A simple and robust LC-MS method for determination of poly(A) tail length using the Orbitrap Exploris MS systems

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INTRODUCTION

The introduction of effective messenger RNA (mRNA) vaccines to combat COVID-19 has accelerated the development of mRNA technologies for other infectious diseases and cancer.[1-3] mRNA-based vaccines not only showed impressive safety and efficacy in clinical trials,[4] but they also offer the potential for faster and more efficient vaccine production as compared to traditional virus-based and protein-based vaccines.[3] Typically, the mRNAs are produced synthetically through in vitro transcription (IVT), and a poly(A) tail is attached to the 3' phosphate of the mRNA transcript to enhance the translational efficiency and mRNA stability. This attachment can be done either through encoding the polyadenosine sequence into the template DNA or by adding the adenosines post-synthesis using a polyadenylase.[5] As a result of this process, sequence variants are generated and thus, the ability to accurately measure the tail length of IVT synthesized mRNA is crucial for evaluation of its impact on protein expression and stability of the mRNA-based therapeutics. Although numerous methods, such as chromatographic methods and PCR-based assays including next-generation sequencing methods, have been reported for measuring the tail length, these methods either lack the resolution or are indirect, as RNAs must be converted to cDNA in PCR-based assays. Here, we report a simple LC-MS method for direct measurement of polyadenylated tails in synthetic mRNA transcripts using high-resolution, accurate mass (HRAM) method developed on the Thermo Scientific™ Orbitrap Exploris™ MS systems. With this method, we were able to profile the poly(A) tail of IVT Cas9 mRNA samples with single nucleoside resolution, with a tail length ranging from 117 to 132 and a medium length around 124-125. In addition, the measured monoisotopic masses are within 5 ppm mass accuracy of the expected monoisotopic masses. We also developed a Thermo Scientific™ Chromeleon™ eWorkflow procedure to streamline the data processing and reporting for ease-of-use, and this procedure was executed on one Thermo Scientific™ Orbitrap Exploris™ 240 mass spectrometer and two Thermo Scientific™ Orbitrap Exploris™ MX mass detectors for automatic determination of poly(A) tail length.

MATERIALS AND METHODS

Sample Preparation

Figure 1 shows the entire sample preparation procedure for the extraction of poly(A) tails. Briefly, IVT Cas9 mRNA was obtained from TriLink Biotechnologies, (San Diego, CA) ~20μg mRNA samples were digested using 1μL of Thermo Scientific™ RNAse T1 at 1000U/μL for 1hr in a thermal mixer set to 37° C and 400RPM. After the digestion, the poly(A) tails were purified using 100μL of Invitrogen™ Dynabeads™ Oligo (dT)25 magnetic beads. Purified samples were dried down under a speed vacuum and then reconstituted in 100μL of 5% solvent B.

HPLC Conditions

10 uL (1μg) of purified sample was injected onto a Thermo Scientific™ DNAPac™ RP column and separated chromatographically using a Thermo Scientific™ Vanquish™ Horizon UHPLC system using the following gradient Table 1.

MS Conditions

For all experiments, data were collected using full scan at a resolution setting of 180,000 at 200 m/z. Intact protein mode with low pressure setting was used for optimal detection and transmission of the poly(A) tail samples. Detailed MS source and scan settings for both Orbitrap Exploris 240 mass spectrometer and Orbitrap Exploris MX mass detector are outlined in Table 2.

Data Processing and Reporting

Enterprise compliance ready Chromeleon CDS 7.3.2 software was used for all instrument control, data acquisition, processing, and reporting.

Figure 1. Schematics for the preparation and purification of the poly(A) tails

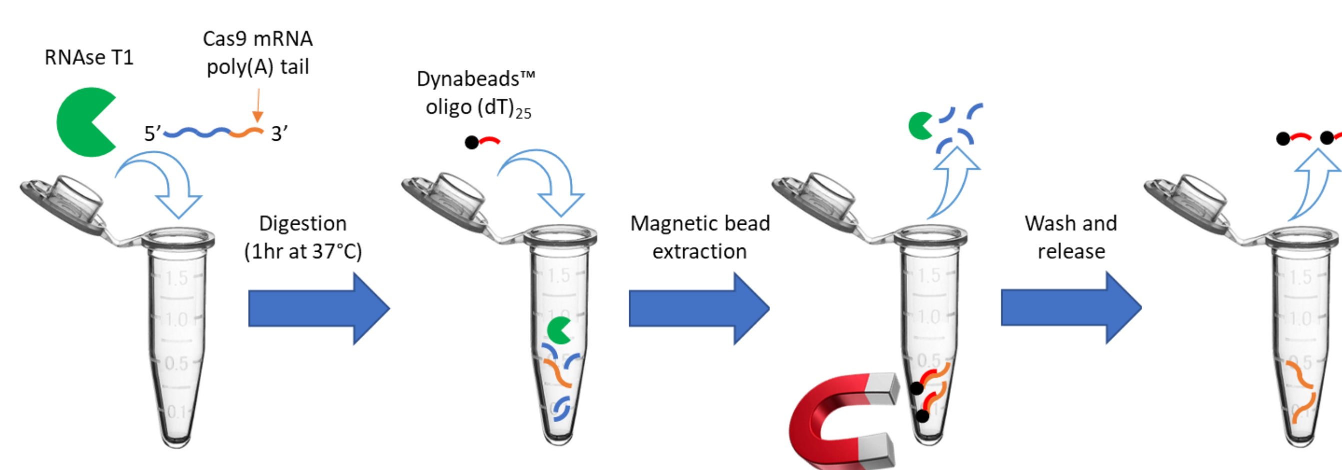


Table 1. LC condition for separation of poly(A) tail samples. Mobile phase is composed of 15mM dibutylamine (DBA) and 25mM 1,1,1,3,3,3-hexafluoro-2-propanol (HFIP) in water (aqueous), and acetonitrile (organic)

HPLC column	DNAPac RP 2.1 x 100 mm, 4 μm	
Flow Rate	0.4 mL/min	
Solvent A	15mM DBA and 25mM HFIP in water	
Solvent B	Acetonitrile	
Gradient	Time (min)	%B
	0	15
	0.5	15
	10	40
	10.5	90
	12	90
	12.5	15
	15	15
Injection Volume	10 μL	
Thermostatting mode	Forced Air	
Column oven temperature	70°C	

Table 2. MS source and scan settings for both Orbitrap Exploris 240 mass spectrometer and Orbitrap Exploris MX mass detector

Instrument	Orbitrap Exploris 240 and Orbitrap Exploris MX
MS source parameters	
Negative ion (V)	3000
Sheath Gas (Arb)	50
Aux Gas (Arb)	10
Sweep Gas (Arb)	1
Ion transfer tube temperature (ºC)	320
Vaporizer temperature (ºC)	320
Full scan parameters	
Application Mode	Intact Protein
Pressure Mode	Low Pressure
Expected LC Peak Width (s)	12
Resolution	180,000
Scan range (m/z)	1000 – 2000
Time range (min)	0 – 10 minutes
RF lens (%)	100
AGC target	3E6
Maximum injection time (ms)	200
Microscan	3

RESULTS

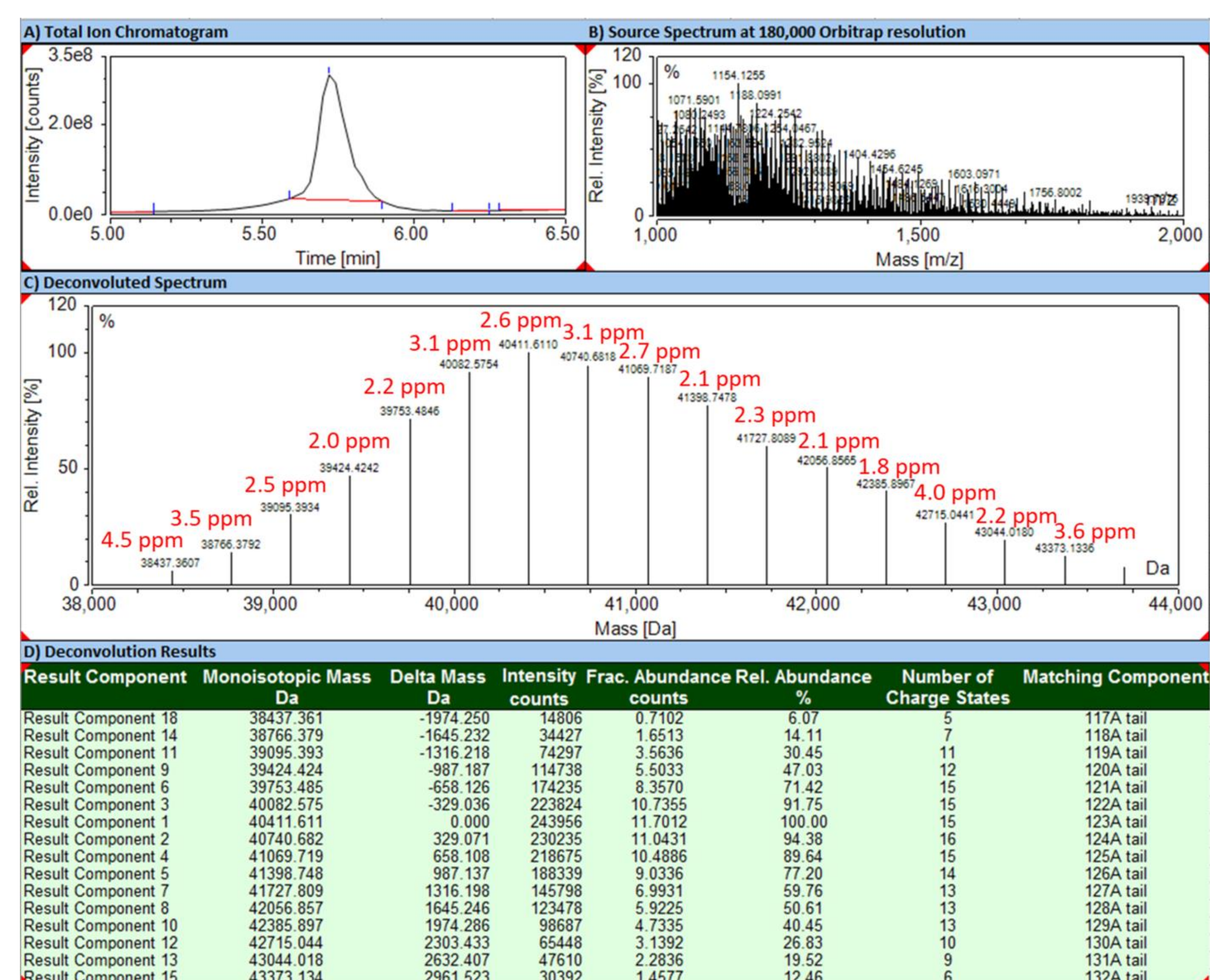
Poly(A) tails preparation

Poly(A) tails were extracted using previously published method.[6] Essentially, IVT Cas9 mRNAs were enzymatically digested by RNAse T1, a naturally occurring endonuclease which specifically cleaves single stranded RNA at guanosine (G) residue. Various small oligonucleotides with 3' G phosphate were generated after complete digestion, while leaving the poly(A) tails intact. A heterogenous population of poly(A) tails were then extracted using oligo(dT)25 magnetic beads as represented in Figure 1.

LC-MS analysis of poly(A) tails

Purified samples were separated using ion-pairing reversed phase liquid chromatography (IPRP-LC) on the DNAPac column. As shown in total ion chromatogram (TIC) in Figure 2, poly(A) tails eluted between 5.6 to 5.9 minutes. The source spectrum was generated by averaging the collected full scans across the retention time window, and then processed using Xtract deconvolution algorithm under the integrated intact protein deconvolution feature within Chromeleon CDS version 7.3.2. This algorithm is specifically designed for processing of isotopically resolved accurate mass data. Despite such complex spectrum with the presence of multiple overlapping charge states, the algorithm was able to detect multiple poly(A) tail variants in the purified samples. As illustrated in Figure 2, a distribution of measured monoisotopic masses ranging from 38437.3607 to 43373.1336 were observed in the deconvoluted spectrum. These measured monoisotopic masses matched the expected monoisotopic masses of 117 to 132 poly(A) tails with less than 5 ppm mass accuracy.

Figure 2. LC-MS analysis of poly(A) tails. A) Sample TIC for the separation of poly(A) tails. B) Sample source spectrum obtained by averaging the full scans across the retention time window from 5.6 to 5.9 minutes. C) Sample deconvoluted spectrum obtained using the Xtract algorithm, both measured monoisotopic masses and mass accuracy were shown. D) Sample deconvolution results showing the measured monoisotopic masses, delta masses, intensity counts, fractional and relative abundances, number of detected charge states, and matching components with less than 5 ppm mass accuracy.



D) Deconvolution Results

Result Component	Monoisotopic Mass Da	Delta Mass Da	Intensity counts	Frac. Abundance counts	Rel. Abundance %	Number of Charge States	Matching Component
Result Component 18	38437.361	-1974.250	14806	0.7102	6.07	5	117A tail
Result Component 14	38766.379	-1645.232	34427	1.6513	14.11	7	118A tail
Result Component 11	39095.393	-1316.218	74297	3.5636	30.45	11	119A tail
Result Component 9	39424.424	-987.187	114738	5.5033	47.03	12	120A tail
Result Component 6	39753.485	-658.126	174235	8.3570	71.42	15	121A tail
Result Component 3	40082.575	-329.036	223824	10.7355	91.75	15	122A tail
Result Component 1	40411.611	0.000	243956	11.7012	100.00	15	123A tail
Result Component 2	40740.682	329.071	230235	11.0431	94.38	16	124A tail
Result Component 4	41069.719	658.108	218675	10.4886	89.64	15	125A tail
Result Component 5	41398.748	987.137	188339	9.0336	77.20	14	126A tail
Result Component 7	41727.809	1316.198	145798	6.9931	59.76	13	127A tail
Result Component 8	42056.857	1645.246	123478	5.9225	50.61	13	128A tail
Result Component 10	42385.897	1974.286	98687	4.7335	40.45	13	129A tail
Result Component 12	42715.044	2303.433	65448	3.1392	26.83	10	130A tail
Result Component 13	43044.018	2632.407	47610	2.2836	19.52	9	131A tail
Result Component 15	43373.134	2961.523	30392	1.4577	12.46	6	132A tail

Chromeleon eWorkflow Procedure

Chromeleon eWorkflow procedure was executed on one Orbitrap Exploris 240 mass spectrometer and two Orbitrap Exploris MX mass detectors for the evaluation of poly(A) tails. Both observed tail length range and median were consistent across 3 MS systems as illustrated in Table 3. Along with auto reporting enabled, this eWorkflow procedure facilitated seamless method transfer and execution across both Orbitrap Exploris 240 and MX systems for poly(A) tail analysis.

Figure 3. Chromeleon eWorkflow procedure for direct method transfer and execution on Orbitrap Exploris 240 mass spectrometer and Orbitrap Exploris MX mass detector

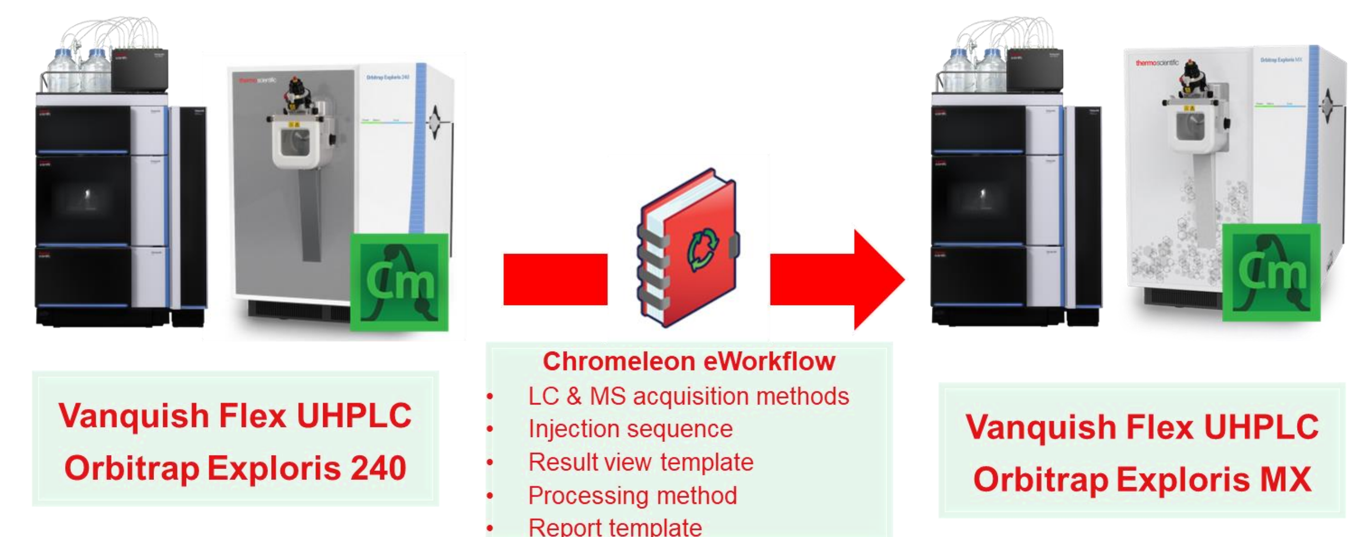


Table 3. Evaluation of poly(A) tails across 3 Orbitrap Exploris MS systems using Chromeleon eWorkflow procedure. Values are based on 3 replicate injections

Poly(A) Tail	Orbitrap Exploris 240	Orbitrap Exploris MX #1	Orbitrap Exploris MX #2
Range	117 – 132	118 – 131	118 – 132
Median	124.5	124.5	125

CONCLUSIONS

- A full scan LC-MS method with 180,000 resolution was developed for accurate monoisotopic mass determination of poly(A) tails
- A report was created for quick identification of the tail lengths by comparing the measured against the expected monoisotopic masses, a matching tail length is shown if the measured mass accuracy is within 5 ppm
- Consistent poly(A) tail attribute measurements (e.g., length range and median) were obtained through executing Chromeleon eWorkflow procedure on one Orbitrap Exploris 240 mass spectrometer and two Orbitrap Exploris MX mass detectors

REFERENCES

1. Clifford T. H. et al, "mRNA-LNP expressing PfCSP and Pfs25 vaccine candidates targeting infection and transmission of Plasmodium falciparum", NPJ Vaccines (2022); 7 (1) DOI: 10.1038/s41541-022-00577-8
2. Neuzil, K. M., "An mRNA Influenza Vaccine—Could It Deliver?", New England Journal of Medicine (2023); DOI: 10.1056/NEJMcibr2215281
3. Qin, S. et al, "mRNA-based therapeutics: powerful and versatile tools to combat diseases", Signal Transduction and Targeted Therapy (2022); volume 7, Article number:166
4. Mulligan, M. J. et al, "Phase I/II Study of COVID-19 RNA Vaccine BNT162b1 in Adults", Nature (2020); 586, 589–593
5. Beverley, M. et al, "Poly A tail length analysis of in vitro transcribed mRNA by LC-MS", Analytical and Bioanalytical Chemistry (2018); 410:1667–1677
6. Thermo Fisher Scientific, Application Note 001183: Characterization of in vitro-transcribed (IVT) mRNA poly(A) tail by LC-HRAM-MS and BioPharma Finder 5.0 software. https://assets.thermofisher.com/TFS-Assets/CMD/Application-Notes/an-001183-bt-orbitrap-exploris-240-ms-mrna-hram-lc-ms-an001183-na-en.pdf

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