Poster Reprint

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# Characterizing the Poly(A) tail in mRNA using Ion-Pairing Reversed-Phase LC/MS (IPRP-LC/MS) and Deconvolution Tools

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## Introduction

Messenger RNA (mRNA) is an increasingly important biotherapeutic that has shown its potential in combating a variety of diseases [1]. Its primary function involves translating from the cell's nucleus to the cytoplasm, where protein machinery reads the mRNA sequence and converts each three-base trinucleotide (codon) into a unique amino acid. An important structural motif that enables this translation while also preventing degradation of mRNA in the cytoplasm is the poly(A) tail. Here, we characterize the length of the poly(A) tail in mRNA after enzymatic digestion with RNase 4 using ion-pairing reversed-phase LC/MS. RNase 4 was selected due to its unique ability to cleave after uridine when followed by purine. This tends to yield more favorable cleavage products that offer a more targeted digestion.

## Experimental

Firefly Luciferase (FLuc) mRNA was purchased from TriLink BioTechnologies (San Diego, CA) and used without further purification. The digestion procedure used the following steps in a thermocycler:

1. Denature mRNA solution by heating at 90°C for 5 minutes (50 µL of mRNA at 1 µg/µL)
2. Cool solution to 4°C for 10 minutes
3. Add 55 µL of master mix containing 50 µL of 2X NEBuffer r1.1 and 5 µL of RNase 4 (250 units)
4. Incubate the digest at 37°C for 45 minutes
5. Add 5 µL of murine quencher and leave at room temperature for 10 minutes
6. Transfer to polypropylene LC/MS vial

### Mobile phase preparation and conditioning

Mobile phases A and B were prepared fresh daily and sonicated for 5 minutes prior to each experiment [2]. To minimize metal adduction, plastic mobile phase bottles were used (p/n 9301-6460) with the GL45 thread adapter (p/n 5043-1192). In addition, Agilent Stay Safe caps (p/n 5043-1222) were used to ensure consistent mobile phase composition. The LC was conditioned with ion pairing reagent for 4 hours prior to recording MS signal from the RNA standard.

### Mass spectrometry detection

The poly(A) tail was detected with an Agilent 1290 Infinity III Bio LC and an Agilent 6230C LC/TOF system. LC/MS signal arising from the poly(A) tail was deconvoluted using Agilent MassHunter BioConfirm software.

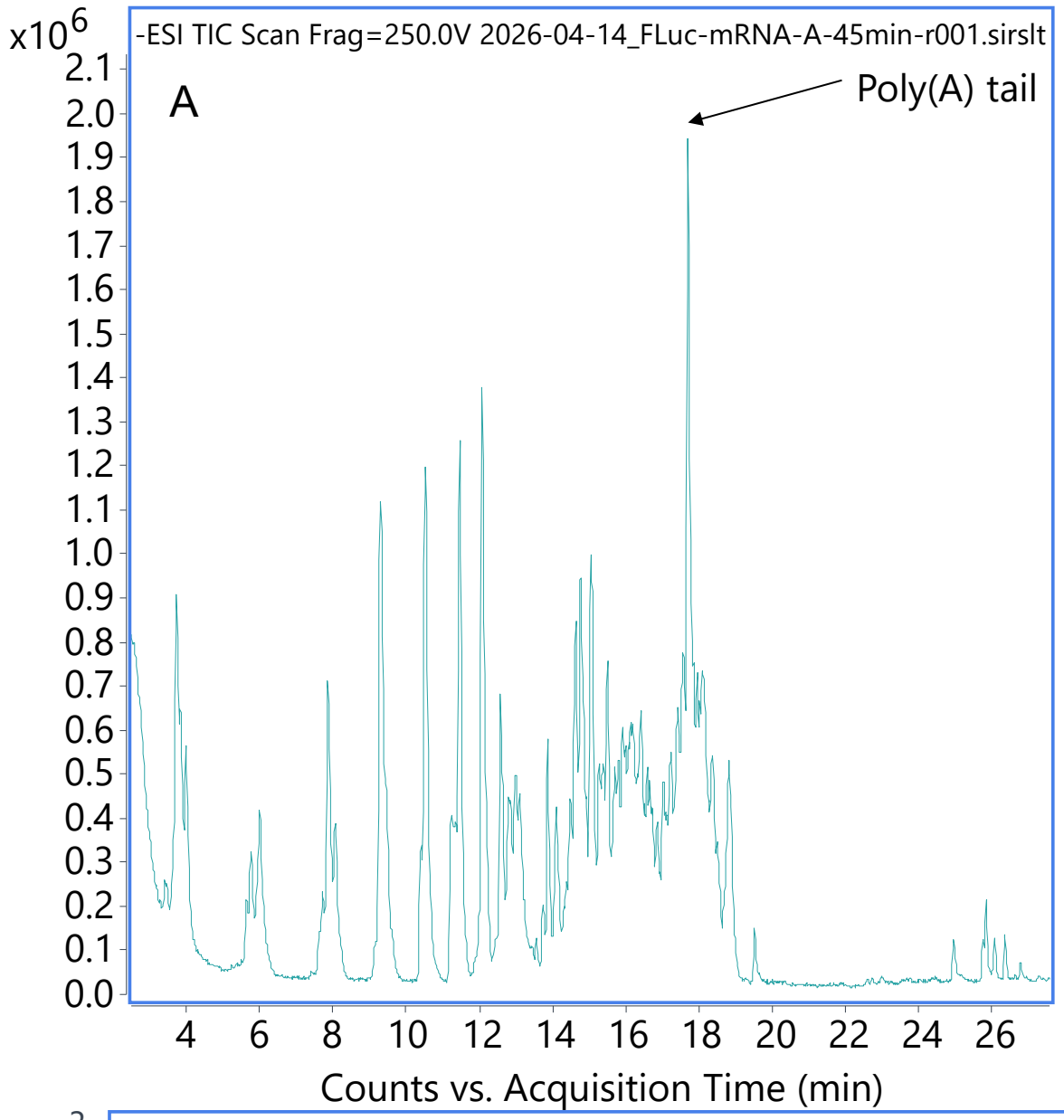
## Experimental

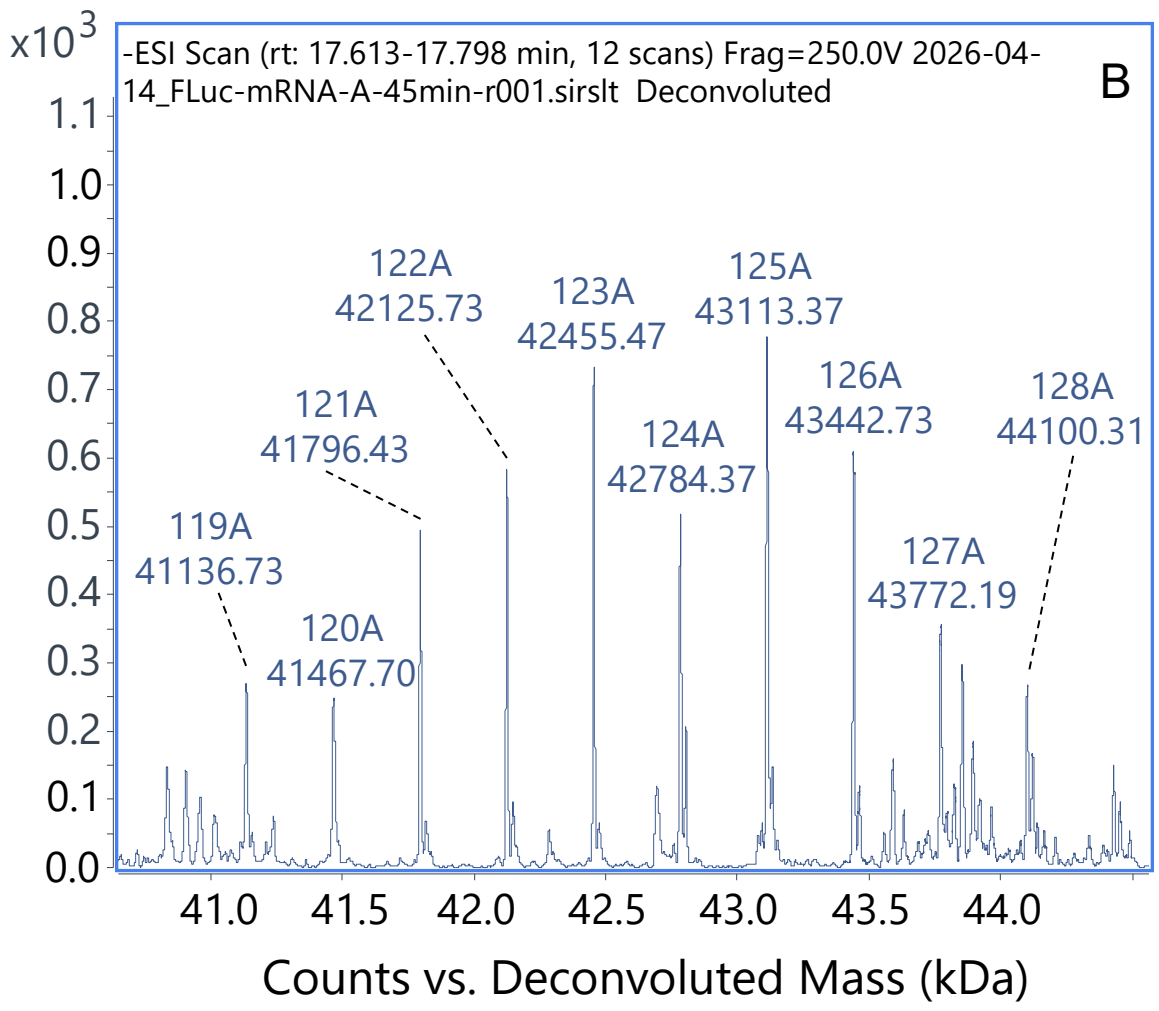
| LC Conditions | |
|---|---|
| Column | Altura Oligo HPH-C18 column, 2.7 µm, 2.1 x 150 mm, p/n 227215-702 |
| Solvent A | 10 mM hexylamine, 50 mM HFIP in 95% $H_2O$, 5% MeOH |
| Solvent B | 10 mM hexylamine, 50 mM HFIP in 97% MeOH, 3% $H_2O$ |
| Solvent C | Acetonitrile |
| Gradient | 0-26.5 min, 20-100% B<br>26.6-30.6 min, 100% B<br>30.65-38.1 min, 100% C (reverse column flush)<br>38.1-46.6 min, 20% B |
| On-column amount | 5.5 µg (12 µL of 0.45 µg/µL) |
| Flow rate | 0.25 mL/min |
| Valve Position | Position 1 at 0 min<br>Position 2 at 30.6 min<br>Position 1 at 45.6 min |
| Column temperature | 60°C |
| **MS Conditions** | |
| Ion Source | Agilent Jet Stream ESI source |
| Polarity | Negative |
| Gas Temperature | 325°C |
| Gas Flow | 12 L/min |
| Nebulizer | 30 psi |
| Sheath Gas Temperature | 275°C |
| Sheath Gas Flow | 10 L/min |
| Capillary Voltage | 4500 V |
| Nozzle Voltage | 1000 V |
| Octopole RF | 750 V |
| Fragmentor | 250 V |
| Skimmer | 65 V |
| Time (ms/spectrum) | 1000 ms |
| MS1 Scan Range | *m/z* 400 to 3200 |
| Reference Ions (*m/z*) | 805.985436, 2533.892301 |

Table 1. LC/MS conditions for polyA tail analysis

## TIC of FLuc mRNA digested with RNase 4, deconvoluted spectrum, and raw mass spectrum

The Poly(A) tail from digested FLuc mRNA can be measured on the Agilent 6230 LC/TOF system. The deconvoluted mass spectrum (Figure 1B) shows a distribution of poly(A) tail lengths between 119 – 128 adenosines. The raw MS spectrum is also shown in Figure 1C.



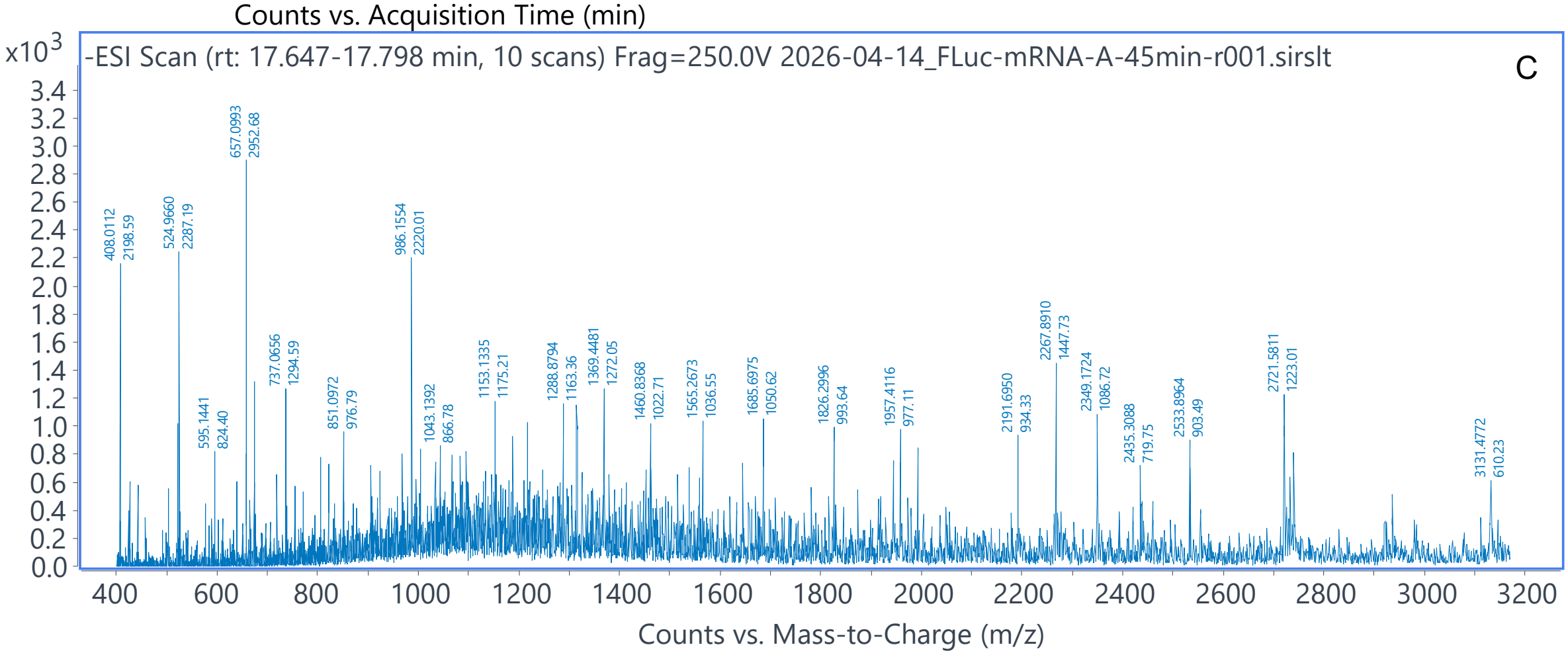
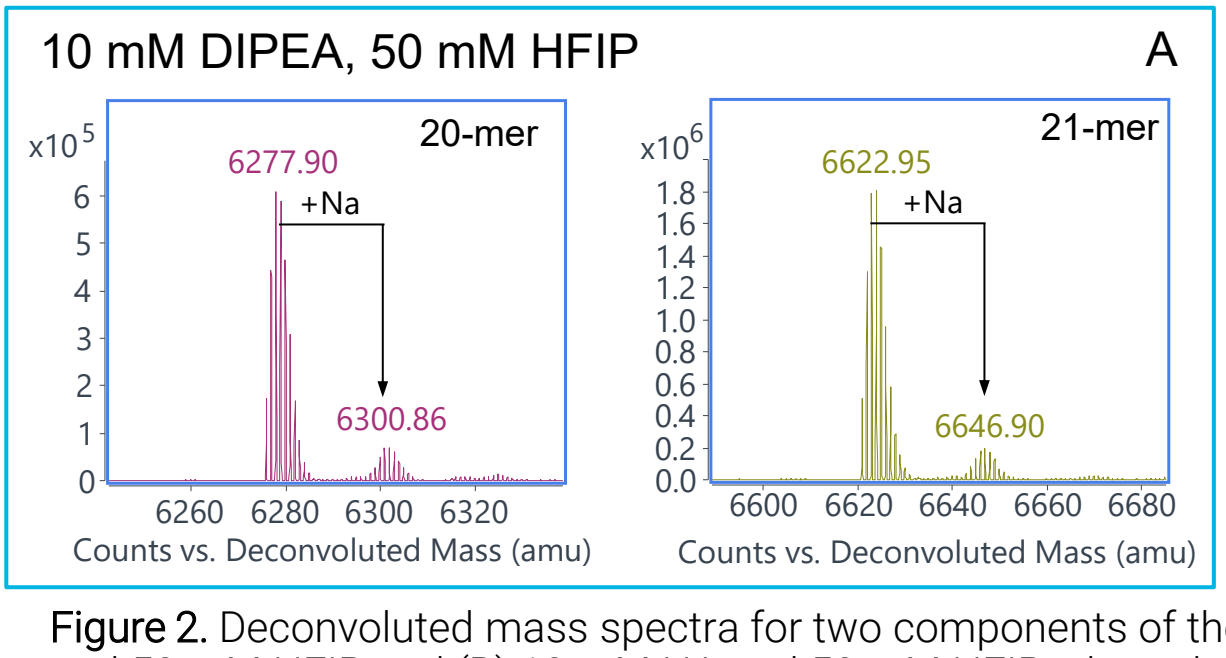




**Figure 1.** (A) TIC for 5.5 µg of FLuc mRNA digested with RNase 4. The poly(A) tail is detected at the end of the gradient. (B) Deconvoluted mass spectrum showing the distribution of poly(A) tail lengths that fall between 119 – 128 adenosines. (C) Raw MS spectrum that serves as the input for deconvolution. The rise in signal beginning at *m/z* 800 shows signal from the poly(A) tail.

## Results and Discussion

### Method development using a System Suitability Standard that precedes mRNA digestion injections

Oligonucleotides are susceptible to metal ions within the LC flow path. The level of $Na^+$ adduction was assessed for all components of the RNA standard (14-mer, 17-mer, 20-mer, and 21-mer) using different ion pairing reagents. Based on deconvoluted mass intensities, the level of $Na^+$ adduction ranged from 1.44 – 3.78% with HA and 10.4 – 13.1% with DIPEA.



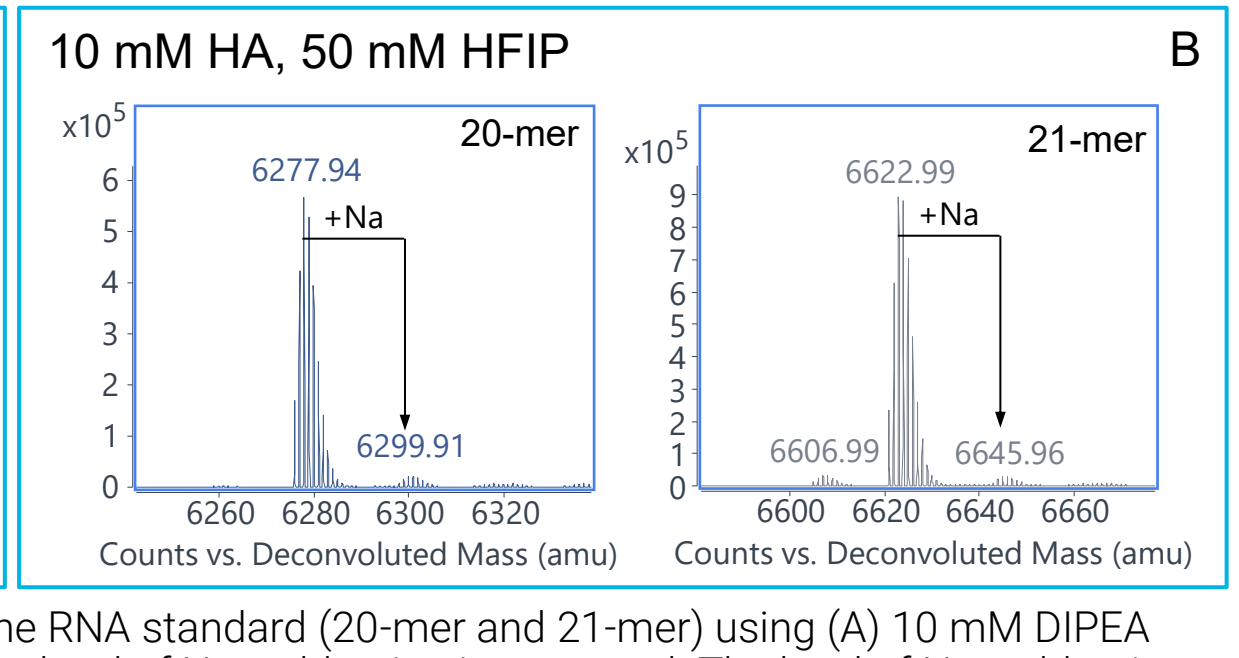




**Figure 2.** Deconvoluted mass spectra for two components of the RNA standard (20-mer and 21-mer) using (A) 10 mM DIPEA and 50 mM HFIP and (B) 10 mM HA and 50 mM HFIP where the level of $Na^+$ adduction is assessed. The level of $Na^+$ adduction is lower with hexylamine, however signal intensity appears higher for the 21-mer with DIPEA.

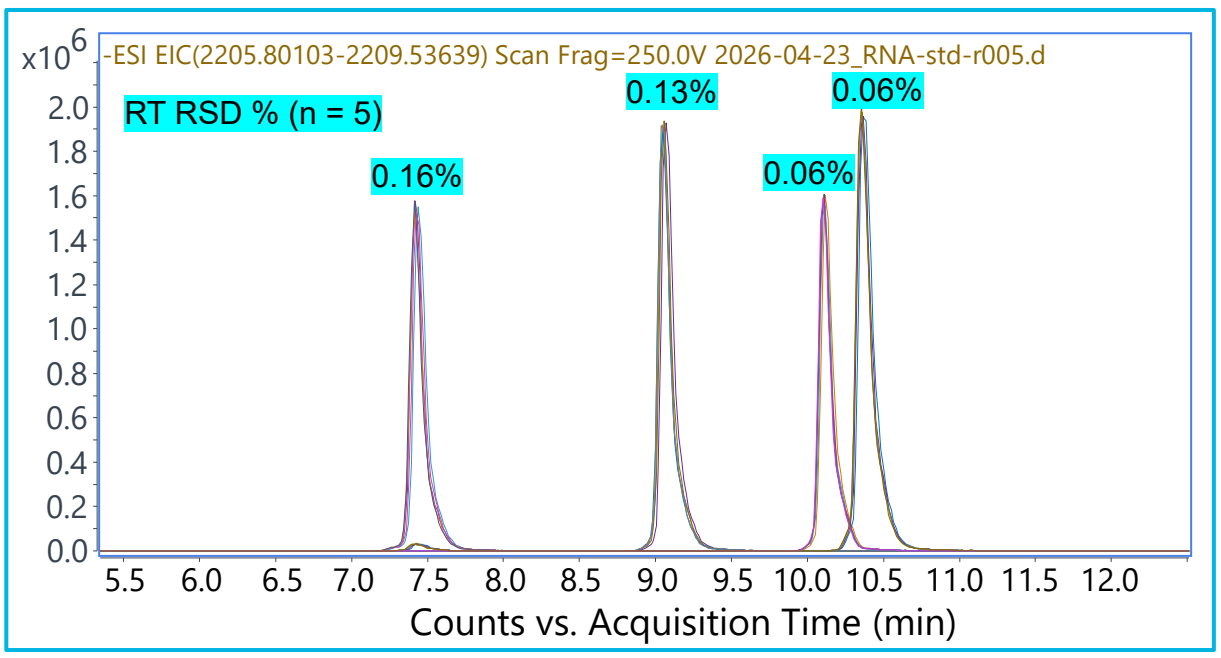



**Figure 3.** Overlay of extracted ion chromatograms from five replicates of the RNA standard prior to detection of the mRNA digest. The retention time RSD % is reported above each peak.

| | 14-mer | 17-mer | 20-mer | 21-mer |
|---|---|---|---|---|
| Run 1 | 1550234 | 1930036 | 1601227 | 1953559 |
| Run 2 | 1574900 | 1920302 | 1605879 | 1976902 |
| Run 3 | 1521230 | 1939204 | 1553282 | 1957457 |
| Run 4 | 1538708 | 1894943 | 1588219 | 1975860 |
| Run 5 | 1575586 | 1914211 | 1591357 | 2008187 |
| EIC RSD % | 1.51 | 0.88 | 1.30 | 1.10 |

**Table 2**. Extracted ion chromatograms peak abundances from five replicates of the RNA standard. The EIC RSD % for each component is less than 2%.

## Conclusions

### The Agilent 6230 LC/TOF system enables detection of the poly(A) tail after RNase 4 digestion

- LC/MS conditions were optimized to detect the poly(A) tail from the FLuc mRNA digest. A distribution of poly(A) tail lengths that fall between 119 – 128 adenosines are detected.
- Method development was performed on an RNA standard with the goal of assessing $Na^+$ adduction, RT RSDs, and EIC RSDs prior to measurement of mRNA digest sample. The level of sodium adduction was lower with hexylamine ion pair (1.44 – 3.78%) for the RNA standard, RT RSDs for all components were less than 0.2%, and EIC peak height RSDs were below 2%.

## References

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