Video Article

An *In Vitro* Assay to Detect tRNA-Isopentenyl Transferase Activity

Antonio E. Chambers*[1], Adam E. Richardson*[1], David F. Read[2], Thomas J. Waller[3], Douglas A. Bernstein[1], Philip J. Smaldino[1]

[1]Department of Biology, Ball State University

[2]Department of Genome Sciences, University of Washington

[3]Department of Molecular, Cellular, and Developmental Biology, University of Michigan

*These authors contributed equally

Correspondence to: Philip J. Smaldino at pjsmaldino@bsu.edu

URL: https://www.jove.com/video/58100
DOI: doi:10.3791/58100

Keywords: Biochemistry, Issue 140, RNA modification, tRNA, isopentenyl transferase, MOD5, TRIT1, i^6A, i^6A37, DMAPP

Date Published: 10/8/2018

Citation: Chambers, A.E., Richardson, A.E., Read, D.F., Waller, T.J., Bernstein, D.A., Smaldino, P.J. An *In Vitro* Assay to Detect tRNA-Isopentenyl Transferase Activity. *J. Vis. Exp.* (140), e58100, doi:10.3791/58100 (2018).

Abstract

N^6-isopentenyladenosine RNA modifications are functionally diverse and highly conserved among prokaryotes and eukaryotes. One of the most highly conserved N^6-isopentenyladenosine modifications occurs at the A37 position in a subset of tRNAs. This modification improves translation efficiency and fidelity by increasing the affinity of the tRNA for the ribosome. Mutation of enzymes responsible for this modification in eukaryotes are associated with several disease states, including mitochondrial dysfunction and cancer. Therefore, understanding the substrate specificity and biochemical activities of these enzymes is important for understanding of normal and pathologic eukaryotic biology. A diverse array of methods has been employed to characterize i^6A modifications. Herein is described a direct approach for the detection of isopentenylation by Mod5. This method utilizes incubation of RNAs with a recombinant isopentenyl transferase, followed by RNase T1 digestion, and 1-dimensional gel electrophoresis analysis to detect i^6A modifications. In addition, the potential adaptability of this protocol to characterize other RNA-modifying enzymes is discussed.

Video Link

The video component of this article can be found at https://www.jove.com/video/58100/

Introduction

At least 163 distinct posttranscriptional RNA modifications have been identified, with these modifications conferring diverse and context-dependent functions to RNAs, directly influencing RNA structure, and affecting interactions of RNA with other molecules[1,2]. As the appreciation for the number and variety of RNA modifications increases, it is critical to develop assays that can reliably interrogate both the RNA modifications and the enzymes that catalyze them.

One of the first RNA modifications to be identified occurs at base 37 in tRNAs, adjacent to the anti-codon on the 3' side[3,4]. An isopentenyl group is transferred from dimethylallylpyrophosphate (DMAPP) to the N6 position of adenosine 37 (i^6A37) [3,5] on a subset of both cytoplasmic and mitochondrial tRNAs. i^6A37 improves translation fidelity and efficiency by increasing the tRNA's affinity for the ribosome[4,6] and i^6A37 is important for stress response in bacteria[7]. The enzymes that perform this modification are termed tRNA isopentenyl transferases and are highly conserved in bacteria[8,9], fungi[10], worms[11], plants[12], and higher eukaryotes[13], including humans[14].

Mutations in the human tRNA isopentenyl transferase gene, *TRIT1*, are associated with human disease. For example, a mutation in *TRIT1* is correlated with a severe mitochondrial disease, likely caused by a defect in mitochondrial protein synthesis[15,16]. Furthermore, *TRIT1* has been described as a tumor suppressor gene[17,18] and is implicated in several types of cancers including melanoma[19], breast[20], gastric[21], and lung cancers[22,23]. Finally, TRIT1 and Mod5 (*Saccharomyces cerevisiae*) isopentenyl transferases are aggregation-prone proteins that form prion-like amyloid fibers[24,25,26]. These observations potentially implicate tRNA isopentenyl transferases in neurodegenerative diseases, although direct evidence for this has not yet been shown.

Given the role that isopentenyl transferases play in translation and disease, methods that directly measure i^6A isopentenyl transferase activity are important for a mechanistic understanding of these enzymes under normal and disease states. An increasing number of methods are available to detect i^6A RNA modifications, including *in vitro* isopentenylation assays, positive hybridization in the absence of i^6A (PHA6) assays, thin layer chromatography (TLC), amino acid acceptance activity assays, and mass spectrometry approaches (Reviewed in Ref. [4]).

An *in vitro* isopentenylation assay has been described that utilizes ^{14}C-DMAPP and unlabeled tRNAs. In this assay, radioactive carbon is transferred to RNA from ^{14}C-DMAPP by the isopentenyl transferase. While this assay is highly sensitive, it is often difficult to determine the specific residue that is modified[9,20,27]. PHA6 assays rely on the bulky i^6A modification interfering with hybridization of a ^{32}P-labeled probe spanning the modified residue. As such, hybridization is greater in the absence of an i^6A modification[18,28,29]. PHA6 assays are highly sensitive, and capable of analyzing total RNA extracted from cellular lysates. Additionally, the ability to design probes specific to the RNA of interest gives

Copyright © 2018 Journal of Visualized Experiments

this method substantial target flexibility. However, PHA6 assays are limited to the characterization of modifications that occur on residues within the targeted region of the probe and therefore are less likely to identify novel modification sites. In addition, as absence of binding is indicative of modification, other modifications or mutations that affect RNA binding will confound data analysis.

Another approach combines benzyl DEAE cellulose (BD) cellulose chromatography with amino acid acceptance activity as a readout of i^6A modifications in tRNA[30]. This approach directly assays the function of the i^6A modification, but it is an indirect approach to detect i^6A modification and lacks resolution to map modifications to a specific residue in the RNA. A TLC approach has been used to detect total tRNA i^6A modifications. In this approach, internally ^{32}P-labeled tRNAs are digested to single nucleotides and two-dimensional TLC analysis is used to identify isopentenylation. This approach is highly sensitive in detecting total i^6A in a given RNA sample but upon digestion, all sequence information is lost; thus, the investigator has no way of determining which residues have been modified[31].

More recently, liquid chromatography-tandem mass spectrometry (LC-MS/MS) methods have been developed that quantitatively compare total RNA modifications between species, cell types, and experimental conditions[32,33,34]. A limitation of this methodology is that it is less able to determine the identity and position within the RNA from which the modified nucleoside was derived[34]. Furthermore, the expertise and equipment necessary to execute these experiments limit the practicality of this approach.

In addition, several next-generation sequencing technologies have been developed to map RNA modifications transcriptome-wide[34]. Immunoprecipitation of RNAs with antibodies specific to a particular modification (RIP-Seq) enable the investigator to identify all sequences containing a specific modification[35,36]. Additionally, reverse transcriptase-based approaches such as Chem-Seq and non-random mismatch sequencing rely on perturbations of the reverse transcription reaction at the modified residues[37,38,39]. Despite the advantage of these techniques to map RNA modifications transcriptome-wide, RIP-seq and Chem-Seq technologies are limited by the lack of reliable antibodies or reactive chemicals available for each specific modification, respectively[34]. Furthermore, reverse transcriptase enzymes required to perform Chem-Seq and non-random mismatch sequencing techniques can be impeded by stable RNA structures. The highly modified and structurally stable nature of tRNAs make them especially difficult to interrogate using these techniques. To date, next-generation sequencing-based technologies have not yet been utilized to map i^6A modifications[34].

Herein, we describe a simple and direct approach to detect i^6A tRNA modifications *in vitro*. This method utilizes incubation of RNAs with recombinant *S. cerevisiae* isopentenyl transferase (Mod5), followed by RNase T1 digestion, and 1-dimensional gel electrophoresis analysis to map i^6A modifications. This approach is direct and requires little specialized expertise to analyze the data. Furthermore, this method is adaptable to other RNA modifying enzymes, including enzymes that covalently change the molecular weight of RNA or an RNA's mobility through a gel.

Protocol

NOTE: The protocol was adapted from Ref. [24].

1. Obtain RNA and Enzyme of Interest

1. Use *in vitro* transcribed RNAs internally labeled with ^{32}P, and recombinant His6-Mod5 expressed in and purified from *E. coli*, as previously described[24].
    1. Introduce *in vitro* transcribed RNAs (section 3) using T7 RNA polymerase in the presence of unlabeled ATP, UTP, CTP, GTP and 10 µCi of gel-purified, ethanol-precipitated α-ATP, resuspended in 1x TE (10 mM Tris-HCl pH 7.5, 0.1 mM EDTA), and stored at -80 °C[24].
2. Alternatively, obtain commercially available fluorescently labeled RNAs of interest. Produce the enzyme(s) of interest in any preferred expression system.
   Caution: Internal fluorescent tags in the RNA can alter RNA structure and recognition by enzymes.

2. Prepare a 20% Polyacrylamide Denaturing Gel

Note: In order to obtain sufficient resolution of RNA fragments, a 40 cm length vertical slab gel is recommended. The width of the gel used is determined by the number of samples to be analyzed.

1. Thoroughly clean glass plates. First, wash with soap and water, rinse well with deionized water, and finally clean with isopropanol and lint-free wipes.
2. Assemble the plates and spacers.
3. Mix the following reagents to make 100 mL of 20% acrylamide, 7.5 M urea, 1x TBE gel: 80 mL of urea gel concentrate (237.5 g/L of acrylamide, 12.5 g/L of methylene bisacrylamide, 7.5 M urea in deionized water), 10 mL of urea gel diluent (7.5M urea in deionized water), and 10 mL of urea gel buffer (0.89 M Tris-Borate-20 mM EDTA buffer pH 8.3 and 7.5 M urea).
   NOTE: The volume of gel solution must be adjusted according to the dimensions of gel.
4. Add 40 µL of N,N,N′,N′-Tetramethylethylenediamine (TEMED) and 800 µL of freshly prepared 10% ammonium persulfate (APS).
5. Draw gel solution up into a large syringe and dispense between glass plates. Tap on glass with fingers while pouring to prevent bubble formation.
6. Allow gel to solidify for 30 min.
7. Clamp solidified gel onto vertical gel apparatus using binder clips.
8. Fill upper and lower buffer chambers with 1x TBE.
9. Two hours prior to loading the gel, pre-run the gel at 20 mA, for 2 h to allow the buffer boundary to outrun the smallest oligonucleotides - otherwise the smallest nucleotides and oligonucleotides tend to collapse at the buffer front.

3. RNA Isopentenylation Assay

1. Prepare reactions in a final volume of 17 µL, containing 58 mM Tris-HCl (pH 7.2), 1.2 mM ATP, 5.8 mM $MgCl_2$, 0.2 mM DMAPP, 10 U of RNase inhibitor (*e.g.*, SuperRNaseIn), 40,000 CPM of internally ^{32}P-labeled RNA, 5.3 µM Mod5, and 1.2 mM 2-mercaptoethanol.
2. Incubate reactions at 37 °C for 1 h.
3. Ethanol-precipitate RNAs using 2.5 volumes (42.5 µL) of 100% ethanol and 1/10 volumes (1.7 µL) of 3.5 M sodium acetate pH 5.5, and place at -20 °C for 1 h or overnight.
4. Centrifuge RNA samples for 20 min at 15,400 x g and 4 °C.
5. Carefully remove the supernatant and wash the RNA pellet with 500 µL of 70% ethanol.
6. Centrifuge samples at for 5 min 15,400 x g and 4°C.
7. Carefully remove the supernatant and air-dry RNA pellets for 15 min, or until all ethanol has evaporated.
8. Resuspend RNA pellets in 10 µL of 8 M urea.
9. Add 150 U of RNase T1 and incubate at 37 °C overnight.
10. Add 2 µL of 6x loading buffer (60% glycerol, 0.1% xylene cyanol).
11. Load 10 µL of each RNA sample on a pre-run, 20% polyacrylamide, 7.5 M urea gel (see section 2 of Protocol).
    NOTE: Radiolabeled RNA size ladders may be included as an additional mobility marker.
12. Run gel for 2 h at 25 mA.
13. Stop gel and remove from apparatus.
14. Break seal between the two glass plates and remove one of the glass plates, with the gel remaining on the "bottom" plate.
    NOTE: Take care not to tear the gel during this step.
15. Place a layer of plastic wrap over the gel and expose on a phosphor screen for 3 h. Alternatively, place gel on chromatography paper and dry with a gel dryer prior to phosphor screen exposure.
16. Image phosphor screen on a phosphor imager.

Representative Results

Mod5 was incubated with a tyrosine tRNA or serine tRNA in the presence or absence of DMAPP. Following the modification reaction, products were RNase T1-digested, which cleaves the 3' end of all guanosines leaving a 3' guanosine monophosphates (GMP)[24] (**Figure 1**). Full digestion of the RNAs produces a predictable pattern of radiolabeled fragments (**Figure 2A**), which are then resolved on a 20% polyacrylamide denaturing gel. The transfer of an isopentenyl group from DMAPP to the RNA causes a mobility shift of the fragment containing the modified residue (**Figure 1**).

This protocol reliably detects isopentenylation of both canonical and non-canonical tRNA residues modified by Mod5[24]. For example, Mod5 is predicted to modify a subset of tRNAs, which contain the previously described AAA_{36-38} sequence requirement[10], including the tyrosine tRNA and serine tRNA used in this study. Mod5 modifies the predicted residue in the presence of DMAPP as is indicated by the shifted 10 nt AAA_{36-38} containing fragment in the tyrosine tRNA (**Figure 2B**). Similarly, when the serine tRNA is incubated with Mod5 and DMAPP, a complete shift of the 10 nt AAA_{36-38} containing fragment is observed (**Figure 2C**). Interestingly, a partial shift of a 7 nt fragment is observed that does not contain the AAA_{36-38} (**Figure 2C**). These data suggest that the AAA_{36-38} sequence and structure are not required for Mod5 *in vitro* activity; however, future studies using LC-MS/MS or other methods are required to confirm the exact chemical nature of the modification.

Copyright © 2018 Journal of Visualized Experiments

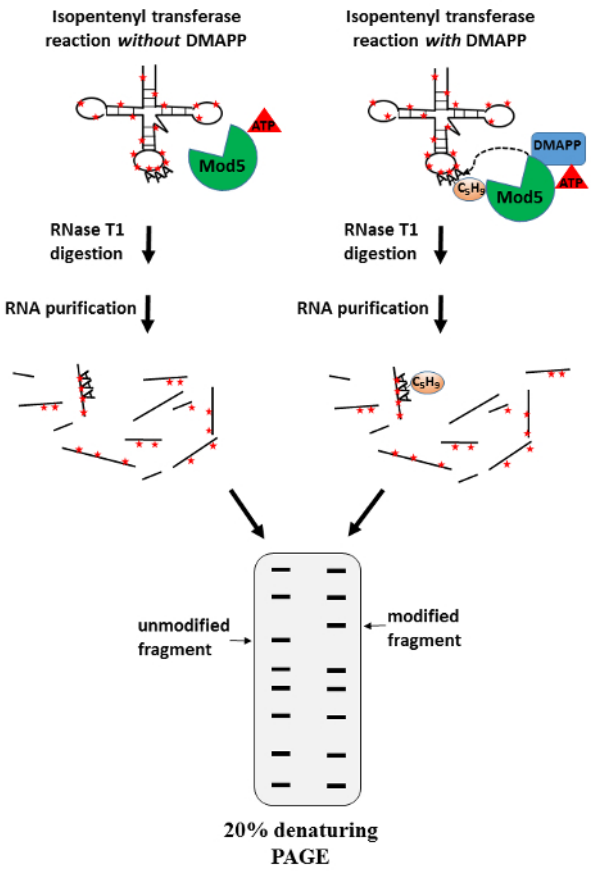

Figure 1: Illustration of isopentenyl transferase assay. tRNA, internally labeled with ^{32}P-adeonsine, is shown incubated with *S. cerevisiae* tRNA isopentenyl transferase (Mod5), and ATP with or without DMAPP. Positions A36-A38 are indicated adjacent to the anticodon region. Red asterisks represent radiolabeled nucleotides. Following incubation, RNAs are digested with RNase T1, extracted, and resolved by 20% denaturing-PAGE. The transfer of an isopentenyl group from DMAPP to the tRNA is indicated by a retarded band during electrophoresis. Please click here to view a larger version of this figure.

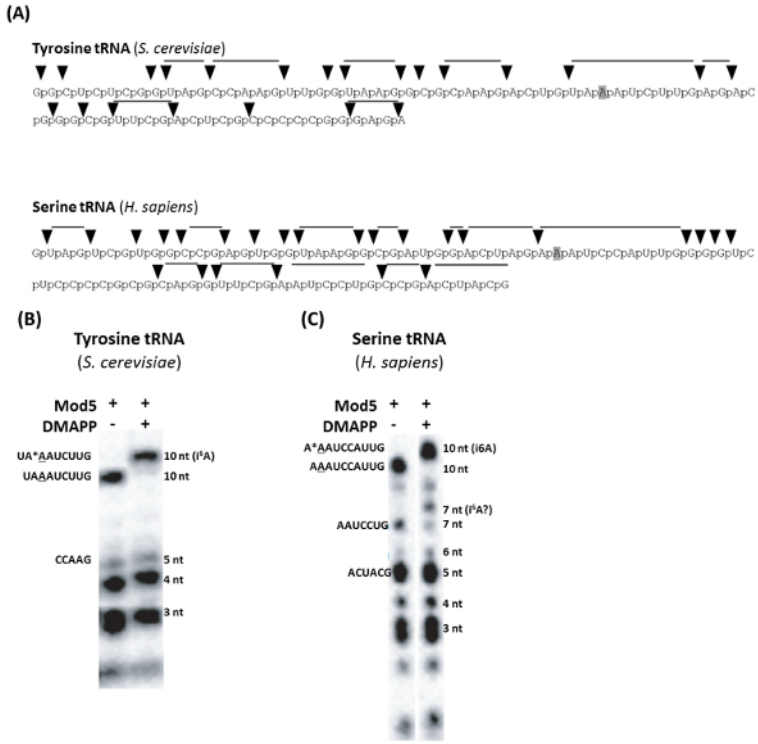

Figure 2: RNase T1 digestion map and representative results of an isopentenyl transferase assay. (A) Black triangles represent RNase T1 cleavage sites, and black lines above the triangles represent resulting fragments that contain at least one ^{32}P-labeled adenosine. Grey highlighted residues are predicted i^{6}A modification sites (*i.e.,* A37). (B) Tyrosine tRNA and (C) serine tRNA were internally labeled with ^{32}P-adenosine. The *S. cerevisiae* isopentenyl transferase, Mod5, was incubated with each RNA in the presence or absence of DMAPP. The RNAs were then digested with RNase T1 and resolved on denaturing-PAGE. Shifted bands dependent on DMAPP indicate the presence of a modified RNA residue. The predicted modification site, A37, is underlined, and modified A37 residues are indicated with an asterisk. An unanticipated and novel modification site is identified and indicated as "i^{6}A?". This figure has been modified from Read *et al.*[24] with permission. Please click here to view a larger version of this figure.

Discussion

RNA modifications continue to be shown to play ever more important and diverse roles in cellular and organismal function. As such, the development of assays to interrogate RNA modifying enzymes is central to better understanding the fundamental aspects of biology. This protocol describes a high-resolution *in vitro* assay to characterize the tRNA modification activity of Mod5.

This protocol has the distinct advantage of providing a direct, and easily interpretable readout of isopentenylation. The protocol described allows for robust biochemical characterization of isopentenyl transferases. Furthermore, this system can be used with enzyme variants or modified RNA substrates, allowing for direct determination of roles that specific residues or domains have on modification. To gain even greater resolution, this protocol could readily be adapted to include parallel digestions with other RNases, such as RNase P1 and/or RNase A, which cleave at all four nucleotides or at C and U, respectively.

A limitation of this assay is that it is relatively low-throughput and thus fewer RNAs that can be practically analyzed compared to RNA-sequencing and mass spectrometry approaches[34]. Therefore, it is not recommended that this protocol be used for those who aim to identify RNA modification sites transcriptome-wide. This protocol is most useful to investigators who are interested in examining a specific RNA modifying enzyme with particular RNAs of interest. However, many transcriptome wide methodologies used to identify RNA modifications require covalent addition of a chemical moiety to a modified nucleotide. This method provides a cheap and efficient assay where one could test modification protocols on an individual substrate to optimize chemical modification before committing to a transcriptome-wide effort[40]. Although this protocol provides a simple and direct assay to detect isopentenyl transferase activity, as it is described here, only the percent modified of the total RNA fragment can be calculated and compared between groups. Researchers interested in making quantitative enzyme activity comparisons must first calculate the units of activity per concentration of enzyme.

Success of this method relies heavily on a few critical steps. It is important that the integrity of the RNA of interest is confirmed prior to the isopentenylation. Degradation of the RNA sample prior to the isopentenyl transferase assay could have a significant effect on RNA modification and confound data interpretation. Furthermore, such degradation is difficult to detect after the RNase T1 digestion has taken place. Therefore, it is recommended that RNA integrity is checked by denaturing PAGE. Furthermore, to fully and accurately characterize the extent of RNA modification, it is essential to ensure RNase T1 digestion has proceeded to completion. Additional RNases, such as RNase P1 and/or RNase A, may be used to digest RNAs in parallel with RNase T1 to increase the resolution of this assay. Radiolabeled oligonucleotide ladders of known length and sequence and undigested RNA samples can be used to assess digestion. Lastly, for some enzymes, the specificity of modification

Copyright © 2018 Journal of Visualized Experiments

depends on the RNA being in the "correctly" folded state. While most tRNAs synthesized *in vitro* fold into structures resembling their *in vivo* structure, this is less certain for other classes of RNAs, particularly with longer RNAs[41].

Although the protocol described is specific to Mod5 isopentenylation of tRNAs, this method could be easily adapted to characterize other RNA-modifying enzymes that covalently add a chemical moiety that significantly alters the molecular weight or gel mobility of the RNA.

Disclosures

None to disclose.

Acknowledgements

We would like to thank Dr. David Engelke for his guidance and helpful comments on this manuscript. PJS - Ball State University laboratory startup funds; DAB - grant 1R15AI130950-01.

References

1. Boccaletto, P. *et al.* MODOMICS: a database of RNA modification pathways. 2017 update. *Nucleic Acids Research.* **46** (D1), D303-d307 (2018).
2. Lewis, C. J., Pan, T., & Kalsotra, A. RNA modifications and structures cooperate to guide RNA-protein interactions. *Nature Reviews: Molecular and Cellular Biology.* **18** (3), 202-210 (2017).
3. Soll, D. Enzymatic modification of transfer RNA. *Science.* **173** (3994), 293-299 (1971).
4. Schweizer, U., Bohleber, S., & Fradejas-Villar, N. The modified base isopentenyladenosine and its derivatives in tRNA. *RNA Biology.* **14** (9), 1197-1208 (2017).
5. Persson, B. C., Esberg, B., Olafsson, O., & Bjork, G. R. Synthesis and function of isopentenyl adenosine derivatives in tRNA. *Biochimie.* **76** (12), 1152-1160 (1994).
6. Urbonavicius, J., Qian, Q., Durand, J. M., Hagervall, T. G., & Bjork, G. R. Improvement of reading frame maintenance is a common function for several tRNA modifications. *EMBO Journal.* **20** (17), 4863-4873 (2001).
7. Aubee, J. I., Olu, M., & Thompson, K. M. The i6A37 tRNA modification is essential for proper decoding of UUX-Leucine codons during rpoS and iraP translation. *RNA.* **22** (5), 729-742 (2016).
8. Caillet, J., & Droogmans, L. Molecular cloning of the Escherichia coli miaA gene involved in the formation of delta 2-isopentenyl adenosine in tRNA. *Journal of Bacteriology.* **170** (9), 4147-4152 (1988).
9. Soderberg, T., & Poulter, C. D. Escherichia coli dimethylallyl diphosphate:tRNA dimethylallyltransferase: essential elements for recognition of tRNA substrates within the anticodon stem-loop. *Biochemistry.* **39** (21), 6546-6553 (2000).
10. Dihanich, M. E. *et al.* Isolation and characterization of MOD5, a gene required for isopentenylation of cytoplasmic and mitochondrial tRNAs of Saccharomyces cerevisiae. *Molecular and Cellular Biology.* **7** (1), 177-184 (1987).
11. Lemieux, J. *et al.* Regulation of physiological rates in Caenorhabditis elegans by a tRNA-modifying enzyme in the mitochondria. *Genetics.* **159** (1), 147-157 (2001).
12. Golovko, A., Sitbon, F., Tillberg, E., & Nicander, B. Identification of a tRNA isopentenyltransferase gene from Arabidopsis thaliana. *Plant Molecular Biology.* **49** (2), 161-169 (2002).
13. Warner, G. J., Rusconi, C. P., White, I. E., & Faust, J. R. Identification and sequencing of two isopentenyladenosine-modified transfer RNAs from Chinese hamster ovary cells. *Nucleic Acids Research.* **26** (23), 5533-5535 (1998).
14. Golovko, A., Hjalm, G., Sitbon, F., & Nicander, B. Cloning of a human tRNA isopentenyl transferase. *Gene.* **258** (1-2), 85-93 (2000).
15. Kernohan, K. D. *et al.* Matchmaking facilitates the diagnosis of an autosomal-recessive mitochondrial disease caused by biallelic mutation of the tRNA isopentenyltransferase (TRIT1) gene. *Human Mutations.* **38** (5), 511-516 (2017).
16. Yarham, J. W. *et al.* Defective i6A37 modification of mitochondrial and cytosolic tRNAs results from pathogenic mutations in TRIT1 and its substrate tRNA. *PLoS Genetics.* **10** (6), e1004424 (2014).
17. Smaldino, P. J., Read, D. F., Pratt-Hyatt, M., Hopper, A. K., & Engelke, D. R. The cytoplasmic and nuclear populations of the eukaryote tRNA-isopentenyl transferase have distinct functions with implications in human cancer. *Gene.* **556** (1), 13-18 (2015).
18. Lamichhane, T. N., Mattijssen, S., & Maraia, R. J. Human cells have a limited set of tRNA anticodon loop substrates of the tRNA isopentenyltransferase TRIT1 tumor suppressor. *Molecular and Cellular Biology.* **33** (24), 4900-4908 (2013).
19. Swoboda, R. K. *et al.* Antimelanoma CTL recognizes peptides derived from an ORF transcribed from the antisense strand of the 3' untranslated region of TRIT1. *Molecular Therapy Oncolytics.* **1,** 14009 (2015).
20. Yue, Z. *et al.* Identification of breast cancer candidate genes using gene co-expression and protein-protein interaction information. *Oncotarget.* **7** (24), 36092-36100 (2016).
21. Chen, S. *et al.* Association of polymorphisms and haplotype in the region of TRIT1, MYCL1 and MFSD2A with the risk and clinicopathological features of gastric cancer in a southeast Chinese population. *Carcinogenesis.* **34** (5), 1018-1024 (2013).
22. Spinola, M. *et al.* Identification and functional characterization of the candidate tumor suppressor gene TRIT1 in human lung cancer. *Oncogene.* **24** (35), 5502-5509 (2005).
23. Spinola, M. *et al.* Ethnic differences in frequencies of gene polymorphisms in the MYCL1 region and modulation of lung cancer patients' survival. *Lung Cancer.* **55** (3), 271-277 (2007).
24. Read, D. F. *et al.* Aggregation of Mod5 is affected by tRNA binding with implications for tRNA gene-mediated silencing. *FEBS Letters.* **591** (11), 1601-1610 (2017).
25. Waller, T. J., Read, D. F., Engelke, D. R., & Smaldino, P. J. The human tRNA-modifying protein, TRIT1, forms amyloid fibers *in vitro. Gene.* **612,** 19-24 (2017).
26. Suzuki, G., Shimazu, N., & Tanaka, M. A yeast prion, Mod5, promotes acquired drug resistance and cell survival under environmental stress. *Science.* **336** (6079), 355-359 (2012).

Copyright © 2018 Journal of Visualized Experiments

27. Soderberg, T., & Poulter, C. D. Escherichia coli dimethylallyl diphosphate:tRNA dimethylallyltransferase: site-directed mutagenesis of highly conserved residues. *Biochemistry.* **40** (6), 1734-1740 (2001).
28. Lamichhane, T. N., Blewett, N. H., & Maraia, R. J. Plasticity and diversity of tRNA anticodon determinants of substrate recognition by eukaryotic A37 isopentenyltransferases. *Rna.* **17** (10), 1846-1857 (2011).
29. Lamichhane, T. N. *et al.* Lack of tRNA modification isopentenyl-A37 alters mRNA decoding and causes metabolic deficiencies in fission yeast. *Molecular and Cellular Biology.* **33** (15), 2918-2929 (2013).
30. Laten, H., Gorman, J., & Bock, R. M. Isopentenyladenosine deficient tRNA from an antisuppressor mutant of Saccharomyces cerevisiae. *Nucleic Acids Research.* **5** (11), 4329-4342 (1978).
31. Etcheverry, T., Colby, D., & Guthrie, C. A precursor to a minor species of yeast tRNASer contains an intervening sequence. *Cell.* **18** (1), 11-26 (1979).
32. Yan, M. *et al.* A high-throughput quantitative approach reveals more small RNA modifications in mouse liver and their correlation with diabetes. *Analytical Chemistry.* **85** (24), 12173-12181 (2013).
33. Su, D. *et al.* Quantitative analysis of ribonucleoside modifications in tRNA by HPLC-coupled mass spectrometry. *Nature Protocols.* **9** (4), 828-841 (2014).
34. Jonkhout, N. *et al.* The RNA modification landscape in human disease. *Rna.* **23** (12), 1754-1769 (2017).
35. Dominissini, D. *et al.* The dynamic N(1)-methyladenosine methylome in eukaryotic messenger RNA. *Nature.* **530** (7591), 441-446 (2016).
36. Meyer, K. D. *et al.* Comprehensive analysis of mRNA methylation reveals enrichment in 3' UTRs and near stop codons. *Cell.* **149** (7), 1635-1646 (2012).
37. Squires, J. E. *et al.* Widespread occurrence of 5-methylcytosine in human coding and non-coding RNA. *Nucleic Acids Research.* **40** (11), 5023-5033 (2012).
38. Schwartz, S. *et al.* Transcriptome-wide mapping reveals widespread dynamic-regulated pseudouridylation of ncRNA and mRNA. *Cell.* **159** (1), 148-162 (2014).
39. Schwartz, S. *et al.* High-resolution mapping reveals a conserved, widespread, dynamic mRNA methylation program in yeast meiosis. *Cell.* **155** (6), 1409-1421 (2013).
40. Behm-Ansmant, I., Helm, M., & Motorin, Y. Use of specific chemical reagents for detection of modified nucleotides in RNA. *Journal of Nucleic Acids.* **2011**, 408053 (2011).
41. Novikova, I. V., Hennelly, S. P., & Sanbonmatsu, K. Y. Tackling structures of long noncoding RNAs. *International Journal of Molecular Sciences.* **14** (12), 23672-23684 (2013).

Copyright © 2018 Journal of Visualized Experiments