

## Comparative Analysis of Ribonucleic Acid Digests (CARD) by Mass Spectrometry

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### Abstract

We describe the comparative analysis of ribonucleic acid digests (CARD) approach for RNA modification analysis. This approach employs isotope labeling during RNase digestion, which allows the direct comparison of a tRNA of unknown modification status against a reference tRNA, whose sequence or modification status is known. The reference sample is labeled with  $^{18}\text{O}$  during RNase digestion while the candidate (unknown) sample is labeled with  $^{16}\text{O}$ . These RNase digestion products are combined and analyzed by mass spectrometry. Identical RNase digestion products will appear in the mass spectrum as characteristic doublets, separated by 2 Da due to the  $^{16}\text{O}/^{18}\text{O}$  mass difference. Singlets arise in the mass spectrum when the sequence or modification status of a particular RNase digestion product from the reference is not matched in the candidate (unknown) sample. This CARD approach for RNA modification analysis simplifies the determination of differences between reference and candidate samples, providing a route for higher throughput screening of samples for modification profiles, including determination of tRNA methylation patterns.

**Key words** Modified nucleosides, RNA sequencing, tRNA, rRNA, Modified bases, Tandem mass spectrometry, LC-MS/MS, MALDI-MS/MS, Isotope labeling, Epitranscriptome

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## 1 Introduction

Transfer RNAs (tRNAs) are adapter molecules that decipher the genetic code via codon-anticodon interaction. In eukaryotes, tRNA is transcribed as a precursor molecule (pre-tRNA) by RNA polymerase III, which undergoes processing to generate the mature tRNA. Among the processing steps, the 5' leader sequence is cleaved by RNase P while the 3' trailer sequence is removed by RNase Z. A conserved CCA sequence is attached to the 3' end, which serves as a site of amino acid attachment [1]. In addition, tRNAs are chemically modified by enzyme-mediated processes either on the nucleobase or on ribose [2, 3]. Modifications can be a simple methylation or can utilize complex multistep pathways involving many enzymes. Each individual tRNA will typically

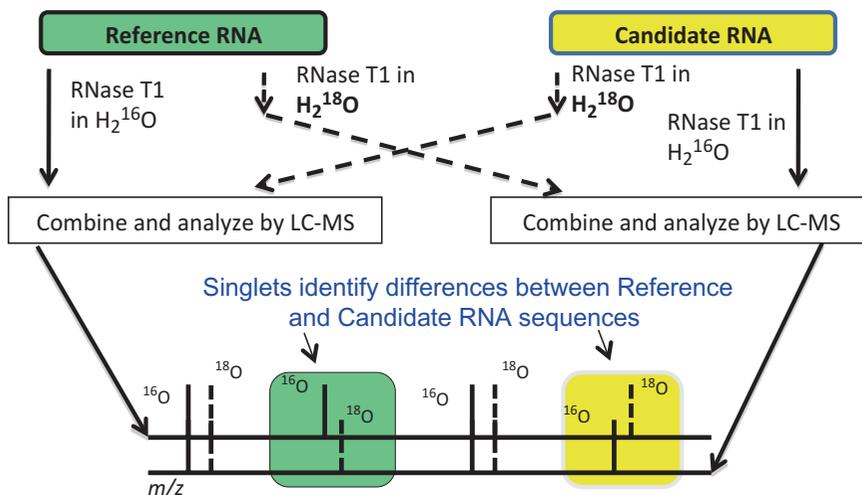
contain multiple modified nucleosides. Depending on where modified nucleosides are located in the sequence of the tRNA, each modification could impact decoding accuracy, help in the proper folding of the tRNA, or serve as determinants of aminoacyl synthetase [4–6]. Recent evidence has also shown that tRNA modifications are dynamic, responding to environmental stress and impacting cellular translation [7, 8].

Several methods have been developed for the characterization of posttranscriptional chemical modifications on tRNAs. These methods include, but are not limited to, reverse transcriptase polymerase chain reaction (RT-PCR) [9, 10], electrophoresis [11], next-generation sequencing (NGS) [12], and mass spectrometry (MS) [13, 14]. Mass spectrometry-based RNA characterization has been shown to be a powerful analytical approach for identifying modified nucleosides and placing each modification in the appropriate tRNA sequence context. Each modification (with the exception of pseudouridine) increases the mass of the canonical nucleoside being modified, allowing mass spectrometry to easily identify the presence and type of such modifications.

The most commonly used mass spectrometry method for placing modified nucleosides onto tRNA sequences is an RNA modification mapping approach [15, 16]. The tRNA is digested to smaller oligonucleotides using a base-specific ribonuclease (RNase), such as RNase T1, which cleaves after unmodified guanosine residues. These oligonucleotides can be separated by reversed-phase high-performance liquid chromatography (RP-HPLC) and detected by mass spectrometry or tandem mass spectrometry (MS/MS). We recently introduced a variant of this RNA modification mapping approach that takes advantage of sequence conservation to readily identify differences in tRNA modification profiles [17–19]. This approach, referred to as the comparative analysis of RNA digest (CARD) method, employs isotope labeling during RNase digestion to differentiate known and unknown tRNA sequences and modification profiles.

A general overview of the CARD method is illustrated in Fig. 1. The RNA endonuclease cleavage mechanism generates a cyclic phosphate intermediate where oxygen from water can be incorporated onto the 3' phosphate. If the RNase digestion is conducted using isotopically labeled water ( $\text{H}_2^{18}\text{O}$ ), the  $^{18}\text{O}$  label gets incorporated to the 3' phosphate, thereby increasing the mass of the digestion product by 2 Da as compared with digestions conducted in normal (light) water ( $\text{H}_2^{16}\text{O}$ ). This comparative approach requires a reference tRNA of known modification status [20–22] or an unmodified version of the tRNA of interest (e.g., an *in vitro* transcript) [23].

The tRNA to be analyzed can be isolated from tissue, bacterial cell pellets, or cultured cells. Typically, the reference tRNA (or tRNA mixture) is labeled with  $^{16}\text{O}$  while the sample tRNA (or tRNA mixture) is labeled with  $^{18}\text{O}$ . If the digestion product

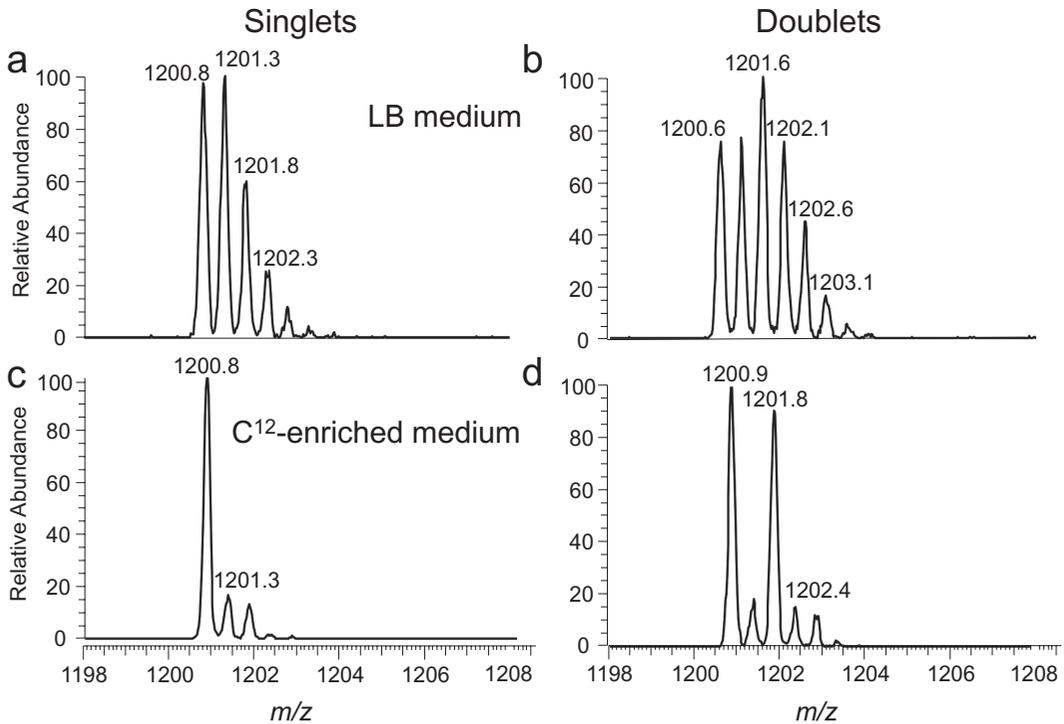


**Fig. 1** Schematic outline of comparative sequencing by isotope labeling and LC-MS using a known reference RNA and a candidate (unknown) RNA to be comparatively sequenced. RNA endonuclease digestion products that are equivalent between the two samples will appear as doublets (separated by 2 Da) in the mass spectral data; digestion products that are different between the two samples will appear as a singlet. Reprinted with permission from “Method for comparative analysis of ribonucleic acids using isotope labeling and mass spectrometry” (2012) *AnalChem*84: 8607–8613. [17] Copyright 2012 American Chemical Society

sequences are identical, those digestion products will have the same mass and appear in the mass spectrum as a characteristic doublet, separated by 2 Da due to the  $^{16}\text{O}/^{18}\text{O}$  mass difference (Fig. 2). The doublet ratio also provides quantitative information relating to each tRNA. When a singlet is detected, then the sequence or posttranscriptional modification status of the sample tRNA differs from that of the reference tRNA.

As singlets often reveal useful information about the state of the sample tRNA, they can be further analyzed to characterize the nature of the singlet. A common step is to reverse the labeling steps, so that the reference is labeled with  $^{18}\text{O}$  and the sample is labeled with  $^{16}\text{O}$ . If the singlet arises from the reference, the mass will increase by 2 Da after reverse labeling, while singlets from the sample will decrease in mass by 2 Da (Fig. 3). Further, singlets can be characterized by MS/MS to confirm the sequence and location of any modification changes.

The CARD approach has been used to characterize total tRNA modification profiles for closely related organisms, using the well-characterized *Escherichia coli* total tRNA pool as the reference. Moreover, by using DNA hybridization probes, the CARD approach can be developed into a targeted assay that would identify populations that lack one or more tRNA modifications. In particular, the CARD approach is a useful tool for rapidly screening for methylation changes present in gene deletion mutants or for examining RNA methylation patterns across samples obtained



**Fig. 2** Improvements in singlet and doublet identification using  $^{12}\text{C}$ -enriched medium as illustrated with the doubly charged *E. coli* total tRNA RNase T1 digestion product A[ms $^{26}\text{A}$ ]AACCGp (MW 2403.4 Da). **(a)** Mass spectrum from sample grown in LB medium and labeled with  $^{16}\text{O}$  during RNase T1 digestion. **(b)** Same sample as in except labeled with both  $^{16}\text{O}$  and  $^{18}\text{O}$  during RNase T1 digestion. **(c)** Mass spectrum obtained when sample grown in  $^{12}\text{C}$ -enriched medium and labeled with  $^{16}\text{O}$  during RNase T1 digestion. **(d)** Same sample as in except labeled with both  $^{16}\text{O}$  and  $^{18}\text{O}$  during RNase T1 digestion. Singlet and doublet identifications are simplified in c and d, respectively, by the use of  $^{12}\text{C}$ -enriched medium. Reproduced from Ref. [19] with permission of Springer

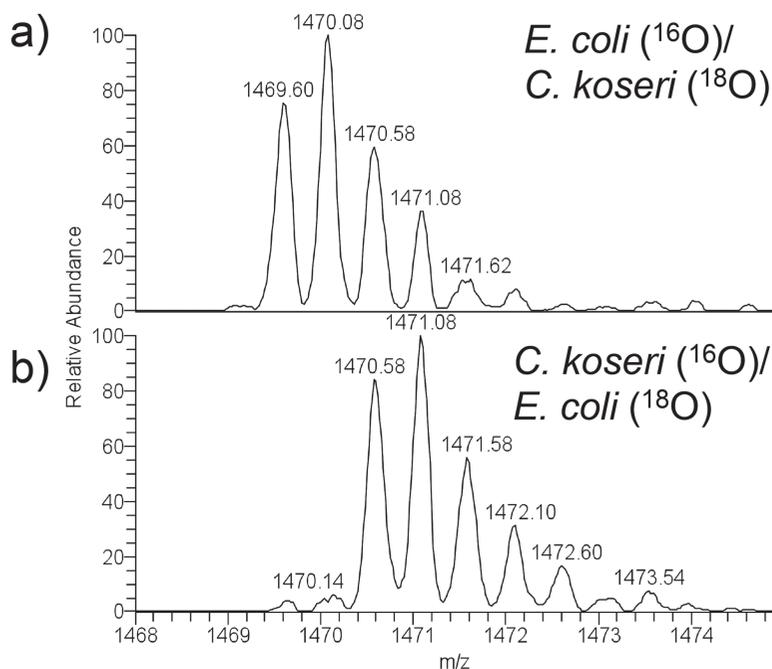
from multiple populations. The protocol below describes the essential sample preparation steps required for successful CARD analysis of RNA methylation or complete modification profiles. Details on the mass spectrometry conditions can be obtained from a number of relevant publications [15, 17–19, 24].

## 2 Materials

All reagents used for RNA extraction and isolation should be reagent grade or higher. Glassware and water should be autoclaved to minimize RNase contamination. Gloves should be worn at all times.

### 2.1 Samples

CARD analysis is applicable to samples isolated from a variety of biological sources. The method is illustrated using both bacterial cell cultures and human tissue samples.



**Fig. 3** Mass spectra corresponding to a detected singlet when (a) total tRNA from *Citrobacter koseri* is labeled with  $^{18}\text{O}$  during RNase T1 digestion and (b) *E. coli* is labeled with  $^{18}\text{O}$ . The singlet U[s<sup>4</sup>U]AACAAAGp ( $m/z$  1469.6, 2- charge) arises from *E. coli* tRNA-Cys(GCA) as confirmed by the +1 increase in the  $m/z$  isotopic envelope after  $^{18}\text{O}$ -labeling of *E. coli*. Reproduced from Ref. [18] with permission from The Royal Society of Chemistry

## 2.2 RNA Extraction and Purification

1. TRI-reagent<sup>®</sup>.
2. Chloroform.
3. MonoExpress medium ( $^{12}\text{C}$  99.95 %): 10× concentrate (Cambridge Isotope Laboratories).
4. Lysozyme buffer: 25 mM Tris-HCl, pH 7.5, 60 mM KCl, 10 mM  $\text{MgCl}_2$ .
5. 2 mm ceramic beads.
6. NucleoBond<sup>®</sup> PC 2000 column.
7. Equilibration buffer: 100 mM Tris-acetate, 15 % ethanol, 200 mM NaCl.
8. Wash buffer: 100 mM Tris-acetate, 15 % ethanol, 400 mM NaCl.
9. Elution buffer: 100 mM Tris-acetate, 15 % ethanol, 750 mM NaCl.
10. Streptavidin agarose beads.
11. 20× saline sodium citrate solution (SSC): 3 M NaCl, 0.3 M sodium citrate. 6× and 3× SSC is diluted from this stock.

12. Specific tRNA elution buffer: 0.1× SSC, 0.1 % SDS.
13. 7.5 M Ammonium acetate.
14. 95 % absolute ethanol.
15. Lysozyme.

### 2.3 CARD Reference Sample: In Vitro Transcribed tRNA

While reference tRNA samples can be generated from the previously characterized sample of known tRNAs [20–22], the method is described using in vitro transcribed tRNA samples.

1. Template DNA, 10 ng/μL.
2. Forward and reverse primer, 100 μM.
3. 10× PCR buffer: 15 mM MgCl<sub>2</sub>, 500 mM KCl, 100 mM Tris-HCl, pH 8.3.
4. *PfuI* DNA polymerase: 2.5 U/μL.
5. 40 mM dNTPs mixture: 10 mM each dNTP.
6. QIAquick® PCR clean up kit.
7. Ampliscribe™ T7 high yield transcription kit, which includes: 10× reaction buffer, 100 mM rNTPs, 100 mM dithiothreitol, Riboguard® RNase inhibitor, T7 enzyme solution.
8. In vitro transcription cleanup (e.g., MEGAClear™ Transcription clean up kit).

### 2.4 RNA Sample Digestion Components

1. Ammonium acetate (NH<sub>4</sub>OAc): 220 mM; mass spectrometry grade or higher.
2. Water-<sup>18</sup>O, 97 %.
3. RNase T1 enzyme. The enzyme is purified by acetone precipitation overnight at –20 °C (300 μL acetone/700 μL enzyme solution). Remove the supernatant and resuspend the enzyme with 1 mL sterile water. Condition the Sep.-Pak C18 column with 3 mL acetonitrile. Equilibrate the column with 3 mL water. Slowly pass the enzyme solution through the column, collecting eluent. Repeat four times with eluent. Wash the column with 1 mL water. Elute the enzyme with 75 % acetonitrile. Aliquot the eluted enzyme (20 μL ~1000 U) in a microcentrifuge tube. Take into dryness and store at 4 °C.
4. Sample RNA (*see* Subheading 2.1).
5. Reference RNA (*see* Subheading 2.1 or 2.3).

### 2.5 HPLC Mobile and Stationary Phases for Mass Spectrometry

1. Solvent A: 200 mM Hexafluoro-2-propanol (HFIP), 8 mM triethyl amine (TEA).
2. Solvent B: 50 % solvent A (final concentration: 100 mM HFIP, 4 mM TEA), 50 % methanol.
3. C18 column, 5 μm pore size; 4.6 mm × 125 mm

## 2.6 Other Instrumentation

1. Mini Bead-Beater.
2. Vortex mixer.
3. Microfuge.
4. Vacuum drying apparatus.
5. UV-vis spectrophotometer.
6. Thermocycler.
7. Liquid chromatography tandem mass spectrometry system.

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## 3 Methods

### 3.1 RNA Isolation

One key to successful CARD mass spectrometry is appropriate sample preparation and purification, due to the confounding nature of salts and buffers on the analysis [25]. The CARD method as described below can be used with RNA isolated from a variety of cell or tissue types. Representative examples of appropriate tRNA sample preparation are provided for bacterial cells and human tissue.

#### 3.1.1 Bacterial Cell Cultures

Bacterial cells can be grown in Luria-Bertani (LB) media following a standard protocol [26]. However, a significant improvement in data analysis has been shown when cells are grown in a  $^{12}\text{C}$  enriched/ $^{13}\text{C}$  depleted medium (MonoExpress Medium; Fig. 2). This approach reduces spectral interferences arising from naturally occurring  $^{13}\text{C}$  isotopes, leading to significantly improved detection of singlets and doublets.

1. Pelletize the harvested bacterial cells and add 4.0 mL lysozyme buffer for every 200 mL culture.
2. Add lysozyme in a 5mg/mL buffer ratio. Incubate at 37 °C for 30 minutes.
3. For every 200  $\mu\text{L}$  of suspension, add 1 mL of TRI-reagent<sup>®</sup>. Vortex the solution and incubate for 10 min at room temperature.
4. Add chloroform (200  $\mu\text{L}$  per 1 mL Tri-Reagent<sup>®</sup>), invert by shaking and then incubate at room temperature for 10 min.
5. Spin the sample in a microcentrifuge for 15 min at 16,000  $\times g$ . The clear, upper layer contains total RNA.
6. Remove the aqueous phase and transfer to a clean, sterile microcentrifuge tube (*see Note 1*).
7. Add an equal volume of isopropyl alcohol.
8. Incubate at -20 °C from 1 h to overnight.
9. Precipitate the RNA by spinning the sample in a microcentrifuge for 5 min at 16,000  $\times g$ . Decant liquid and then wash the pellet with 1–2 volumes 75 % ethanol to remove residual salts.

10. Remove the ethanol and allow pellet to air dry.
11. Dissolve pellet in minimal amount of sterile water (~500  $\mu\text{L}$ ). Quantify the RNA isolated by checking the absorbance at  $A_{260}$  and  $A_{280}$  nm (*see Note 2*).

### 3.1.2 Tissue Samples (Placenta)

1. Remove the umbilical cord and other connective tissues. Slice into smaller pieces using sterile scalpel (*see Note 3*).
2. Wash with 100 mM  $\text{CaCl}_2$  to remove blood clots.
3. For every ~2 mg placenta, add 600  $\mu\text{L}$  of TRI-reagent<sup>®</sup>. Transfer in a tube containing 1.4 mm ceramic beads. Homogenize in a bead beater for 15 min at room temperature or below. Spin the sample in a microcentrifuge for 20 min at  $16,000 \times g$  and 4 °C.
4. Transfer the pinkish layer to a clean 1.5 mL microcentrifuge tube, add 200  $\mu\text{L}$  chloroform and vortex to facilitate mixing. Let it stand for 20 min at room temperature.
5. Spin the sample in a microcentrifuge for 15 min at  $16,000 \times g$ . The clear, upper layer contains total RNA.
6. Remove the aqueous phase and transfer to a clean, sterile microcentrifuge (*see Note 1*).
7. Add an equal volume of isopropyl alcohol.
8. Incubate at  $-20$  °C from 1 h to overnight to precipitate RNA.
9. Precipitate the RNA by spinning the sample in a microcentrifuge for 5 min at  $16,000 \times g$ . Decant liquid and then wash the pellet with 1–2 volumes 75 % ethanol to remove residual salts.
10. Remove the ethanol and allow pellet to air dry.
11. Dissolve pellet in minimal amount of sterile water (~1000  $\mu\text{L}$ ). Quantify the RNA isolated by checking the absorbance at  $A_{260}$  and  $A_{280}$  nm (*see Note 2*).

### 3.2 Transfer RNA Enrichment

The following enrichment procedure has been optimized for a Nucleobond<sup>®</sup> 2000 RX column, which has a binding capacity of ~2000 mg RNA.

1. Condition the column with 50 mL of equilibration buffer.
2. Dilute total RNA sample with two volumes of equilibration buffer (20 mL equilibration buffer/10 mL total RNA solution).
3. Load sample onto column and collect the flow through. Repeat this step twice to maximize binding and yield.
4. Add  $2 \times 50$  mL of the wash buffer.
5. Elute transfer RNA from the column by adding  $3 \times 20$  mL of a warm elution buffer (*see Note 4*).
6. Precipitate tRNA by adding an equal volume of isopropyl alcohol to the collected sample (column eluent) and incubate at  $-20$  °C from 1 h to overnight.

7. Pelletize the tRNA by spinning the sample in a centrifuge for 20–30 min at  $16,000 \times g$ . Decant liquid and then wash the pellet with 1–2 volumes 75 % ethanol to remove residual salts.
8. Remove the ethanol and allow pellet to air dry. For CARD analysis using total tRNA samples, *see* **Note 5**.
9. Dissolve pellet in minimal amount of sterile water ( $\sim 1000 \mu\text{L}$ ). Quantify the RNA isolated by checking the absorbance at  $A_{260}$  and  $A_{280}$  nm (*see* **Note 2**).
10. Check the integrity of the sample by analyzing 2  $\mu\text{g}$  of the tRNA on a 1.5 % agarose gel electrophoresis.

### **3.3 Isolation of a Single tRNA by Oligonucleotide Affinity Probe (Optional)**

For CARD analyses focused on single tRNA species, an oligonucleotide affinity probe can be used to isolate the target tRNA from total RNA or total tRNA prepared above.

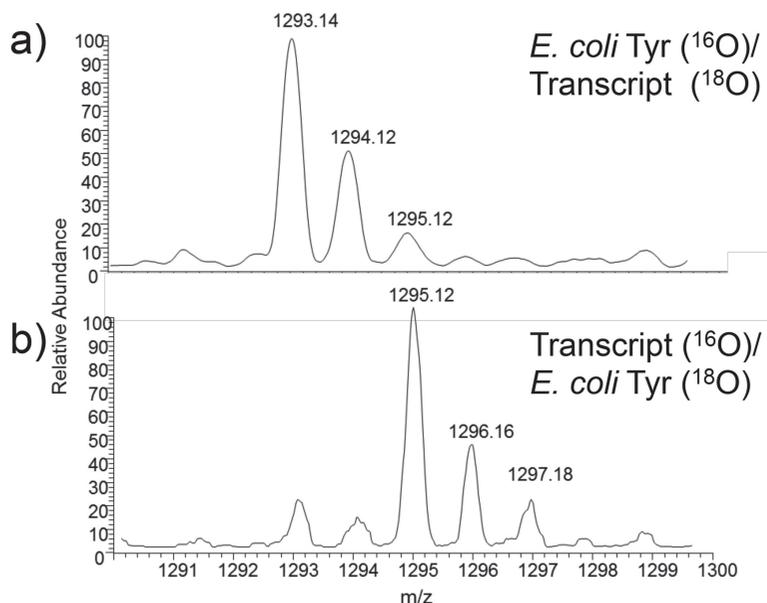
1. Wash 125  $\mu\text{L}$  of streptavidin agarose beads with 750  $\mu\text{L}$  of  $6\times$  SSC. Spin using a microfuge for 10 min and remove the supernatant. Repeat three times. Resuspend the beads in 375  $\mu\text{L}$   $6\times$  SSC.
2. Heat at 70 °C for 5 min a solution containing up to 10  $\mu\text{g}$  of the 5'-biotinylated oligodeoxynucleotide probe, designed to be complimentary to a region of the target tRNA of interest (*see* **Note 6**).
3. Add the heated probe to the washed beads. Incubate at room temperature using a shaker for 1 h.
4. Remove the unbound probe by washing the beads twice with  $6\times$  SSC.
5. Prepare the sample RNA by adding sufficient  $20\times$  SSC to create a final concentration of  $6\times$  SSC. Warm the sample RNA to 85 °C for 10 min and then lower to 70 °C.
6. Add the beads from **step 4** to the RNA sample. Hold solution at 70 °C for 10 min.
7. Cool the mixture to 37 °C and incubate from 2 h to overnight.
8. After incubation, spin sample using a microfuge for 10 min and remove the supernatant containing unbound RNA.
9. Wash the beads with 750  $\mu\text{L}$  of  $3\times$  SSC and 750  $\mu\text{L}$   $1\times$  SSC for increased stringency in hybridization.
10. Elute the bound tRNA by incubating the beads at 80 °C in 500  $\mu\text{L}$  elution buffer for 5 min.
11. Remove the beads by spinning the sample in a microfuge at  $16,000 \times g$  for 10 min. Decant 400  $\mu\text{L}$  of the supernatant containing the elution buffer and probe-purified RNA (*see* **Note 7**). Save sample for further analysis.
12. (Optional) **Steps 10** and **11** can be repeated up to five times to increase the amount of probe-purified tRNA. If steps are repeated, add only 400  $\mu\text{L}$  of the elution buffer before the 5 min incubation at 80 °C. Combine all samples.

13. Precipitate tRNA by adding 1/3rd volume 7.5 M ammonium acetate and two volumes absolute ethanol. Incubate overnight at  $-80\text{ }^{\circ}\text{C}$ .
14. Spin the samples in a microcentrifuge for 20–30 min at  $16,000 \times g$ . Decant liquid and then wash the pellet with 1–2 volumes 75 % ethanol to remove residual salts.
15. Remove the ethanol and allow pellet to air dry.
16. Dissolve pellet in minimal amount of sterile water ( $\sim 50\text{ }\mu\text{L}$ ). Quantify the tRNA isolated by checking the absorbance at  $A_{260}$  and  $A_{280}\text{ nm}$  (*see Note 2*). As necessary and if sufficient sample is available, check sample integrity by gel electrophoresis.

### 3.4 Reference tRNA from *in Vitro* Transcription (Optional)

The reference RNA sample for CARD analysis can be from a variety of sources. For applications examining the modification status of a particular tRNA, an *in vitro* transcript of the tRNA sequence serves as an appropriate unmodified reference (Fig. 4). The template used for the protocol described below is the PCR product from the amplification of a gene block. The gene of interest is downstream the T7 RNA polymerase promoter sequence.

1. Design a gene block with the following: forward primer sequence, T7 RNA polymerase promoter region, gene sequence of a specific RNA, and reverse primer.



**Fig. 4** A modified RNase T1 digestion product from *E. coli* tRNA Tyr detected as a singlet during CARD analysis using an *in vitro* transcript of *E. coli* tRNA Tyr as the reference. **(a)** Mass spectrum arising from the RNase T1 digestion product [ $m^5\text{U}$ ] [ $\Psi$ ]CGp ( $m/z$  1293.1 Da,  $-1$  charge state) when labeled with  $^{16}\text{O}$ . **(b)** The same singlet detected after reverse labeling using  $^{18}\text{O}$  for the unknown. The  $+2$  Da shifts confirm that this singlet is modified and arises from the *E. coli* tRNA Tyr

2. Prepare a PCR master mix from the following recipe (*see Note 8*).
  - 33  $\mu\text{L}$  sterile water.
  - 4  $\mu\text{L}$  10 $\times$  PCR buffer.
  - 0.8  $\mu\text{L}$  40 mM dNTPs.
  - 0.4  $\mu\text{L}$  Forward primer.
  - 0.4  $\mu\text{L}$  Reverse primer.
  - 0.4  $\mu\text{L}$  DNA polymerase (*PfuI*).
3. In one tube, add 1  $\mu\text{L}$  of the gene block template. For the negative control, add 1  $\mu\text{L}$  of sterile water.
4. Perform standard PCR reaction.
5. Run an aliquot of the PCR reaction on a 1.5 % agarose gel with standard DNA marker. Visualize the DNA with ethidium bromide staining under UV (*see Note 9*).
6. Purify the remaining PCR products using a commercial clean-up kit following manufacturer's instruction. The amplified gene block is used as the template for T7 RNA transcription.
7. Prepare the transcription reaction by assembling the components as listed. A single reaction has a total volume of 20  $\mu\text{L}$  and is maintained by addition of sterile water.

Sterile water.

- 1  $\mu\text{g}$  DNA template (PCR product).
  - 2  $\mu\text{L}$  10 $\times$  reaction buffer.
  - 1.5  $\mu\text{L}$  100 mM ATP.
  - 1.5  $\mu\text{L}$  100 mM GTP.
  - 1.5  $\mu\text{L}$  100 mM CTP.
  - 1.5  $\mu\text{L}$  100 mM UTP.
  - 2  $\mu\text{L}$  100 mM dithiothreitol.
  - 0.5  $\mu\text{L}$  RNase inhibitor.
  - 2  $\mu\text{L}$  T7 RNA polymerase (2.5 U/ $\mu\text{L}$ ).
8. Incubate the reaction at 37 °C for 2 h.
  9. Purify and elute the transcripts using a commercial clean-up kit following manufacturer's instruction.
  10. Resuspend the purified transcript in sterile water (~ 20  $\mu\text{L}$ ) and verify integrity of transcript using 1.5 % agarose gel.

### **3.5 CARD Sample Preparation and Enzymatic Digestion**

1. Add 1  $\mu\text{g}$  reference tRNA (if single tRNA) or 10  $\mu\text{g}$  reference tRNA (if RNA mixture such as total tRNA) to a 500  $\mu\text{L}$  microcentrifuge tube, add half total volume of 220 mM ammonium acetate, and take to dryness. Resuspend reference in 5  $\mu\text{L}$   $\text{H}_2^{18}\text{O}$ .

2. Add 1  $\mu\text{g}$  sample tRNA (if single tRNA) or 10  $\mu\text{g}$  sample tRNA (if RNA mixture such as total tRNA) to a 500  $\mu\text{L}$  microcentrifuge tube, add half total volume of 220 mM ammonium acetate and take to dryness. Resuspend the sample in 5  $\mu\text{L}$   $\text{H}_2^{16}\text{O}$ .
3. Transfer the contents into separate tubes, each containing RNase T1 enzyme (50 U enzyme/ $\mu\text{g}$  RNA ratio) (*see Note 10*). Incubate for 2 h at 37 °C, take to dryness, and then reconstitute in 10  $\mu\text{L}$  HPLC solvent A. Store at 4 °C until analysis by HPLC-MS/MS
4. The labeling approach (**steps 1 and 2**) can be reversed (reference in  $^{16}\text{O}$  and sample in  $^{18}\text{O}$ ) as needed.

### 3.6 HPLC-MS/MS Analysis

The LC-MS/MS conditions are described for standard microbore LC connected in-line with a mass spectrometer. Any commercial LC-MS/MS system is compatible with CARD MS, although best results are obtained using linear ion trap, q-TOF, or FT mass spectrometers.

1. The flow rate of the HPLC should be at 30  $\mu\text{L}/\text{min}$ .
2. Prepare the LC system by equilibrating the column in 95 % solvent A and 5 % solvent B.
3. The gradient is as follows: start with 5 % B and then increase to 20 % after 5 min, hold at 20 % B for 2 min, and then increase to 50 % B at 0.7 % B/min. Reequilibrate the column at 5 % B for at least 20 min before the next sample injection.
4. Combine 2.5  $\mu\text{L}$  of reference tRNA digest with 2.5  $\mu\text{L}$  sample tRNA digest and inject total amount (5  $\mu\text{L}$ ) on column.
5. Instrument appropriate mass spectrometer settings should be used for the negative polarity analysis of RNase T1 digestion products. When possible, data-dependent acquisition of MS/MS spectra should be acquired for confirmation of detected singlets and doublets (as needed) [24].
6. Vendor-specific software can be used for data processing and analysis. Singlets and doublets can be identified manually or using open source software (e.g., MZMine [27]).

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## 4 Notes

1. Observe caution in removing the aqueous phase. It is best to remove only ~95 % of the upper layer so as not to introduce contamination of genomic DNA.
2. For a 1-cm path length, one  $A_{260}$  unit is equal to 40  $\mu\text{g}/\text{mL}$  RNA. Pure RNA will have an  $A_{260}/A_{280}$  ~2.0. Protein or phenol contamination will have a higher  $A_{260}/A_{280}$  ratio.
3. It is best to work with fresh tissue as much as possible. However, if immediate extraction is not possible, the tissue can be “flash frozen” with liquid nitrogen and stored at -80 °C.

4. Long RNAs can be eluted from the column using a flush buffer (100 mM Tris–acetate, 15 % ethanol, 1500 mM NaCl). The column can also be reused several times. After the flush buffer, wash the column with distilled water and follow **step 1** again. Store the column in 50 % ethanol at 4 °C.
5. Salt adducts (i.e., Na<sup>+</sup> or K<sup>+</sup>) confound mass spectrometry analysis. They can be minimized by re-precipitating the RNA solution with ammonium acetate (NH<sub>4</sub>OAc). For every 100 μL volume of the solution, add 33.3 μL of 7.5 M NH<sub>4</sub>OAc and 250 μL ethanol. Incubate the sample at –80 °C. Spin the sample in a microcentrifuge for 25 min at 16,000 × *g*. Repeat twice.
6. A 25–30 nucleotide long DNA probe is recommended for high specificity to the target tRNA.
7. Be very cautious in removing the supernatant so as not to disturb the agarose beads. If bead contamination is suspected, spin the sample in a microcentrifuge for 15 min at 16,000 × *g*. The beads will pelletize at the bottom. Carefully remove the supernatant and proceed to **step 13**.
8. The PCR buffer has to be warmed up at 37 °C for at least 5 min. All the other components should be on ice.
9. In some cases, there can be more than one product generated in the PCR or in vitro transcription reaction. If this happens, load the entire PCR reaction to an agarose gel and separate. The full-length product (based on the DNA marker) can be excised from the gel and eluted using commercial kits.
10. Back-exchange is typically not a problem during LC-MS/MS analysis of RNase T1 digestion products. However, if back-exchange is suspected, RNase T1 can be taken to dryness prior to the addition to the <sup>18</sup>O–containing solution. This is most easily accomplished by drying the required amount of RNase T1 in a microcentrifuge tube and adding the RNA and H<sub>2</sub><sup>18</sup>O into the tube containing dried RNase T1.

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