Novel sample preparation platform coupled with LCMS for mRNA mass mapping

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Introduction

The emergence of large RNA, including synthetic messenger RNA (mRNA), as a vital class of therapeutics has garnered significant attention. Liquid chromatography coupling with mass spectrometry is a potent analytical tool for mRNA characterization, providing direct sequence mapping and post-translational modification identification. Enzymatic digestion with site-specific ribonuclease is applied to generate short oligoribonucleotides that are more amenable to LC separation and MS detection. In this study, we developed an automated sample preparation workflow that enables controlled mRNA digestion reaction with uridine-specific human endoribonuclease hRNase 4 [1], which is coupled online with LCMS for mRNA mass mapping, as illustrated in Fig 1.

Methods

The CleanCap EPO mRNA (TriLink Biotechnologies) was denatured and loaded onto an Elute UHPLC combined with the SampleStream® Platform (IPT) using an 8 kDa molecular weight cut-off membrane. Subsequently, hRNase 4 and T4 PNK (New England Biolabs) were loaded onto the flow cell. Both the mRNA and the enzyme were incubated at an elevated temperature, with the focusing buffer mobilizing the digested products across the membrane continuously. The digested products were concentrated onto an AdvanceBio Oligonucleotide guard column (Agilent) and analyzed by LC-MS/MS using a timsTOF fleX (Bruker) and a BioPharma Compass prototype (Bruker).

Results

The feasibility of utilizing a flow cell with a MWCO membrane as the chamber for the digestion reaction depends on the size of the MWCO. A recovery study showed that the 8K MWCO membrane was able to effectively retain the large mRNA and the enzyme, allowing the in-flow-cell digestion.

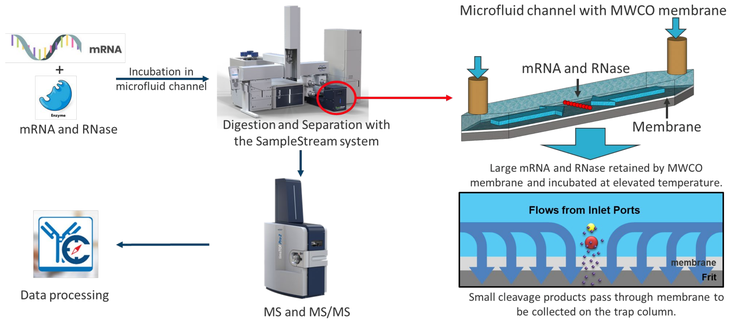

Fig. 1 mRNA sequence mapping workflow with the SampleStream platform

The small cleavage products, typically less than 20nt, were able to pass through the MWCO membrane. Fig 2 shows the recovery of a short 20nt RNA (Integrated DNA Technologies), with more than 80% recovery achieved with 2000 µL of the focusing buffer through the membrane.

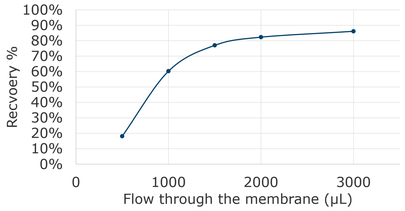

Fig. 2 Recovery rate of the 20 mer RNA vs the volume of the flow through the membrane

HRNase 4 cleaves RNA primarily after U prior to purines, while T4 PNK dephosphorylates the 3’ end of the RNA. Here 2’,3’-hydroxylated cleavage products are searched for sequence coverage. The optimization of the digestion condition was carried out, including the sample/enzyme ratio, flow rate, incubation time, and incubation temperature, using a one-factor-at-a-time approach as presented in Table 1. Varying the loading amount of mRNA or the hRNase 4 enzyme while maintaining the other constant did not lead to significant changes in sequence coverage. Incubation times below 20 minutes resulted in lower sequence coverage. Furthermore, the incubation temperature was found to have an impact on the sequence coverage, with an optimized temperature of 50°C. The results of the mRNA mass mapping at the optimized conditions for all parameters are depicted in Fig 3. A sequence coverage of 82% was achieved for the EPO mRNA with multiple overlapping annotations for the same sequence.

Digestion method	mRNA	hRNAse4	T4PNK	Flow volume	Flow Rate	Reaction Time	Temperature	Sequence Coverage
SampleStream	2 µg	0.5 µL	6.4 µL	2000 µL	38 µL/min	30 min	45 ºC	74.20%
		1.5 µL						79%
		3 µL						77.70%
	0.5 µg	0.5 µL	6.4 µL	2000 µL	38 µL/min	30 min	45 ºC	75.40%
	1 µg							70.60%
	2 µg							74.20%
	1 µg	0.5 µL	6.4 µL	2000 µL	38 µL/min	30 min	45 ºC	69.60%
					57 µL/min	20 min		68.70%
					75 µL/min	15 min		57.60%
	1 µg	0.5 µL	6.4 µL	2000 µL	38 µL/min	30 min	37 ºC	67.20%
							45 ºC	71.80%
							50 ºC	74.60%
							55 ºC	69.10%
							60 ºC	70.40%
In solution	2 µg	1.5 µL	6.4 µL	2000 µL	38 µL/min	30 min	50 ºC	82%
	2 µg	0.5 µL	6.4 µL			60 min	37 ºC	83.30%

Table 1. Summary of the parameters optimized for the digestion condition.

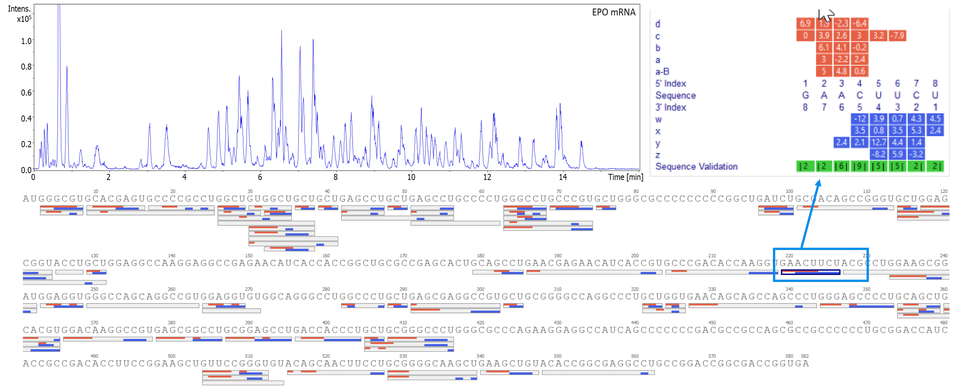

Fig 3. Base peak chromatogram and sequence mapping results of EPO mRNA digestion (2’,3’-hydroxylated products) with the SampleStream equipped system

Conclusion

- Automated sample preparation workflow using the SampleStream platform enables a controlled mRNA digestion reaction with site-specific ribonuclease.
- Various parameters of digestion conditions are optimized for the Elute SampleStream with a high sequence coverage of 82% achieved for EPO mRNA.

Sample preparation for mRNA mass mapping

[1] Wolf, E. J., Grünberg, S., Dai, N., Chen, T. H., Roy, B., Yigit, E., & Corrêa Jr, I. R. (2022). Human RNase 4 improves mRNA sequence characterization by LC–MS/MS. Nucleic Acids Research, 50(18), e106-e106.