Optimization of a Liquid Chromatography-Tandem Mass Spectrometry mRNA Sequence Mapping Workflow

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SCAN FOR POSTER

Introduction

Structural characterization is necessary to ensure safety and efficacy of therapeutic mRNA oligonucleotides. LC-MS/MS bottom-up sequence mapping is a powerful solution for assessment and confirmation of mRNA oligonucleotide primary structure and detection of modifications. However, this technique faces several challenges including development of the endoribonuclease digest, separation of oligonucleotide digest products, assignment of MS/MS spectra, distinguishing isomers, and efficient data analysis and reporting. In this study we investigate the primary structure of an eGFP mRNA sequence (RiboPro) using a novel oligonucleotide mapping workflow.

Methods – Sample Preparation

- hRNase4 (New England Biologics) cleaves at U|G and U|A.
- T4 PNK (New England Biologics) creates uniform hydroxylated 3' ends.
- 40-minute digestion at 37ºC.
- Sample is injected directly and desalted with a 5-minute hold at 3% B.

Methods – IPRP-MS/MS

Table. 1: Ion Pairing Reverse Phase (IPRP) Method Summary

Mobile Phase A	95 mM HFIP, 5.7 mM DIPEA in water
Mobile Phase B	7.1 mM HFIP, 2.1 mM DIPEA in 80% methanol
Column	Phenomenex bioZen 2.6 µm Oligo 150 x 2.1 mm PN: 00F-4790-AN
Column Temp.	75ºC
Injection Vol.	5 µL
Flow Rate	300 µL/minute

Gradient	
Time (min)	**%B**
0	3%
5	3%
50	45%

MS Analysis: Thermo Q-Exactive Plus using Data Dependent Acquisition in negative mode.

Methods – Data Processing

- The data processing workflow used in this study is a Protein Metrics beta product still under development.
- Data analysis was performed using Byos™ and a repurposed version of Byonic® for MS/MS oligonucleotide spectrum matching (OSM).
- The solution accepts all major MS vendor data formats.

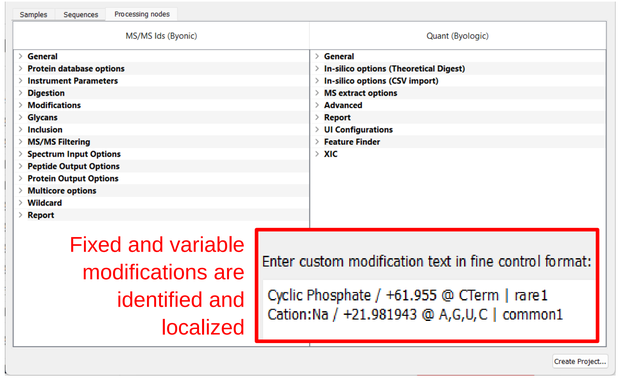

Fig. 1: Project creation window allows users to define expected sequence and processing parameters for their specific instrument and analysis.

- Workflow includes Wildcard Search™, Modification Fine Control, OSM scoring, and Automatic/Custom Reporting.

Results – IPRP Separation

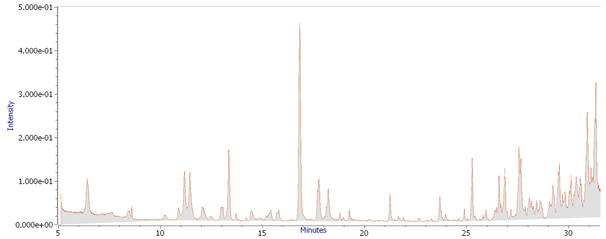

Fig. 2: Total Ion Current Chromatogram of hRNAse4 digested eGFP mRNA separated with Phenomenex bioZen 2.6 um Oligo 150 x 2.1 mm column.

Results – Distinguishing Isomers

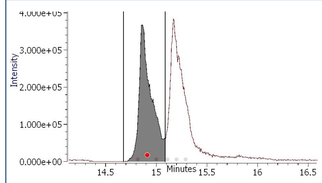

Fig. 3: XIC of two resolved isomeric oligonucleotides. In Byos red dots represent OSMs assigned to the currently selected oligonucleotide while grey dots represent OSMs assigned to unique isomers.

Isomers with distinct mRNA sequences are common for oligonucleotide mapping. Confident assignment of isomers is critical and challenging.

An example isomer pair is separated by IPRP and automatically assigned using our data processing solution (Figures 3 and 4).

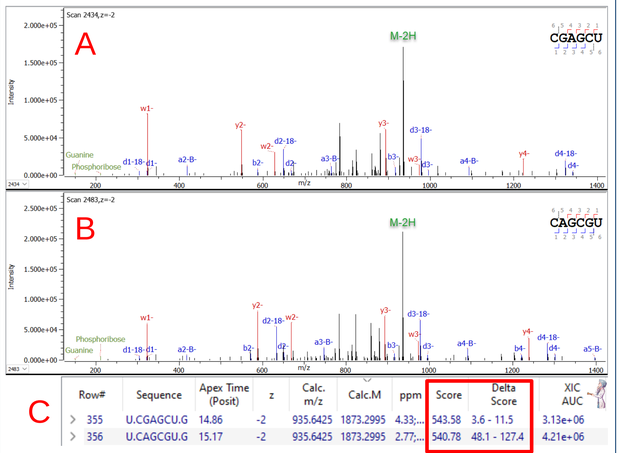

Fig. 4: Select screenshots of the Byos project investigation view used for manual inspection of assignments. Oligonucleotide spectra for each isomer are annotated with assigned fragment ions (A, B). Some columns from the oligonucleotide table are shown (C).

- Isomer distinguishing fragment ions y2, w2, y4, b2, d2-18, and d4-18 are observed.
- **Delta Score:** OSM score difference between the best (assigned) and next best assignment.
- Intelligent OSM scoring and IPRP separation are critical.

Results – Automatic Reporting

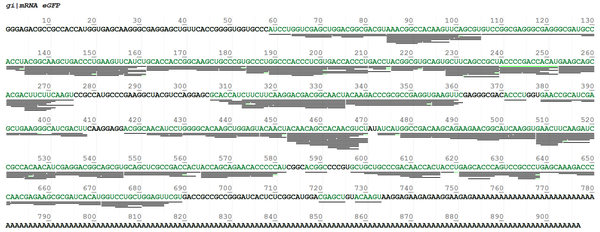

Fig. 5: eGFP sequence coverage map with black lines representing oligonucleotide IDs. **80% sequence coverage excluding the PolyA tail.**

OSM Assignment Table: Score with Delta Score Heatmap

Start AA ↑	End AA ↑	Sequence (unformatted) ↑	z ↑	Calc. m/z ↑	MS Alias name ← eGFP hRNase4
	72	GGUCGAGCU	-2	1433.7	445
64	84	GGUCGAGCUGGACGGCGA CGU	-9	756.655	180
			-8	851.363	231
67	72	CGAGCU	-2	935.642	544
73	101	GGACGGCGACGUAAACGG CCACAAGUUCA	-12	784.435	227
			-11	855.839	277

Fig. 6: A report table with numerical OSM Scores and heatmap color code illustrating Delta Scores, valuable for assessing reliability of assignment.

Variable Modification: 2',3'-Cyclic Phosphate

Sequence (unformatted) ↑	Apex Time (Posit) ↑	Mod. Summary ↓	MS Alias name ← eGFP hRNase4 (%)
ACAACAGCCACAACGUCU	27.5698		93.1
	28.0611	CTerm(Cyclic Phosphate/61.9550)	6.9
ACAACU	16.8051		98.6
	19.3696	CTerm(Cyclic Phosphate/61.9550)	1.4
ACAAGACCCGCGCCGAGGU	27.6605		90.2
	28.1019	CTerm(Cyclic Phosphate/61.9550)	9.8

Fig. 7: A report table with assigned variable modifications. Relative abundance of modified vs. unmodified is calculated automatically.

Conclusions

- 80% eGFP seq. coverage (-PolyA) with a single digest.
- Beta OSM tool (repurposed Byonic) assigns OSMs with intelligent scoring and detection of modifications.
- Workflow separates and automatically detects isomers.

Acknowledgments
We gratefully acknowledge Roxana Eggleston-Rangel and Phenomenex for preparing samples and acquiring data.