Oligonucleotide Workflow Incorporating Nuclease-Conjugated Beads with LC-MS/MS

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Abstract

This research aims to demonstrate a sample preparation workflow for partially digesting large RNA molecules using an immobilized nuclease on magnetic beads, specifically RNase T1, for subsequent mass spectrometric (MS) analysis. The study compares the efficiency and control of reaction progression of RNA digestion using enzyme-conjugated beads versus in-solution digestion. After incubation of the enzyme-substrate, the reaction was terminated either by removing beads using a strong magnet, or by addition of $MgCl_2$ for enzyme-in-solution experiments. The cleavage site of RNase T1 on conjugated beads matched the predicted digestion products for all analyzed RNA samples, indicating that the coupling of RNaseT1 with the beads did not affect the specificity of the enzymatic reaction. When compared to in solution reactions, on-bead RNaseT1 digestions were better able to produce partial digestion products. This allows the facile generation of larger RNA fragments for improved sequencing by MS.

Introduction

The success of mRNA COVID vaccines during the COVID-19 pandemic has propelled a wave of RNA innovations both in industry and academia. These advances in technology have opened the door to new treatment methodologies previously unavailable for many diseases. This has led to the need for testing various higher molecular weight RNA samples using a fast, precise, and accurate method of analysis. Many nucleases such as RNase T1 possess predictable but high frequency cleavage motifs which results in very small digestion products following complete digestion. Control over the extent of digestion is therefore critical for generating reproducible and informative sequencing results via tandem mass spectrometry. As a result, we have developed a sample preparation workflow (Figure 1) for partially digesting RNA molecules using an immobilized nuclease on magnetic beads (Dynabeads™) for subsequent mass spectrometric analysis.

Materials and methods

Sample Preparation

Figure 1 illustrates the entire sample preparation procedure for the digestion of RNA. Briefly, a simplified model RNA of 50 bases in length with known cleavage motifs (3Gs in the middle) was obtained from Integrated DNA Technologies (IDT). ~1µg RNA samples were digested using 5µl of Thermo Scientific™ RNase T1 (in-solution) or Thermo Scientific™ Dynabeads™ (RNaseT1 on beads) each at 1unit/µL for 10min in a thermal mixer set to 37°C and 1400 rpm. After the digestion, reactions were quenched either by removing the immobilized enzyme beads using a magnet or through the addition of 100mM $MgCl_2$ to the solution digestions.

HPLC Conditions

2 µL (80ng) of each digested sample was injected onto a Thermo Scientific™ DNAPac™ RP column and separated chromatographically using IP-RP (ion pair reverse phase) buffer systems on Thermo Scientific™ Vanquish™ Horizon™ UHPLC system. The mobile phase was composed of 10mM dibutylamine (DBA) and 25mM hexafluoro-2- propanol (HFIP) in water (mobile phase A) and acetonitrile (mobile phase B) and used a column temperature of 80°C. A fourteen min active gradient of 0 to 37.5% B was used for chromatographic separation.

MS Conditions

For all experiments, data was collected in negative mode using full scan at a resolution setting of 120,000. Eluting RNA was isolated and fragmented data dependently using resonant CID at a normalized collision energy of 25% at an activation *q* of 0.15. Subsequent MS^2 spectra were collected using a decision tree method[1]. Precursor ion target and number of microscans was optimized for each analyte based on their size by using m/z and charge state filters

Data Analysis

Thermo Scientific™ BioPharma Finder™ (Version 5.2) was used for data interpretation. Assignments were performed using tandem MS and searching spectra with a ±10 ppm mass tolerance along with filters to show only theoretical digest matching sequences.

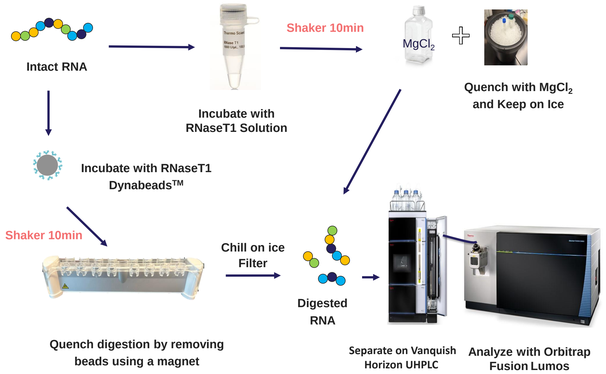

Figure 1. Oligonucleotide digestion workflow for RNase T1 immobilized on Dynabeads™ vs in-solution. One µg of RNA was incubated with 5 units of enzyme for 10min at 37°C while shaking continuously. Cleavage of RNA and isolation of cleaved fragments by magnetic beads after 10 min for Dynabeads™ was followed by 0.2-micron centrifuge filtration and $MgCl_2$ termination for in-solution was performed before LC-MS analysis.

Results and Discussion

To understand the full extent of enzymatic digestion, we designed a simplified model RNA of 50 bases in length with 3 cleavage motifs. The model was designed to have an uncharacteristically low guanine content to better generate large digestion products even when the digestion is run to completion. Figure 2 illustrates the possible digestion products from this model system. Complete digestion can produce 4 digested products (blue) while missed cleavages from partial digestion will produce an additional 5 partial digestion products (green). All product ions are sufficiently large such that they can be reasonably monitored in an LC-MS experiment.

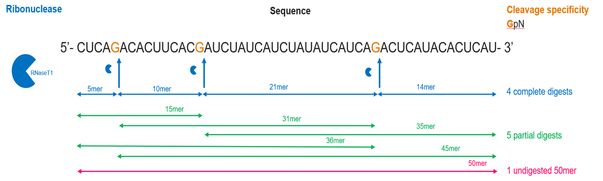

Figure 2. Theoretical digest of model RNA. Complete digestion products (blue) are produces when all theoretical cleavage sites are digested whereas partial products (green) include one or more missed cleavages and undigested 50mer (pink)

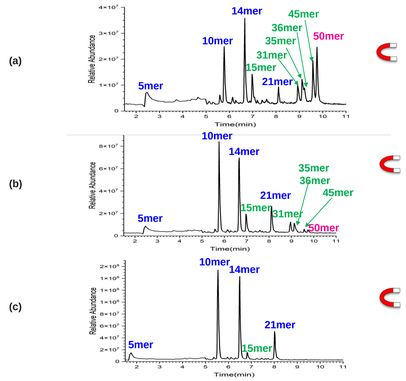

Figure 3. Representative chromatograms for (a) 10 minute (b) 30 minute and (c) 120 minute digestions using Dynabead™ RNase T1. Note the absence of most of the incomplete digestion products (green) in 120 minute.

Using this model system, we evaluated the ability to produce partial digestion product by modifying the incubation time with RNase T1 conjugated beads. Figure 3 depicts annotated chromatograms resulting from analyzing digestions performed at different bead incubation times. Shorter incubation times show clear evidence of larger, partial digestion products. These products are ideal because these larger digestion products are both more likely to be unique, retain on an LC column, and partially overlap within the sequence, all of which contribute to improved identification of the original sequence[2]. For longer reaction times, the reaction is driven to completion and are comparatively absent of partial digestion products (Figure 3c), demonstrating that controlling the incubation time is required to produce the partial digestion.

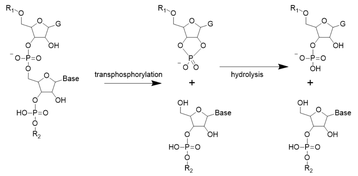

Figure 4. Mechanism of cleavage of RNaseT1. Cleavage mechanism for RNase T1. Digestion occurs 3' to guanine residues to produce one 5' hydroxyl reaction product and one 3' monophosphate.

Notably, the resulting digestion products from Dynabeads™ were consistent with an incomplete cleavage mechanism from RNase T1. This mechanism (Figure 4) canonically proceeds through two steps, the first of which produces a cyclic phosphate through transphosphorylation of the phosphate 3' to guanine and the second of which hydrolyzes the cyclic phosphate to form a 3' terminal phosphate[3]. Although all expected cleavage sites were observed within the analysis, all RNA fragments cleaved at their 3' were observed in their cyclic phosphate form rather than the terminal phosphate (Figure 3) produced by the full T1 mechanism. Even the longest digestion time which produced otherwise complete digestion products still produced exclusively cyclic phosphate products. This suggests that even at the longer digestion conditions, the reaction is still proceeding in a controlled fashion such that the final hydrolysis step is prevented. This is critical for correct data interpretation following this protocol.

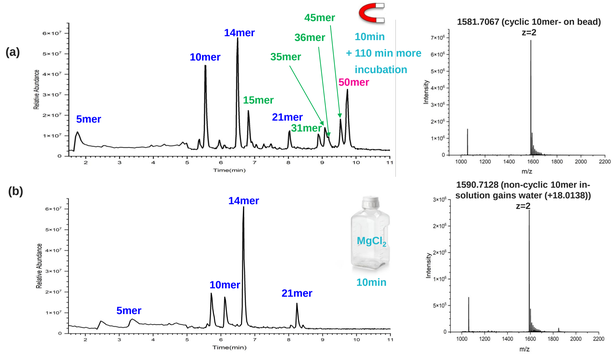

Figure 5: Chromatograms for RNase T1 digestions performed a) on Dynabead™ and b) in solution and quenched after 10 minutes by either removing the beads or adding MgCl2, respectively, and incubated for 110 more minutes.

To better evaluate the control over the digest, the ability to quench the on-bead digestion was compared to in-solution digestion. A major limitation in performing limited digestions is the ability to reproducibly arrest the reaction. This can lead to irreproducibility or a complete inability to stop the reaction. Figure 5a depicts the resulting chromatogram following a 10 minute digestion and 110 minute post-digestion quench. The on-bead digestion demonstrates fine control over the digestion; the chromatogram is nearly identical to the previous 10 minute digestion, indicating that the digestion can be effectively quenched and is highly reproducible. Conversely, the in-solution digest resulted in a complete digestion in 10 minute. Importantly, this digestion proceeded so completely that most observed products were in their terminal phosphate form rather than the cyclic phosphate seen even in the longest on-bead incubation times. This may be the result of a failed (or incomplete) quench by $MgCl_2$ or a marked difference in digestion kinetics between on-bead and in solution digestions. Further experiments using shorter digestion times or lower enzyme concentrations will be required to disentangle these possibilities.

Conclusions

- RNase immobilized on beads offers a much better control of digestion, enabling limited digestion for larger digestion products.
- Removal of RNase at the end of digestion (by magnet) easily quenches the reaction and eliminates system contamination. Current RNA in-solution digestion is difficult to stop.
- Future prospect include conjugation of other types of RNases on Dynabeads™

References

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