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The basis for breakthroughs

# LCMS Assays for mRNA CQAs: A Development Story

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# Analytical Methods for mRNA DS CQAs

- Current 5’ cap method is HPLC-UV based
  - Method needs to be suitable for many different constructs
  - Limitations
    - Long gradient (~100 minutes) required for best separation of fragments
    - Long development time
    - Length of fragments generated by the digestion
    - Degraded mRNA from stability studies interfere with separation
- This seems like a job for Mass Spec!

| Quality | Attribute | Method |
|---|---|---|
| **Integrity** | mRNA intactness | Capillary electrophoresis [D] |
| | | Capillary gel electrophoresis (CGE) [D] |
| | | Agarose gel electrophoresis |
| **Purity** | mRNA purity | Ion pair reversed-phase high-performance liquid chromatography (IP-RP-HPLC) |
| | 5’ capping efficiency | Reverse-phase liquid chromatography mass spectroscopy (RP-LC-MS/MS) [D] |
| | | Ion pair reversed-phase high-performance liquid chromatography (IP-RP-HPLC) |
| | | Liquid chromatography mass spectroscopy (LC-MS/MS) [D] |
| | 3’ poly(A) tail length | Liquid chromatography mass spectroscopy (LC-MS/MS) [D] |
| | | Ion pair reversed-phase high-performance liquid chromatography (IP-RP-HPLC) |
| | Product related impurities - dsRNA | Immunoblot |
| | | Enzyme-linked immunosorbent assay (ELISA) |
| | Product related impurities - aggregate quantitation | Size exclusion-high-performance liquid chromatography (SEC-HPLC) [D] |
| | Product related impurities - percentage of fragment mRNA | Reversed-phase HPLC (RP-HPLC) [D] |
| | Process related impurities - residual DNA template | quantitative PCR (qPCR) |
| | Process related impurities - quantitation of free/non-incorporated nucleosides | Reverse-phase liquid chromatography mass spectroscopy (RP-LC-MS/MS) [D] |
| | Process related impurities - residual NTP and capping agent | Anion exchange high-performance liquid chromatography (AEX-HPLC) [D] |
| | Process related impurities - residual T7 RNA polymerase content | Enzyme-linked immunosorbent assay (ELISA) |
| Potency | Expression of target protein | Cell-based assay |
| Safety | Endotoxin | USP <85> |
| | Bioburden | USP <61>, <62>, <1115> |
| Other | Appearance | USP <790> |
| | Residual solvents | USP <467> |
| | pH | USP <791> |

1. Analytical Procedures for Quality of mRNA Vaccines and Therapeutics: Draft Guidelines: 3rd Ed.

# Aldevron's High-Res Mass Spectrometry Lab

## Thermo Fisher Exploris™ 480 (Orbitrap)

- Vanquish™ Horizon UHPLC Front-End
- MS/MS workflow for 5' cap Fragment Sequence ID
- BioPharma Finder™ software for nucleic acid workflows*

## Agilent Advance Bio 6545XT (QTOF)

- Agilent 1290 Bio Binary Pump UPLC Front-End
- Capable of poly(A) distribution for both enzymatic >180 As and encoded tails
- Capable of automated 5' cap analysis using BioConfirm. Most "GMP-ready"

## Waters Xevo™ G3 (QTOF)

- Waters™ ACQUITY Premier UPLC Front-End
- Characterization of poly(A) tail and 5' cap digestion products
- waters_connect™ for nucleic acid workflows

## SCIEX ZenoTOF 7600 (QTOF)

- Waters ACQUITY Premier UPLC Front-End
- Electron Activated Dissociation (EAD) – allows for unique fragmentation for lipid analysis

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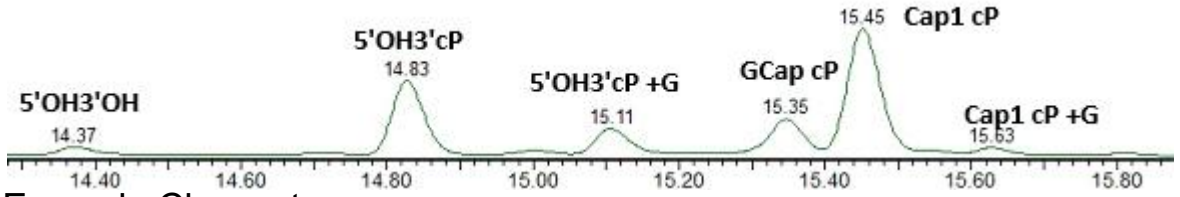
# 5’Cap LCMS POC Data

| Xevo G3 (QTOF) | QDa (Single Quad) |
|---|---|
| Scan & ID | Targeted |



Example Chromatogram

| | Run 1 | | Run 2 | | Run 3 | |
|---|---|---|---|---|---|---|
| Sample name | %Purity | % Recovery | %Purity | % Recovery | %Purity | % Recovery |
| 100% capped | 93.7 | N/A | 93.4 | N/A | 93.5 | N/A |
| 99% capped | 92.2 | 99.4 | 92 | 99.5 | 92.1 | 99.5 |
| 95% capped | 86.7 | 97.4 | 86.5 | 97.5 | 86.5 | 97.4 |
| 85% capped | 75.9 | 95.3 | 75.8 | 95.5 | 75.9 | 95.5 |
| 70% capped | 59.2 | 90.3 | 58.7 | 89.7 | 59.4 | 90.7 |
| 100% capped 2x Dilution | 96.4 | 99.9 | 97.5 | 100.4 | 97.1 | 100.1 |
| 100% capped 3x Dilution | 97.5 | 99.9 | | | | |
| 100% capped 5x dilution | 98.7 | 100.1 | 99.0 | 100.1 | 99.1 | 100.3 |
| 100% capped 10x dilution | | | 99.8 | 100.4 | 99.6 | 100.2 |
| 100% capped 20x dilution | | | 100.0 | 100.1 | 99.8 | 99.9 |

- DNAzyme based digestion
- Tested set of capped material with uncapped spiked in
  - 1% - 30% Uncap spikes
- Perform 3 reps with fresh digests on different days.

- 89.7 – 99.5% Recoveries across 3 analytical runs
- 0.8% RSD n=3 for Capped Sample
- Dilutional Linearity – $R^2 = 0.9995$

# 3’ Poly(A) Tail POC with QDa

- Single Quads like the QDa struggle with poly(A)

| Tail Length | Theoretical Monoisotopic Mass (Da) | Observed Deconvoluted Mass (Da) | Mass Difference (Da) |
|---|---|---|---|
| 20A | 6519.09 | 6524.2 | 5.1 |
| 30A | 9809.613 | 9815.1 | 5.5 |
| 40A | 13100.135 | 13105.4 | 5.3 |
| 50A | 16390.658 | 16400.1 | 9.4 |
| 60A | 19681.181 | 19688.2 | 7.0 |
| 70A | 22971.704 | 23021.6 | 49.9 |
| 80A | 26262.227 | 26286.4 | 24.2 |
| 90A | 29552.75 | 29508.9 | -43.8 |
| 100A | 32843.273 | 32800.8 | -42.5 |

- Ask was to move only one instrument to QC
- Shift focus to QTOF

# 5’ Cap POC on the Agilent 6545XT QTOF

- Same GFP mRNA as with the QDa
- Initial runs of the spike recovery experiments were underwhelming

| | Run 1 | | Run 2 | |
|---|---|---|---|---|
| Sample name | %Purity | % Recovery | %Purity | % Recovery |
| 100% capped | 84.1 | 100 | 90.9 | 100 |
| 99% capped | 81.4 | 97.8 | 88.7 | 98.6 |
| 95% capped | 74.7 | 93.5 | 81.9 | 94.8 |
| 85% capped | 55.8 | 78.1 | 64.2 | 83.1 |
| 70% capped | 37.7 | 64.0 | 41.5 | 65.2 |

- Possible parameters to investigate
  - Bioconfirm software calculations
    - Selection and summation of charge states
    - Manually selected charge states and integrated XICs. No change
  - MS source parameters

# 5’ Cap POC on the Agilent 6545XT QTOF

- Agilent Jet Stream Technology (AJT)
  - Dual Stream AJT Source

- Started with Agilent’s recommended source parameters for oligos
  - Nozzle Voltage: 1000
  - Capillary Voltage: (-)4000 V
  - Fragmentor Voltage: 225 V
  - Skimmer: 65 V

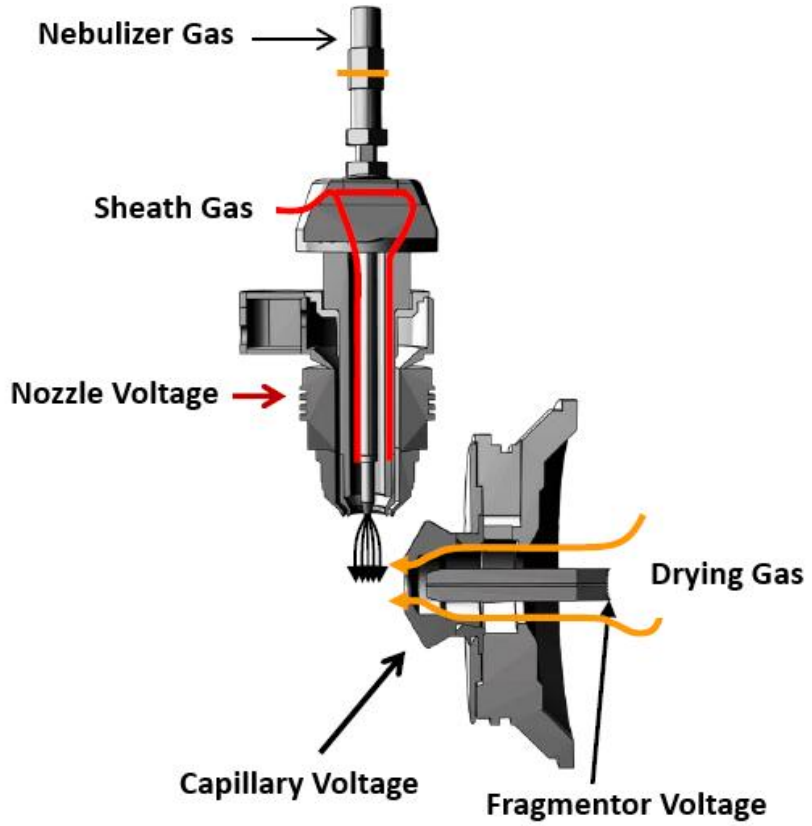
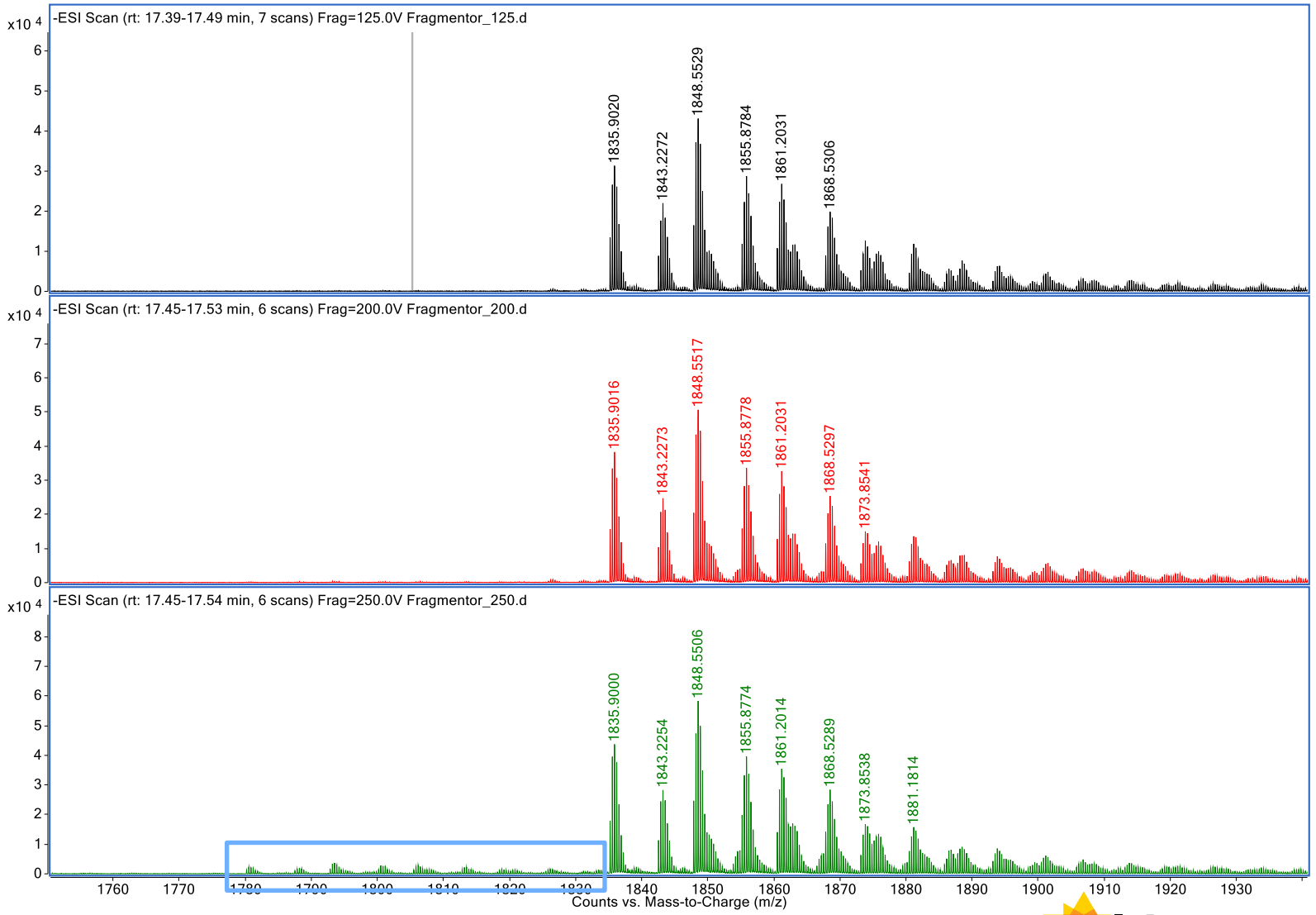



Figure adapted from Agilent

# 5’ Cap POC on the Agilent 6545XT QTOF

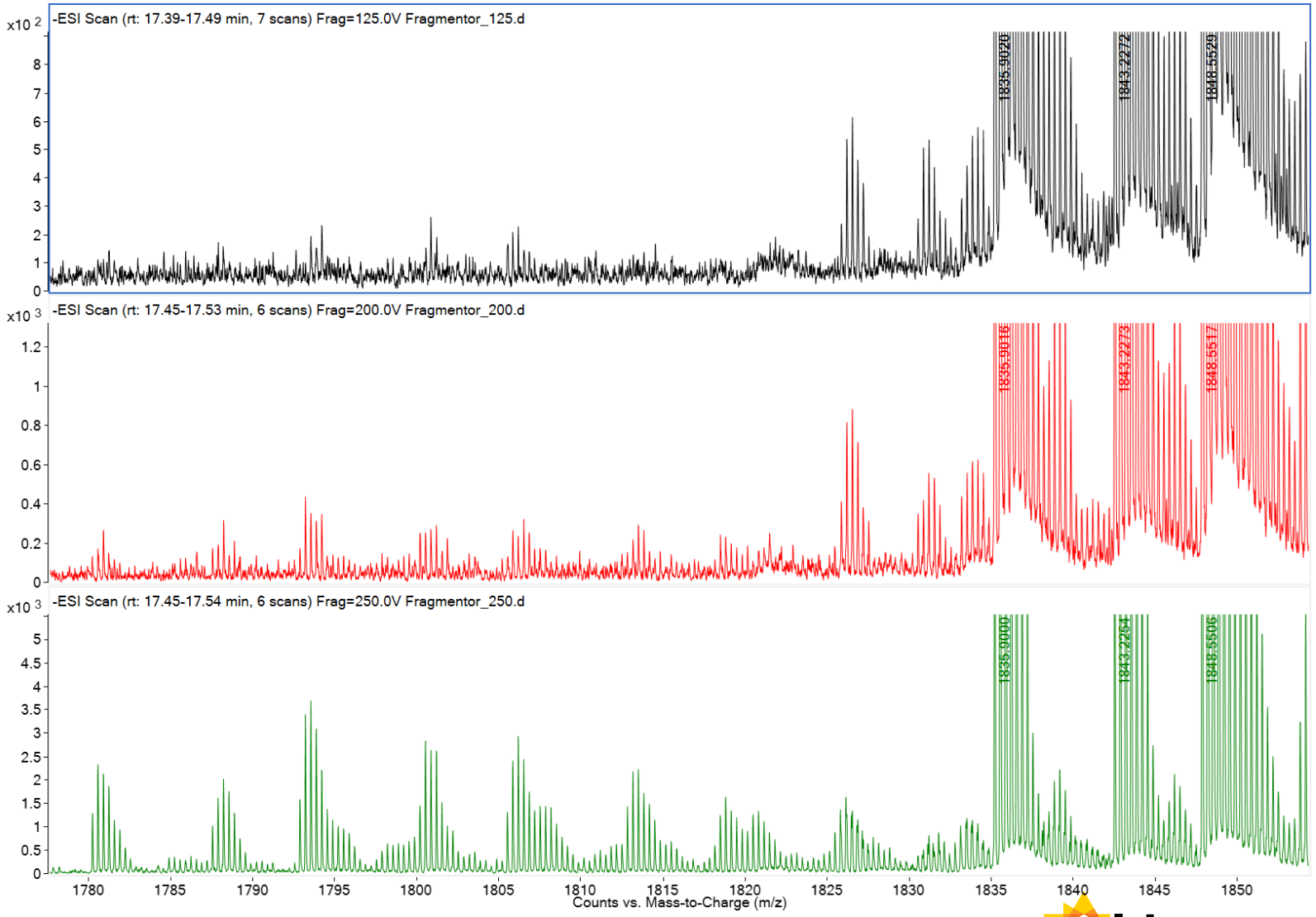
- After investigation of the mass spectra, we noticed lots of lower-than-expected m/z clusters. Also have significant K+ adducts

- Suspected fragmentation was occurring



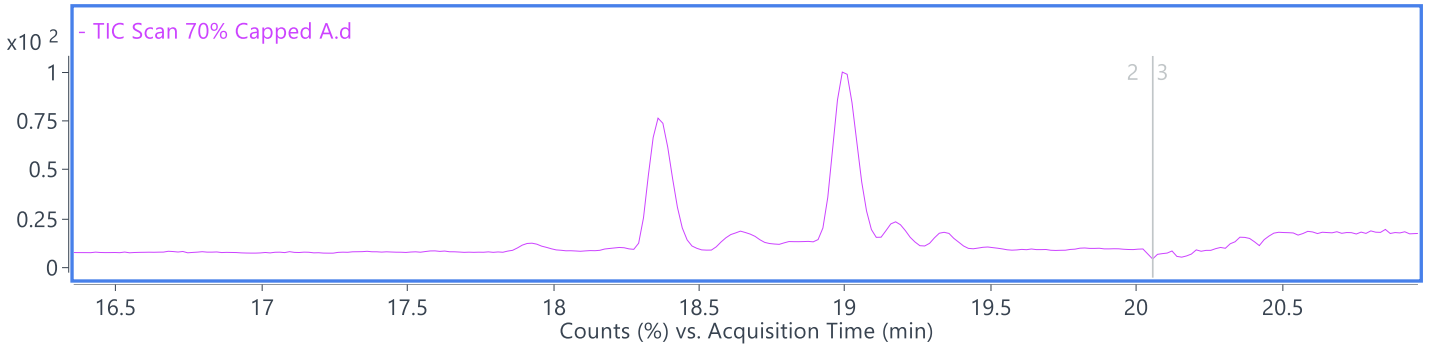
# 5’ Cap POC on the Agilent 6545XT QTOF

- After investigation of the mass spectra, we noticed lots of lower-than-expected m/z clusters. Also have significant K+ adducts
- Suspected fragmentation was occurring
- Saw the fragmentation was eliminated at FragV = 125V



# 5’ Cap POC on the Agilent 6545XT QTOF

- Now run triplicate digests on different days and run method
- Recoveries ranging from 99.4 – 80.9%
  - Appears to be a bias for uncapped material with drops in recovery appearing for the 85% and 70% capped samples
- %RSD <2% for each spike level
- %RSD <0.2% for the capped sample




| | Run 1 | | Run 2 | | Run 3 | |
|---|---|---|---|---|---|---|
| Sample name | %Purity | % Recovery | %Purity | % Recovery | %Purity | % Recovery |
| 100% capped | 87.5 | | 87.3 | | 87.2 | |
| 99% capped | 84.7 | 98.7 | 84.6 | 98.9 | 85.0 | 99.4 |
| 95% capped | 80.6 | 96.9 | 80.7 | 97.3 | 79.8 | 96.4 |
| 85% capped | 66.1 | 88.9 | 68.0 | 91.7 | 68.1 | 91.9 |
| 70% capped | 49.5 | 80.9 | 49.7 | 81.4 | 49.7 | 81.5 |

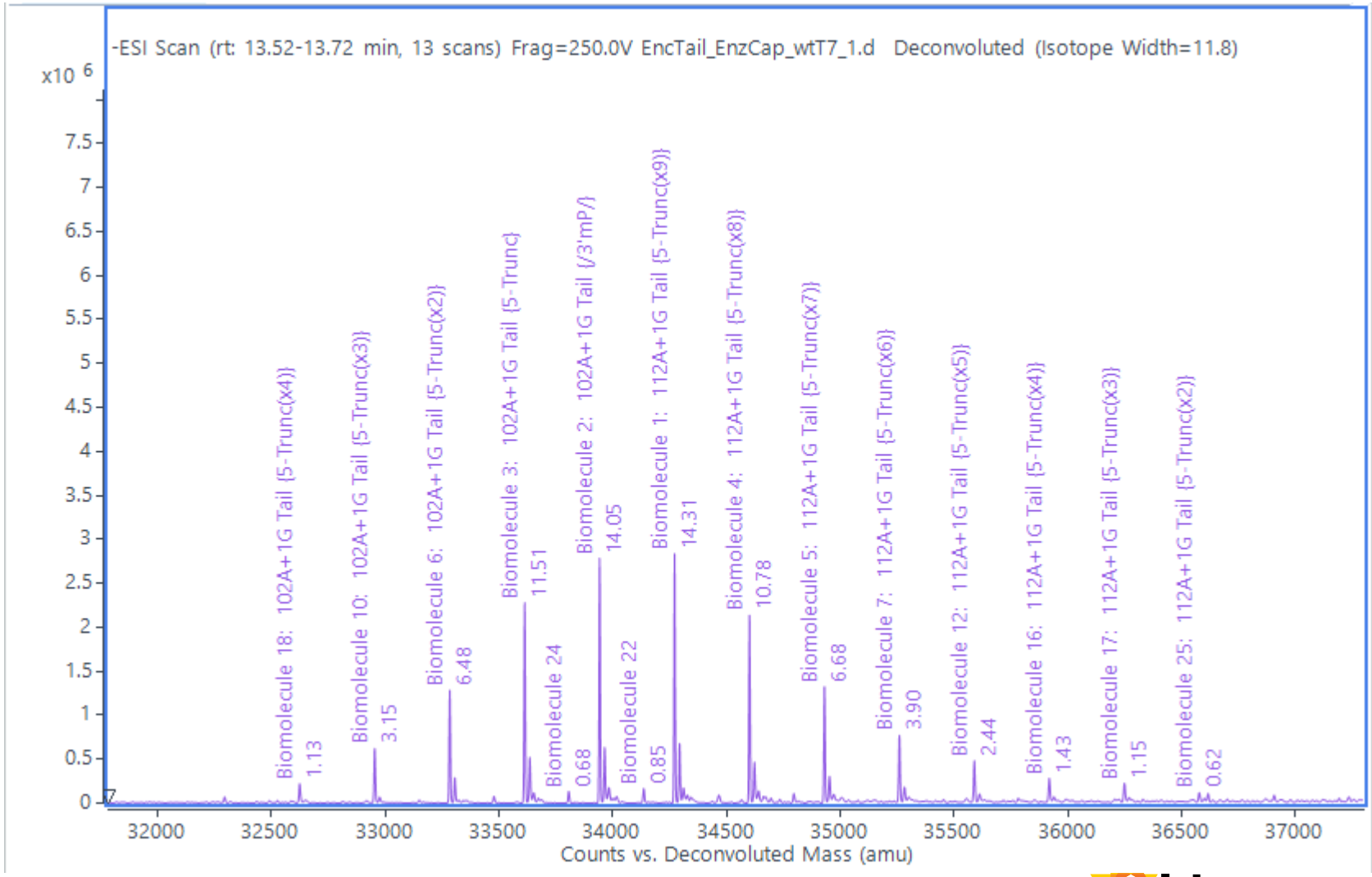
# Bonus Poly(A) Tail Enzymatic vs Encoded

- Enzymatic poly(A) tails create a challenge for MS based analysis due to the broad poly(A) tail range and heterogeneity present inherently in enzymatic added tails
- Encoded tails are much less diverse and are more easily deconvoluted

- Method
  - Enzymatic tail dropoff with RNAse T1
  - Platform LCMS using IPRPLC gradient

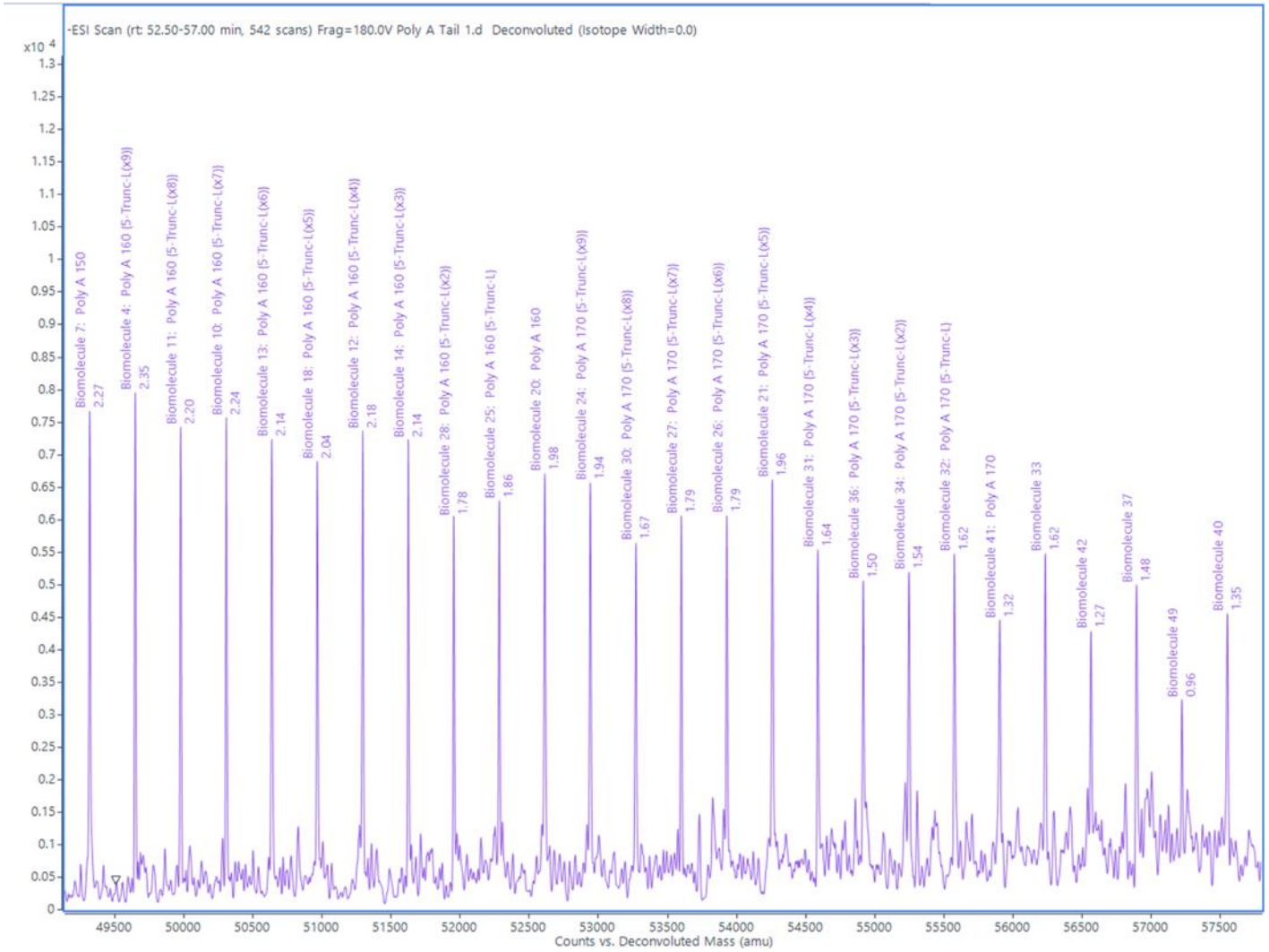
# Encoded Poly(A) Tail Analysis Example

- Method
  - Enzymatic tail dropoff with RNAse T1
  - Platform IPRPLC gradient
- Target 102As
- Distribution from 98-110 A with the most abundant length by peak area being 102A



# Enzymatic Poly(A) Tail Analysis Example

- Method
  - Enzymatic tail dropoff with RNAse T1
  - Platform IPRPLC gradient
- Target 180As
- Distribution from 125-170As with the most abundant length by peak area being 140A



# Summary

- We were able to show a 5’ cap LCMS-based method which can provide valuable insights to capping efficiency for process development. Our method showed acceptable accuracy, linearity, and reproducibility on both a Waters QDa and an Agilent 6545XT QTOF.

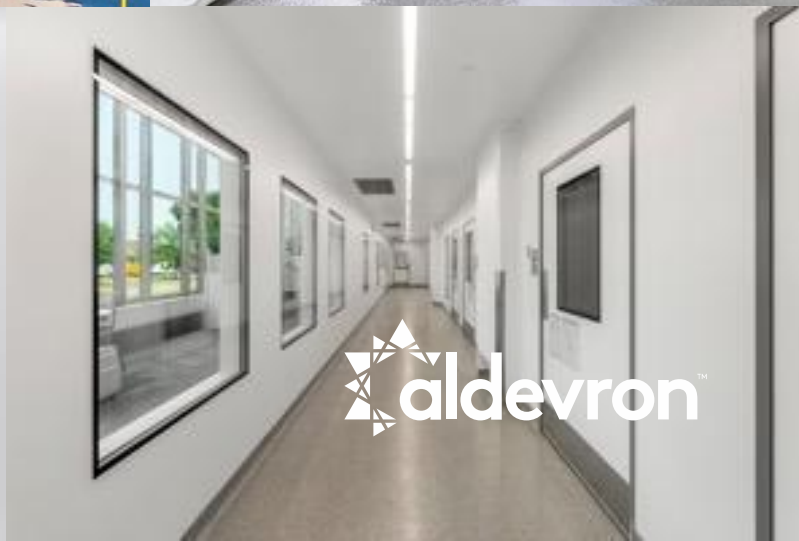
- We also showed an accurate mass Proof-of-Concept LCMS-based poly(A) tail length and heterogeneity method, which can report median length and range for both encoded and enzymatically added tails.

# Acknowledgements


- My Aldevron AMD Team
  - **Wes Mosher**
  - Sid Pradhan
  - Babak Mamnoon
  - Alexzandrea Woudenberg
  - Sheng Feng
  - Tajnin Sultana
  - Autumn Dinnel*
  - Anders Lund



ÄKTA readyflux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