Using Novel RNases to Measure 5' Cap and Poly(A) Tail Modifications during Oligonucleotide Mapping LC-MS of messenger RNA

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INTRODUCTION

Accelerated development of RNA therapeutics require capable analytical approaches that produce more comprehensive data sets in less time. Confirmation of identity, purity, and modifications of sgRNA and mRNA through oligonucleotide mapping is a widely accepted strategy. Enzymatic hydrolysis of RNA generates a mixture of oligonucleotide digestion products for a subsequent chromatographic separation; and hyphenation with mass spectrometry provides a detailed molecular fingerprint. The RNase T1 is the most frequently used RNase although it generates relatively small oligonucleotides that make it difficult to obtain high confidence information across the RNA full length. The new RapiZyme™ MC1 and Cusativin RNases (**Figure 1**) exhibit complementary enzyme specificity to fill the gap by providing high confidence sequence information. MC1 cleaves RNA at [A/U/C]-p-U bonds and Cusativin prefers C-p-[U/A/G] bonds (1). Although low frequency cuts are possible at other sites (**Figure 2**), partial cleavage of RNA is the norm under optimal digestion conditions.

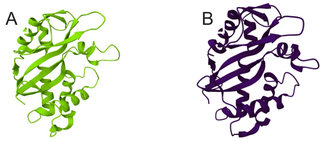


Figure 1: AlphaFold Protein structure entries for MC1—P23540 (A) and Cusativin– A0A0A0LC37 (B) (2-3).

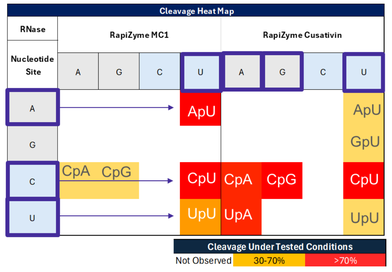


Figure 2: MC1 and Cusativin cleavage preferences

METHODS

Sample preparation: HPRT1 sgRNA (IDT), mRNA (Biosynthesis, GenScript) and lyophilized recombinant MC1 and recombinant Cusativin (Waters RapiZyme RNases) were studied. 3.2 µg (sgRNA) or 10 µg (mRNA) in 10 µL of 200 mM ammonium acetate, pH 8 (MC1) or pH 9 (Cusativin) were denatured (90 °C for 2 min) then cooled at 4 °C. 50 U (sgRNA) or 150 U (mRNA) of MC1 or Cusativin were added, incubated at 30 °C/30 min and heat inactivated at 75 °C/15 min.

LC-MS data acquisition: Xevo™ G3 MS or BioAccord™ MS connected to ACQUITY™ Premier LC system and ACQUITY Premier OST, 2.1 x 150 mm Column with BEH™ C18 130 or 300 A°, 1.7µm at 60 °C were used at 300 µL/min flow rate. The mobile phases include Solvent A: 10 mM DPA (dipropylamine), 40 mM HFIP (Hexafluoroisopropanol) in DI water, pH 8.5 **or** 0.1% DIPEA (Diisopropylethylamine) 1% HFIP in water and Solvent B: 10 mM DPA (dipropylamine), 40 mM HFIP in 50 methanol **or** 0.0375% DIPEA, 0.075% HFIP in 35% water and 65% acetonitrile with 5 µL injection and gradient. The oligonucleotides were detected at 260 nm and in negative ion ESI-MS mode with mass range of 400 – 4,000 / 5,000 Da, cone Voltage of 40 V and 2 Hz acquisition rate using waters_connect™ Software (Figure 3).

Time (min)	Flow rate (mL/min)	Solvent A composition (%)	Solvent B composition (%)	Curve profile
0.00	0.3	100	0	Initial
45.00	0.3	50	50	6
45.10	0.3	15	85	6
49.10	0.3	15	85	6
50.00	0.3	100	0	6
60.00	0.3	100	0	6

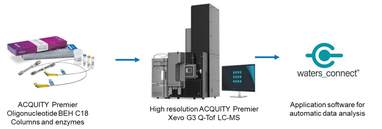


Figure 3: LC-MS sample data acquisition workflow.

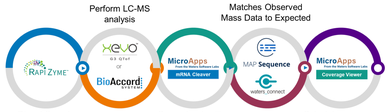


Figure 4: Oligonucleotide mapping workflow tools for confident sequence assignment.

RESULTS AND DISCUSSION

Oligonucleotide mapping of HPRT1 sgRNA by RapiZyme RNases

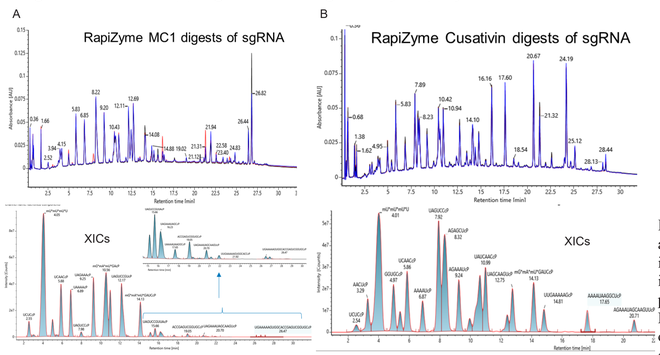


Figure 5: Oligonucleotide mapping of HPRT1 sgRNA following digestion with MC1 (A) and Cusativin (B). Top panels shows the UV chromatograms of sgRNA digests from 3 independent batches (biological replicates) of enzymes. Bottom panels depict the extracted ion chromatograms (XICs) of the oligonucleotide m/z values (mass error <20 ppm) predicted from the mRNA cleaver. Overlay of the observed digestion products on the HPRT1 sgRNA sequence is shown in C. Note the overlapping digestion product pattern of oligonucleotides resulting in partial cleavages leading to end-to-end or 100% sequence coverage including the 5' and 3'end modified oligonucleotides (4).

Messenger RNA characterization by RapiZyme RNases

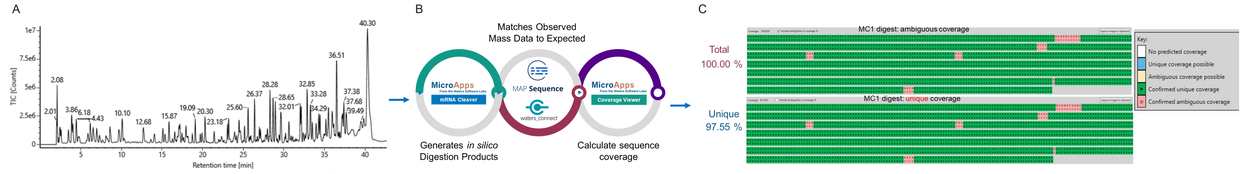
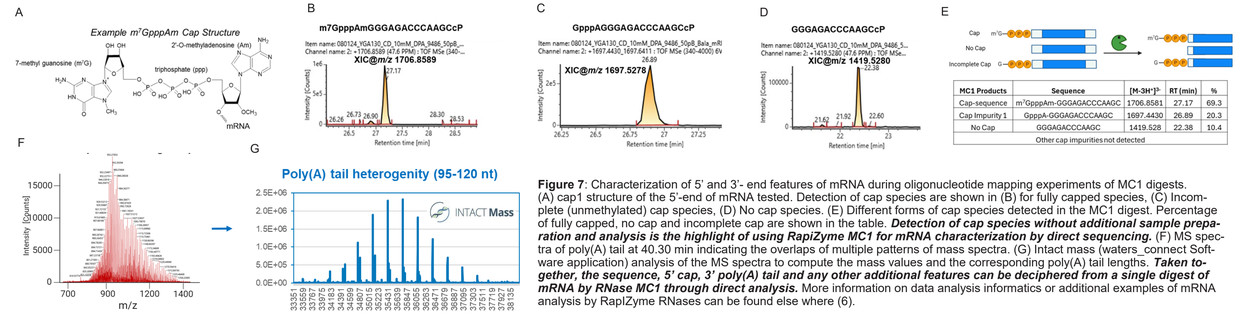


Figure 6: mRNA Oligonucleotide mapping workflows involving LC-MS data acquisition (A), data analysis software (B), and the sequence coverage output (C). An example GFP mRNA was digested with RapiZyme MC1 and the resulting oligonucleotides were characterized by LC-MS. *In silico* digestion products with 4 cleavage skips are predicted by mRNA cleaver, matched with the experimentally observed products by MAP sequence and the coverage computed for both unique digestion products (fingerprint) and unique+ambiguous for total coverage (5).



MC1 Products	Sequence	[M-3H+]3-	RT (min)	%
Cap-sequence	m7GpppAm-GGGAGACCCAAGC	1706.8581	27.17	69.3
Cap Impurity 1	GpppA-GGGAGACCCAAGC	1697.4430	26.89	20.3
No Cap	GGGAGACCCAAGC	1419.528	22.38	10.4
Other cap impurities not detected				

Figure 7: Characterization of 5' and 3'- end features of mRNA during oligonucleotide mapping experiments of MC1 digests. (A) cap1 structure of the 5'-end of mRNA tested. Detection of cap species are shown in (B) for fully capped species, (C) Incomplete (unmethylated) cap species, (D) No cap species. (E) Different forms of cap species detected in the MC1 digest. Percentage of fully capped, no cap and incomplete cap are shown in the table. ***Detection of cap species without additional sample preparation and analysis is the highlight of using RapiZyme MC1 for mRNA characterization by direct sequencing.*** (F) MS spectra of poly(A) tail at 40.30 min indicating the overlaps of multiple patterns of mass spectra. (G) Intact mass (waters_connect Software application) analysis of the MS spectra to compute the mass values and the corresponding poly(A) tail lengths. ***Taken together, the sequence, 5' cap, 3' poly(A) tail and any other additional features can be deciphered from a single digest of mRNA by RNase MC1 through direct analysis.*** More information on data analysis informatics or additional examples of mRNA analysis by RapIZyme RNases can be found else where (6).

CONCLUSION

- Unique dinucleotide specificity of RapiZyme MC1 and Cusativin for complementary digestion
- Partial cleavage of RNA under optimal conditions with batch-to-batch reproducibility
- Longer digestion products with unique masses for improved or even complete sequence coverage at MS1 level
- Overlapping digestion products for increased redundancy and multiple points of confirmation
- Simple sample preparation protocol without requiring additives to inactivate the enzyme
- Compliance ready informatics for streamlined RNA sequencing mapping
- Higher sequence coverage (>95%) by unique digestion products dramatically decreasing the ambiguity of sequence assignments
- Cap analysis of mRNA 5'-ends feasible from oligonucleotide mapping digests
- Capping efficiency estimation without additional sample preparation
- Poly(A) tail analysis possible from same sample prep.
- Complete characterization of sgRNA or mRNA feasible from a single digest

References

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