5’ mRNA Analysis by Microchip CE-MS Using an Internal Cleavage Motif for RNase H Digestion

Adi Kulkarni[1] • Robert L. Ross[2] • Min Du[2] • Kate Yu[1]
[1]908 Devices, Inc., Boston, MA • [2]Thermo Fisher Scientific, Lexington, MA

REPLIGEN

Repligen Corporation now owns the life sciences PAT product portfolio of 908 Devices Inc. Please contact Repligen for further inquiries.

Highlights

- Microchip CE/MS characterization of mRNA 5’ cap with PATsmart™ ZipChip®/QE
- Easy and clean analysis: no ion pairing reagents, no method development, minimum sample preparation

Introduction

In vitro transcribed messenger RNA (mRNA) is a new class of biotherapeutics. A key quality attribute in production of IVT mRNA is its 5’ cap efficiency. Qualitative analysis can be performed through complementary probe hybridization, followed by RNase H digestion. The RNase H enzyme will cleave the RNA strand in an RNA:DNA duplex. By designing a probe complementary to the 5’ end of the mRNA, it is possible to cleave the 5’ end from the intact mRNA and then isolate using a biotinylated leader and magnetic beads. Recently, this assay is performed with a 25mer probe having a 5-nucleotide motif on the 3’ end of the probe to produce a 20–22mer for analysis. Here, our goal was to investigate an internal DNA motif to produce a shorter digestion product while simultaneously maintaining the length of the probe at 25nt for hybridization specificity. The shorter oligo results in easier detection using Microchip CE-MS approach.

Materials and Methods

Samples

All oligonucleotides were purchased as lyophilized standards from Integrated DNA Technologies Inc. (IDT). **Intact analysis:** The standards were reconstituted with the Oligos diluent (Repligen) to appropriate concentrations prior to analysis. A 25 nt 3’ Bioteg RNA probe (IDT-DNA) was designed complementary to the 5’ end of a commercially available mRNA. The probe contained an internal sequence of five DNA nucleotides with the rest of the strand 2’-O-methylated. Hybridization between probe and mRNA (100 pmol) was performed followed by digestion with 5U RNase H (Thermo Fisher Scientific). 5’ digestion products were purified by streptavidin mag-beads (New England Biolabs), dried, resuspended and analyzed.

Instrumentation

A ZipChip® Device was used for all analyses (Repligen). High Resolution Bare (HRB) Glass Chip and the Oligos BGE (Repligen) were used. An on-chip injection volume was 1 nL with separation run at a field strength of 500 V/cm. MS analysis was performed on a Thermo Fisher Orbitrap QExactive HF mass spectrometer, and data acquisition mode was by ESI+.

Data Processing

The data was visualized using the Freestyle 1.8 data analysis software (Thermo Fisher Scientific). Intact Mass analysis was performed using BioPharma Finder 5.2 (Thermo Fisher Scientific).

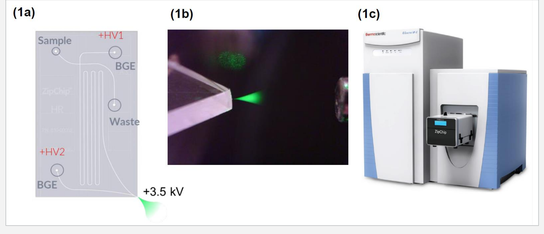

Figure 1. Schematic of the microchip capillary-electrophoresis devices (a); chip spray corner spray illuminated with a green laser (b); ZipChip interface mounted on an Orbitrap Qexactive HF mass spectrometer (c).

Background

RNA is described by its nucleobase joined to a ribose sugar by a glycosidic bond to form a nucleoside. In forming ssRNA, the nucleosides are joined 5’ to 3’ by way of a phosphodiester linkage. During the electrospray process, the oligonucleotide transitions from the liquid phase into the gas phase as an ion.[1] This process is thermodynamically unfavorable as the electronegative backbone makes the oligonucleotide highly hydrophilic and results in the oligonucleotide signal in the mass spectrometer to be suppressed, either through metal adducts left over from droplet evaporation, or from incomplete desolvation. This effect is exaggerated as the oligonucleotide length increases. For mRNA 5’ cap analysis, the use of RNase H digestion has been established using complementary probes ~25 nts in length.[2] Here, we show as a proof of principle, the use of an internal DNA sequence to generate a smaller capped oligonucleotide through RNase H digestion (**Figure 2**).

Results

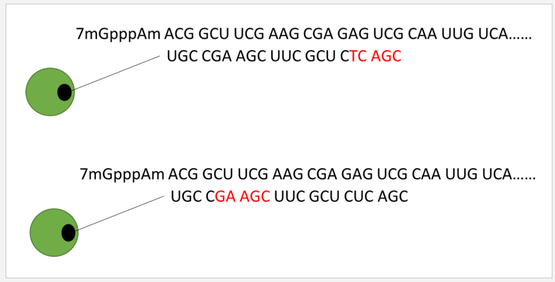

Figure 2. Cartoon showing two 3’ biotinylated probes used for mRNA 5’ capping efficiency assay. The top is representative of a probe for conventional 5’ capping assay using RNase H, where the DNA motif is on the terminal end. The bottom probe is a representation of the one used to generate the work shown here, with the DNA sequence 4 nucleotides down strand (4nt leader) from the 5’ end of the RNA.

The microchip-based ZipChip CE-MS analysis enables a fast (~ 5 min) method for characterization of oligonucleotides in positive ESI mode. Since the Oligos BGE does not denature the oligonucleotides, the dried samples were resuspended in DMSO to ensure denaturation of the cleavage products to enable complete dissociation between complementary strands. This sample was loaded directly on the chip and an on-chip injection volume of 1 nL was used for analysis. By using an internal cleavage motif in the hybridization probe, we were able to generate shorter cleavage products from the 5’ end containing the Cap(1) modifications.

Generation of the shorter digestion product facilitated data analysis through wide separation between target, probe, and any probe failure sequences that may have been carried over in the purification. As an additional advantage, shorter cleavage products also showed lower tendency towards adduction caused by sodium and potassium, improving the sensitivity of the protonated adduct in positive ESI mode.

No additional method development was necessary. Secondly, the ZipChip Oligos BGE does not contain any ion-paring agents which could contaminate a shared instrument’s front end, reducing instrument downtime required for cleaning the mass spectrometer prior to switching applications.

Previously, we have shown that a DNA recognition motif restricted to five nucleotides limits multiple digestion products generated by RNase H at the cleavage site. When restricting the number of nucleotides upstream from the DNA motif to four nucleotides we generate a single cleaved product six nucleotides in length where the RNAse H cleaves between the second and third nucleotide in the DNA;RNA duplex. No other cleaved products were detected. **Figure 3** shows the isotopic peak envelope for the 6mer oligonucleotide generated using the four nucleotide leader in the probe. Clear baseline separation allows for easy deconvolution.

Results (cont.)

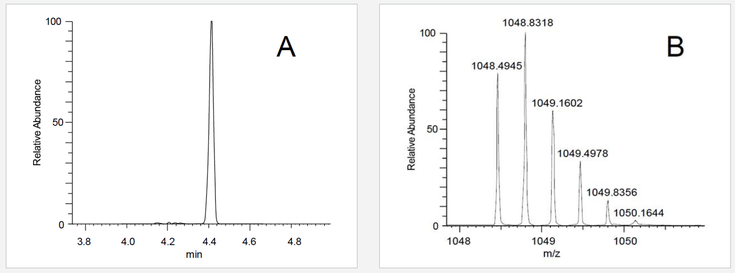

Figure 3. A) Extracted ion chromatogram for mass 1048.49-1050.16 Da. B) Isotopic peak envelope of the 5’ capped oligonucleotide using a DNA cleavage motif four nucleotides from the cap. RNase H cleavage generates a single 6mer oligonucleotide.

Table 1 lists the results from BioPharma Finder Intact Mass analysis of the four as well as a five nucleotide leader. Both short generated oligonucleotides were detected and measured with a matched mass error of ~5 ppm. The five nucleotide digestion product was also cleaved between the 2nd and 3rd DNA nucleotide in the DNA motif strongly implying that the coupling between enzyme and dsRNA is unidirectional. More experiments are planned to test this theory.

When relative abundance between the two generated digestion products was compared there was a clear difference between abundances, suggesting, for this approach, the use of the shorter leader for digestion is preferred. More work is needed to verify this observation.

Component	Digestion Product	Monoisotopic Mass	Theoretical Mass (Da)	Matched Mass Error (ppm)
4 nt leader	6 mer	3142.466	3142.449	5.4
5 nt leader	7 mer	3471.521	3471.501	5.7

Table 1. BioPharma Finder 5.2 Component Table showing comparison results between a 6 mer and a 7 mer digestion product.

Conclusions and Future Work

- mRNA 5’ cap oligonucleotides can be generated using an internal DNA sequence.
- Isotopic peaks from positive mode ZipChip introduction allows for accurate deconvolution.
- RNase H cleavage is possibly unidirectional when cleavage motif is restricted both in length of nucleotide as well as placement near dsRNA terminus.
- Next Step: Investigation of RNase H cleavage near dsRNA terminal.

References

1. Kebarle, P., Tang, L. From Ions in Solution to Ions in the Gas Phase - the Mechanism of Electrospray Mass Spectrometry. *Anal. Chem.* **1993**, 65 (22), 972A-986A.
2. Beverly, M., Dell, A., Parmar, P., Houghton, L. Label-Free Analysis of MRNA Capping Efficiency Using RNase H Probes and LC-MS. *Anal Bioanal Chem* **2016**, 408 (18), 5021–5030.
3. Ross, R. L., Murphy, K., Zhang, Y., Du, M. Characterization of MRNA 5’ Capping Products Using an LCHRAM-MS/MS Analytical Platform and Thermo Scientific BioPharma Finder Software Solution. https://view.highspot.com/viewer/64755cfd3f017e8f75b89c7e.

Learn more at **repligen.com/zipchip**