A simple and robust LC-MS method for determination of poly(A) tail length using the Orbitrap Explotra 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 therapeutics for other infectious diseases and cancer.1-3 mRNA-based vaccines not only showed impressive safety and efficacy in clinical trials but also provided an efficient vaccine production compared to traditional viral-based and protein-based vaccines.1,4 Typically, the mRNAs are produced synthetically through in vitro transcription (IVT), and a poly(A) tail is added to the 3’ phosphite of the mRNA transcript to enhance the transfection efficiency and mRNA stability. This attachment can be done either by encoding the polyadenosine sequence into the template DNA or by adding the adenine post-synthesis in a polyadenylated mRNA. 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 vital for evaluation of its impact on protein expression and stability of the mRNA-based therapeutics. Although numerous methods, such as chromatographic 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® Explotra™ MS systems. With this method, we were able to profile the poly(A) tail of IVT Cas9 mRNA samples with single nucleotide resolution, with a tail length ranging from 117 to 132 and a median 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™ Chromatofinder™ workflow to streamline the data processing and reporting for casexol-like, and this procedure was executed on the Thermo Scientific™ Orbitrap® Explotra™ 240 mass spectrometer and two Thermo Scientific™ Orbitrap® Explotra™ 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 from mRNA. mRNA was obtained from Invitrogen™ (Carlsbad, CA, USA) and reconstituted with a volume of 15 µL of water. After the digestion, the poly(A) tails were purified using 10 µL of oligo(dT)20 beads (Invitrogen™). The poly(A) tail samples were then eluted with 25 µL of water, and the eluted samples were dried down under a vacuum and then reconstituted with 150 µL of 5% acetonitrile.

HPLC Conditions

The purified poly(A) sample was injected into a Thermo Scientific™ Dionex™ RF column and separated chromatographically using a Thermo Scientific™ Vanquish™ 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 205 nm mass with positive scan set for optimal detection and transmission of the poly(A) tail samples. Detailed MS source and scan settings for both Thermo Scientific Vanquish™ UHPLC mass spectrometer and Orbitrap Explotra MX mass detector are outlined in Table 2.

RESULTS

Poly(A) tails preparation

Poly(A) tails were extracted using previously published method.1 Essentially, IVT Cas9 mRNA was enzymatically digested by RNase T1, a naturally occurring endonuclease which specifically cleaves single-stranded RNA at pyrimidine (G) residues. Various small oligonucleotides with 3’ G phosphate were generated after complete digestion, while leaving the poly(A) tails. A heterogenous population of poly(A) tails were then extracted using oligo(dT)25 magnetic beads as representation in Figure 1.

LC-MS analysis of poly(A) tails

Poly(A) tails samples were separated using ion-pairing reverse phase liquid chromatography (IPRP-LC) on the Dionex™ column. A full scan ion chromatography (IC) in Figure 2 (poly(A) tails eluted between 5.6 to 9.9 minutes). The source spectrum was generated by averaging the collected full scans across the retention time window, and then processed using Sculptor algorithm within the integrated high precision deconvolution feature within Chromatograph 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. A similar to Figure 1, a distribution of measured monoisotopic masses ranging from 14511.1527 to 14710.4332 was 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.

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

<table>
<thead>
<tr>
<th>Time</th>
<th>Condition</th>
<th>Flow (µL/min)</th>
<th>Detector</th>
</tr>
</thead>
<tbody>
<tr>
<td>0-10</td>
<td>Buffer A</td>
<td>5</td>
<td>MRM</td>
</tr>
<tr>
<td>10-100</td>
<td>Buffer B</td>
<td>5</td>
<td>MRM</td>
</tr>
</tbody>
</table>

Table 2. MS source and scan settings for both Thermo Scientific Vanquish™ UHPLC and Orbitrap Explotra MX mass detector.

<table>
<thead>
<tr>
<th>Source</th>
<th>Mass Range (m/z)</th>
<th>Collision Energy (eV)</th>
<th>Dynamic Exclusion (s)</th>
<th>Resolution (m/Δm)</th>
</tr>
</thead>
<tbody>
<tr>
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<td>10</td>
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<td>100000</td>
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<tr>
<td>ESI</td>
<td>100-1000</td>
<td>30</td>
<td>30</td>
<td>100000</td>
</tr>
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</table>

REFERENCES

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.

Continuous poly(A) tail attribute measurements (e.g., length, range and median) were determined through executing Chromatofinder™ procedure on one Orbitrap Explotra 240 mass spectrometer and two Orbitrap Explotra MX mass detectors.

TRADEMARKS/LICENSES

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

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 9.9 minutes. C) Sample deconvoluted spectrum obtained using the Sculptor algorithm, both measured monoisotopic masses and mass accuracy were shown. D) Sample deconvolution results showing the measured monoisotopic masses, delta masses, ion counts, fractional relative abundances, number of detected charge states, and matching components with less than 5 ppm mass accuracy.

Figure 3. Chromatofinder™ workflow procedure for direct method transfer and execution on Orbitrap Explotra 240 mass spectrometer and Orbitrap Explotra MX mass detector.

Table 3. Evaluation of poly(A) tails across 3 Orbitrap Explotra MS systems using Chromatofinder™ workflow procedure. Values are based on 3 replicate injections.