant Poly(A) Tail peak that eluted last in both the UV and TIC chromatograms. The data presented in this figure was recorded for a single LC-MS assay used for measuring the 5′-Capping Efficiency as well as the Poly(A) Tail measurements. Figure 6 presents the combined ESI-MS spectrum acquired for all co-eluting polyadenosine components of the Fluc mRNA Poly(A) Tail generated by RNase T1 digestion. Multiple charge states, ranging from −24 to −36, were observed for the major oligonucleotide species (19.3% relative abundance), identified as a 102-mer with the composition $C(A)_{101}$. Closer inspection of the most intense charge state, $[M-34H]^{34-}$, demonstrated that the Xevo mass spectrometer (at 100,000 mass resolution) was able to resolve the isotopic distribution of this highly charged ion. Charge states corresponding to additional Poly(A) Tail components were similarly well resolved (data now shown). Although the monoisotopic peak corresponding to this high-molecular-weight oligonucleotide was not detected because of its extremely low abundance, as indicated by the blue line in Figure 7, the resolved isotopic distributions nevertheless enabled highly accurate mass measurements for all identified Poly(A) Tail species (see Figure 9) with mass accuracy errors under 10 ppm.

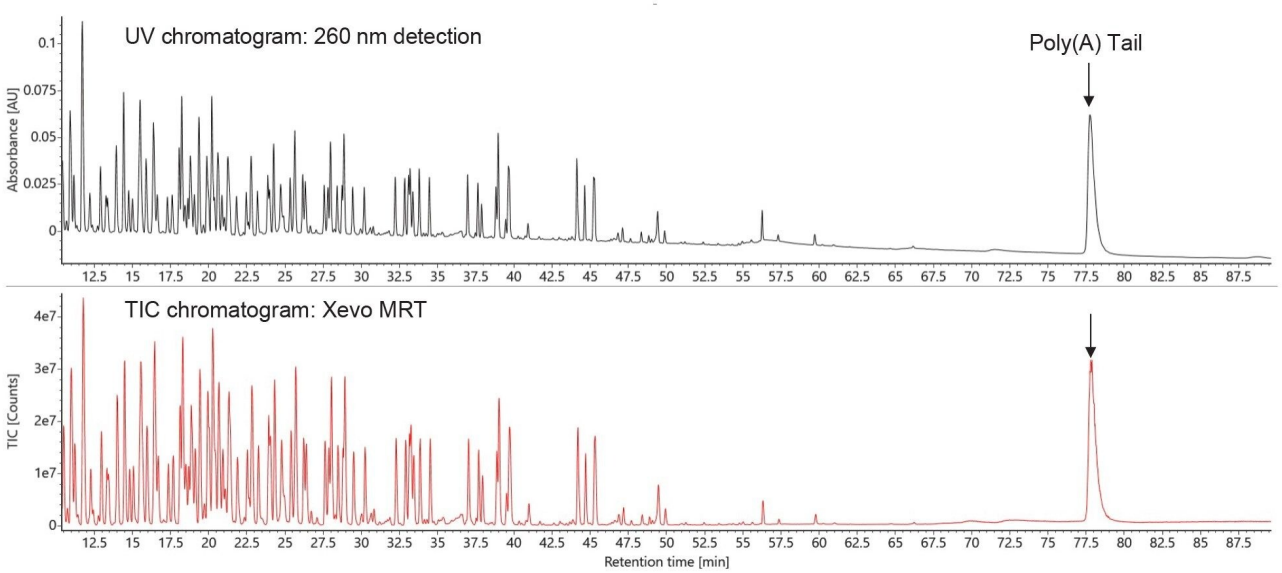


Figure 5. Separation of the RNase T1 digest of Fluc mRNA: (A) UV chromatogram recorded at 260 nm; (B) TIC chromatogram recorded on the Xevo MRT QTOF Mass Spectrometer. All poly(A) tail oligonucleotide species co-elute in a single chromatographic peak with a retention time of 78 minutes, well separated from all the other shorter digestion products.

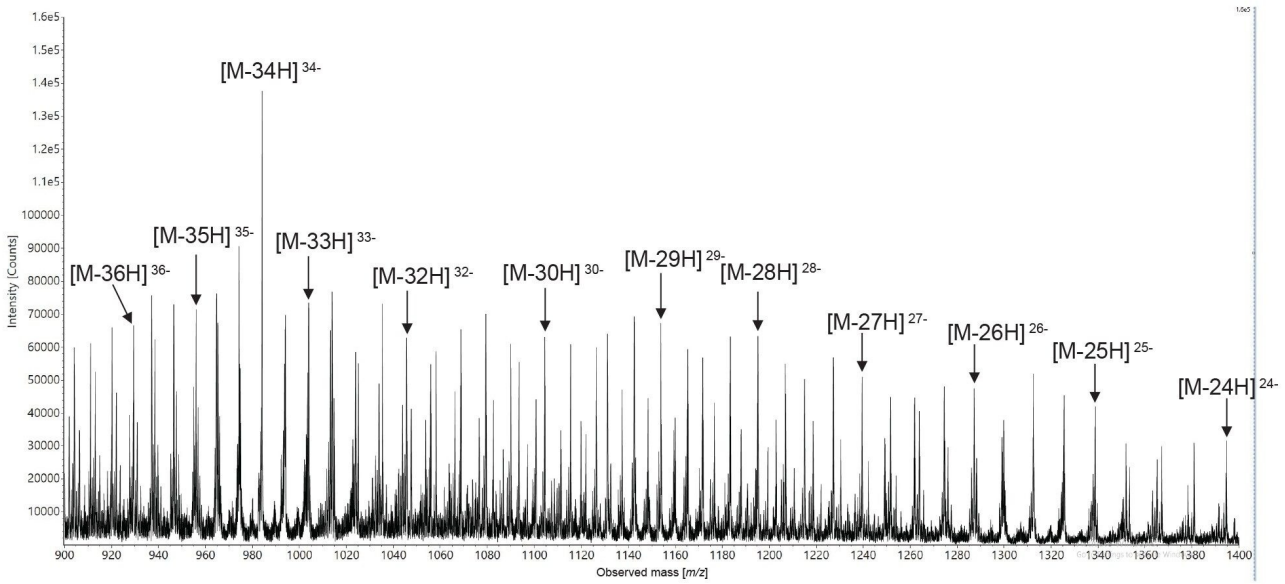


Figure 6. Combined ESI-MS spectrum recorded for all coeluting polyadenosine components of the Fluc mRNA poly(A) tail produced by RNase T1 digestion. Several charge states (in the range of -24 to -36) belonging to the major oligonucleotide component (19.3% relative abundance), a 102-mer with the composition $C(A)_{101}$, are annotated.

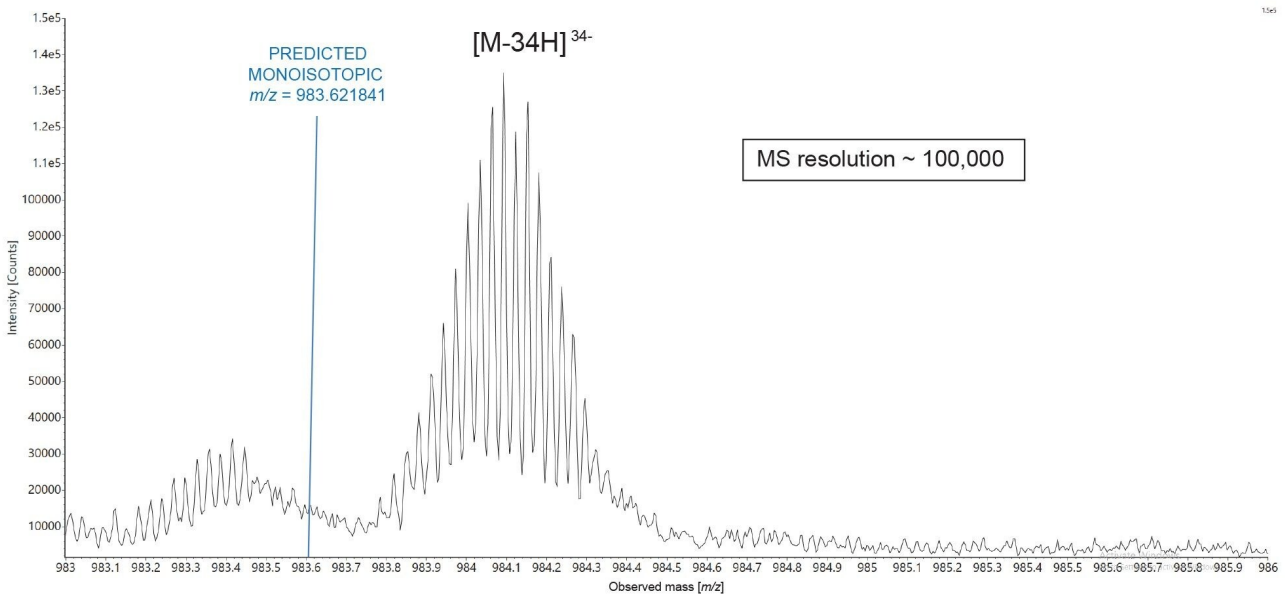


Figure 7. Isotopically resolved $[M-34H]^{34-}$ charge state of the most abundant 102-mer poly(A) tail oligonucleotide with the sequence $C(A)_{101}$, resulted from RNase T1 cleavage of Fluc mRNA. With ~ 100,000 MS resolution, the Xevo MRT QTOF is capable of resolving this highly charged precursor produced by a large (~ 35 kDa MW) oligonucleotide. The corresponding charge states, produced by other poly(A) tail components, were resolved as well, contributing to excellent mass accuracy for measuring all nine species, as illustrated by the INTACT Mass Application screenshot from Figure 8.

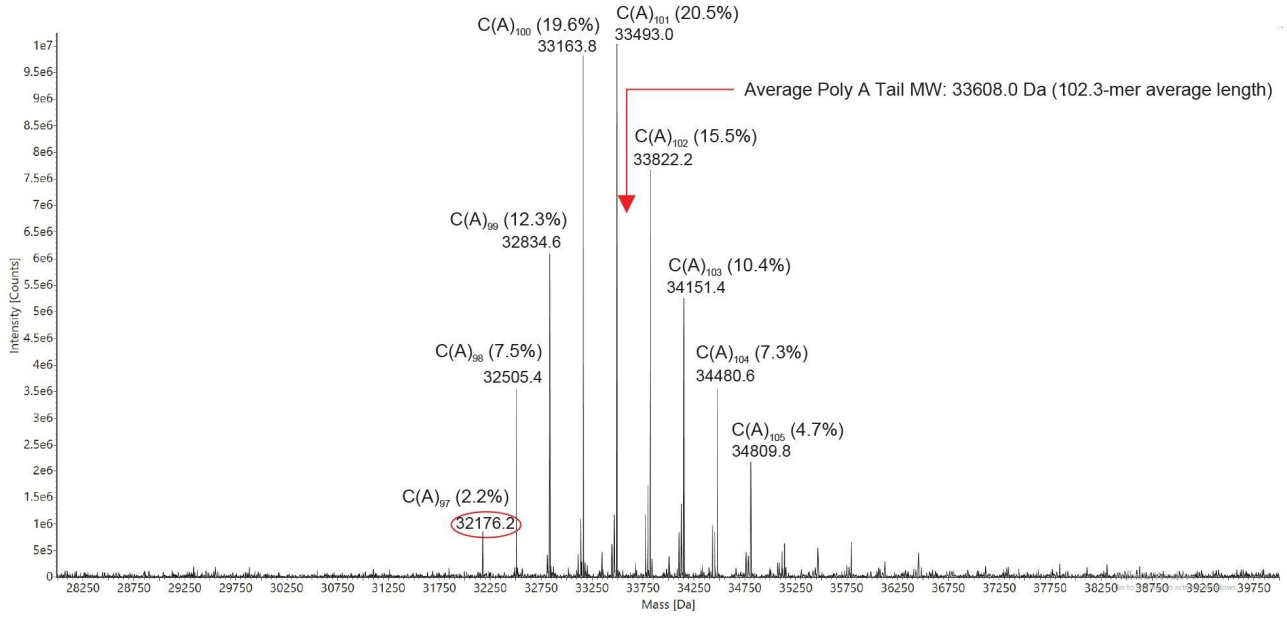
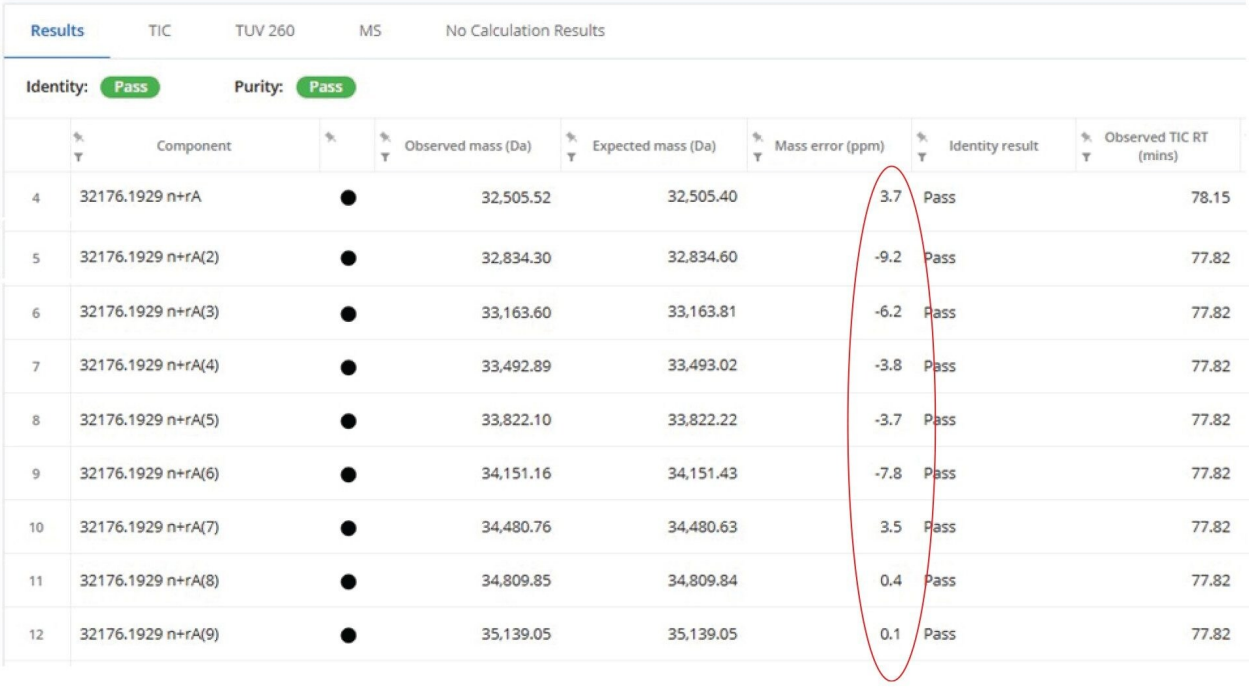


Figure 8. Deconvolved ESI-MS spectrum showing the average molecular weight of the nine poly(A) tail oligonucleotide variants detected and their relative abundances calculated based on the corresponding ESI-MS signal intensities. The calculated average poly(A) tail length was 102.3-mer (33,608.0 Da average MW), after taking into account the relative abundances of each poly(A) species detected above 2% relative abundance.



Results | TIC | TUV 260 | MS | No Calculation Results

Identity: Pass Purity: Pass

	Component		Observed mass (Da)	Expected mass (Da)	Mass error (ppm)	Identity result	Observed TIC RT (mins)
4	32176.1929 n+rA	●	32,505.52	32,505.40	3.7	Pass	78.15
5	32176.1929 n+rA(2)	●	32,834.30	32,834.60	-9.2	Pass	77.82
6	32176.1929 n+rA(3)	●	33,163.60	33,163.81	-6.2	Pass	77.82
7	32176.1929 n+rA(4)	●	33,492.89	33,493.02	-3.8	Pass	77.82
8	32176.1929 n+rA(5)	●	33,822.10	33,822.22	-3.7	Pass	77.82
9	32176.1929 n+rA(6)	●	34,151.16	34,151.43	-7.8	Pass	77.82
10	32176.1929 n+rA(7)	●	34,480.76	34,480.63	3.5	Pass	77.82
11	32176.1929 n+rA(8)	●	34,809.85	34,809.84	0.4	Pass	77.82
12	32176.1929 n+rA(9)	●	35,139.05	35,139.05	0.1	Pass	77.82

Figure 9. Screenshot showing the INTACT Mass App processing results. Very good mass accuracy (< 10 ppm) was obtained on the Xevo MRT QTof for the measurement of all nine detected poly(A) tail species.

The INTACT Mass App was used for automated data processing of the Xevo Poly(A) dataset. The ESI-MS spectrum generated from all coeluting Poly(A) Tail oligonucleotides (shown in Figure 6) was deconvolved using the MaxEnt1 algorithm[15] implemented in INTACT Mass App v1.9[16], and the resulting spectrum is presented in Figure 8. Deconvolution enabled the identification of nine distinct Poly(A) Tail species, ranging in length from 98 to 106 nucleotides. Relative abundances were determined from the intensities of the deconvolved spectra, with the most abundant component corresponding to a 102-mer oligonucleotide of composition C(A)101, accounting for 19.3% of the total MS signal. Based on the combined contributions of all nine polyadenosine species detected at relative abundances above 2.0%, the average molecular weight of the Poly(A) Tail was determined to be 33,608.0 Da, corresponding to an average tail length of 102.3 nucleotides. Both Poly(A) heterogeneity/dispersity, as well as the average mass measurement are important quality attributes for therapeutic mRNA molecules. Figure 9 presents a screenshot of the INTACT Mass processing results. As summarized in the table, nine consecutive Poly(A) Tail species were putatively identified with mass accuracies better than 10 ppm. The high MS resolution (~100,000) of the Xevo MRT mass spectrometer enabled highly accurate mass measurements for

all Poly(A) Tail oligonucleotides through resolution of the isotopic distributions of their highly charged ions.

Thus, the single LC-MS assay developed for Capping Efficiency analysis also proved highly effective for the characterization of Poly(A) Tails enzymatically cleaved from prophylactic and therapeutic mRNAs.

Conclusions

Data Summary

- The Xevo MRT Mass Spectrometer, paired with waters_connect SYNTHETIC Library, MAP Sequence, and INTACT Mass Applications, deliver a high-impact, streamlined LC-MS solution for mRNA CQA analysis by combining exceptional mass resolution, accuracy, and sensitivity with outstanding usability.
- A single LC-MS assay simultaneously quantified two pivotal CQAs – 5′-capping efficiency and average poly(A) tail length/heterogeneity of Fluc mRNA – accelerating method development, reducing runtime, and lowering costs.
- Applied to Fluc mRNA, the workflow demonstrated 100% capping efficiency, based on detection of two 5′-Cap1 structures, and measured an average poly(A) tail length of 102.3-mer (average mass 33,608.0 Da) after accounting for the nine most abundant species, providing precise, actionable data to support product quality, process optimization, and regulatory readiness.

Impact on Gene Therapy Drug Development and Manufacturing

- LC-MS workflows developed by Waters have the potential to move gene therapy and mRNA drug development from exploratory characterization toward routine, scalable commercialization by enabling faster confirmation of sequence, purity, and critical quality attributes. Waters LC-HRMS systems, waters_connect workflows, and mRNA-focused sample preparation and software tools support more reproducible LC-MS analysis, reduce manual data processing, and make it easier for development, QC, and release-testing laboratories to monitor mRNA product quality with confidence.

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Xevo MRT <https://www.waters.com/nextgen/global/products/mass-spectrometry/mass-spectrometry-systems/xevo-mrt.html>

ACQUITY Premier System < https://www.waters.com/nextgen/global/products/chromatography/chromatography-systems/acquity-premier-system.html>

waters_connect Informatics Platform <https://www.waters.com/nextgen/global/products/informatics-and-software/waters_connect/waters-connect-informatics-platform.html>

INTACT Mass Application Software

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