SHIMADZU ProtaGene

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Improving LC-MS Analysis of mRNA: Enzymatic Digestion and LC-MS Analysis for Characterization of mRNA Modifications and Sequence Integrity

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1. Introduction

- The rapid advancements in mRNA synthesis and delivery technologies have enabled the development of various mRNA-based vaccines and therapeutics, offering significant benefits in combating human diseases[1]. RNA modifications are crucial for ensuring the efficacy of in vitro transcribed therapeutic mRNAs, as they enhance translation and stability while reducing immunogenicity[2].
- This study shows the integrated analysis of the mRNA encoding the SARS-CoV-2 (COVID) spike protein based on enzymatic digestion with site-specific endoribonucleases to generate unique oligonucleotides, which can be mapped to the expected mRNA sequence during data analysis (**Fig. 1**).

Fig. 1 Workflow of this research

2. Methods

Based on an in-silico digest of mRNA encoding the COVID spike protein with various RNases (T1, MC1, Cusativin, 4, A, U2), most appropriate RNases were selected for enzymatic reactions. Inactivation of RNases was performed by magnetic bead removal (RNase T1) or heat (RNase MC1).

The resulting oligonucleotides were separated using ion-pair reversed-phase HPLC with 0.1% TEA and 1% HFIP (Shimadzu Nexera XSi) and introduced into a Q-TOF mass spectrometer (Shimadzu LCMS-9050) running in DDA negative mode. MS-conditions were adjusted for effective ion transfer and fragmentation of oligos of about 10-30 nucleotides in length.

Data analysis for confident oligo MS/MS identification and mRNA sequence confirmation was performed with Protein Metrics Byos using the Digested Oligos Workflow. 2'3' cyclic phosphate modifications were allowed as variable modifications at the 3' terminal.

3. Results

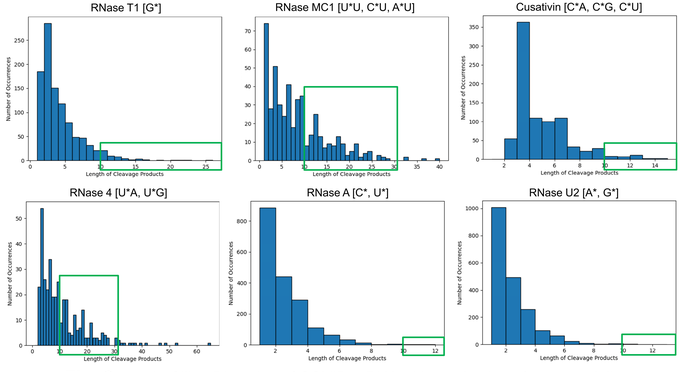

Fig. 2 In-Silico Digestion of COVID mRNA with a selection of commercially available RNases. Highlighted in green are the cleavage products ranging from 10 to 30 nucleotides, most relevant for further analysis.

- RNases U2, A and Cusativin were not considered for digestion of COVID mRNA due to short length of expected oligonucleotides
- RNase MC1 was selected as the first digestion enzyme as it theoretically generates most oligos with preferred length (**Fig. 2**)
- As the second enzyme an RNase T1 - magnetic bead complex was selected over RNase 4 because of the possibility to easily remove the enzyme from the digestion mix and thus stop the enzymatic reaction

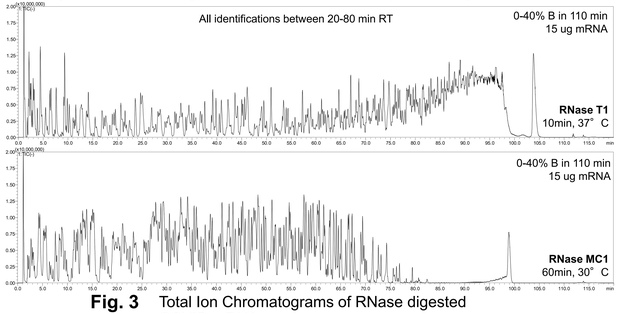

Fig. 3 Total Ion Chromatograms of RNase digested COVID mRNA

- Highest identification rates were observed for RNase T1 after digestion for 10 min at 37 °C and for RNase after 60 min at 30 °C (**Fig. 3**).
- 397 oligonucleotides could be identified (174 for the T1 Digest and 223 for the MC1 Digests) which corresponds to 78% sequence coverage
- Only unique oligonucleotides with at least 8 nucleotides in length, at least 2 observed charge states and a Search Engine Score of >400 were considered

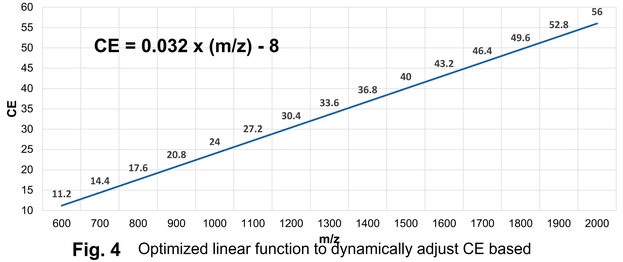

Fig. 4 Optimized linear function to dynamically adjust CE based on precursor mass

- We established a correlation between oligonucleotide precursor mass and the CE required for optimal fragmentation along the oligonucleotide backbone (**Fig. 4**).
- Our data indicate that these optimized conditions resulted in a 30% increase in sequence coverage. Furthermore, the systematic adjustment of CE reduced non-specific fragmentation occurrences, leading to cleaner spectra and fewer ambiguous sequence assignments (**Fig. 5**).

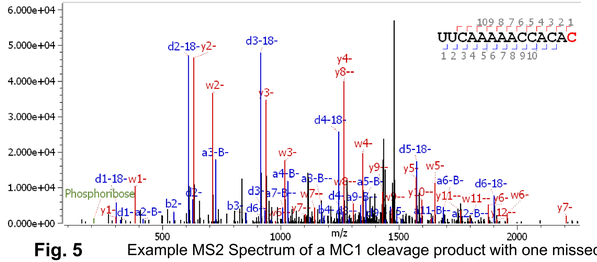

Fig. 5 Example MS2 Spectrum of a MC1 cleavage product with one missed cleavage eluting at 57.5 min. Precursor m/z: 1479.202 (z = -3).

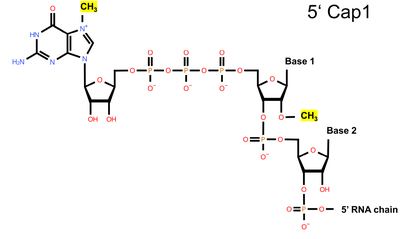

Fig. 6 XIC for the 5' Cap1 Structure of the COVID mRNA

- In order to verify the successful capping of the mRNA, XICs were generated for the expected m/z values considering the enzymes used (**Fig. 6**)
- In the T1 digested sample the Cap1 structure could be identified via specific precursor and fragment ion masses

4. Conclusion

- Our findings underscore the significance of tailored enzymatic digestion and dynamic CE optimization in enhancing the quality of LC-MS analysis of mRNA.
- Overall, our approach effectively addresses the technical challenges associated with full-length mRNA characterization and significantly improves both sequence coverage and the detection of modifications, enabling more comprehensive and reliable analysis.

Reference
1) The Lancet (2024), Vol. 403, 1192–1204, Parhiz & Atochina-Vasserman, *et al.*
2) Nucleic Acids Research (2016) Vol. 44, 7511–7526, Ramanathan, *et al.*

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