**TITLE:**

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

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**SHORT ABSTRACT:**

Here, we describe a protocol for the biochemical characterization of the yeast RNA-modifying enzyme, Mod5, and discuss how this protocol could be applied to other RNA-modifying enzymes.

**LONG ABSTRACT:**

N6-isopentenyladenosine RNA modifications are functionally diverse and highly conserved among prokaryotes and eukaryotes. One of the most highly conserved N6-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 i6A 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 i6A modifications. In addition, the potential adaptability of this protocol to characterize other RNA-modifying enzymes is discussed.

**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 molecules1,2. 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’ side3,4. An isopentenyl group is transferred from dimethylallylpyrophosphate (DMAPP) to the N6 position of adenosine 37 (i6A37) 3,5 on a subset of both cytoplasmic and mitochondrial tRNAs. i6A37 improves translation fidelity and efficiency by increasing the tRNA’s affinity for the ribosome4,6 and i6A37 is important for stress response in bacteria7. The enzymes that perform this modification are termed tRNA isopentenyl transferases and are highly conserved in bacteria8,9, fungi10, worms11, plants12, and higher eukaryotes13, including humans14.

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 synthesis15,16. Furthermore, *TRIT1* has been described as a tumor suppressor gene17,18 and is implicated in several types of cancers including melanoma19, breast20, gastric21, and lung cancers22,23. Finally, TRIT1 and Mod5 (*Saccharomyces cerevisiae*) isopentenyl transferases are aggregation-prone proteins that form prion-like amyloid fibers24-26. 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 i6A 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 i6A RNA modifications, including *in vitro* isopentenylation assays, positive hybridization in the absence of i6A (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 14C-DMAPP and unlabeled tRNAs. In this assay, radioactive carbon is transferred to RNA from 14C-DMAPP by the isopentenyl transferase. While this assay is highly sensitive, it is often difficult to determine the specific residue that is modified9,20,27. PHA6 assays rely on the bulky i6A modification interfering with hybridization of a 32P-labeled probe spanning the modified residue. As such, hybridization is greater in the absence of an i6A modification18,28,29. 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 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 i6A modifications in tRNA30. This approach directly assays the function of the i6A modification, but it is an indirect approach to detect i6A modification and lacks resolution to map modifications to a specific residue in the RNA. A TLC approach has been used to detect total tRNA i6A modifications. In this approach, internally 32P-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 i6A 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 modified31.

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 conditions32-34. 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 derived34. 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-wide34. Immunoprecipitation of RNAs with antibodies specific to a particular modification (RIP-Seq) enable the investigator to identify all sequences containing a specific modification35,36. 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 residues37-39. 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, respectively34. 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 i6A modifications34.

Herein, we describe a simple and direct approach to detect i6A 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 i6A 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**

1.1 Use *in vitro* transcribed RNAs internally labeled with 32P, and recombinant His6-Mod5 expressed in and purified from *E. coli*, as previously described24.

1.1.1. 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 °C24.

1.2. 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.

2.1 Thoroughly clean glass plates. First, wash with soap and water, rinse well with deionized water, and finally clean with isopropanol and lint-free wipes.

2.2 Assemble the plates and spacers.

2.3 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.

2.5 Add 40 µL of N,N,N′,N′-Tetramethylethylenediamine (TEMED) and 800 µL of freshly prepared 10% ammonium persulfate (APS).

2.6 Draw gel solution up into a large syringe and dispense between glass plates. Tap on glass with fingers while pouring to prevent bubble formation.

2.7 Allow gel to solidify for 30 min.

2.8 Clamp solidified gel onto vertical gel apparatus using binder clips.

2.9 Fill upper and lower buffer chambers with 1x TBE.

2.10 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**

3.1 Prepare reactions in a final volume of 17 µL, containing 58 mM Tris-HCl (pH 7.2), 1.2 mM ATP, 5.8 mM MgCl2, 0.2 mM DMAPP, 10 U of RNase inhibitor (*e.g.*, SuperRNaseIn), 40,000 CPM of internally 32P-labeled RNA, 5.3 µM Mod5, and 1.2 mM 2-mercaptoethanol.

3.2 Incubate reactions at 37 °C for 1 h.

3.3 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.

3.4 Centrifuge RNA samples for 20 min at 15,400 x g and 4 °C.

3.5 Carefully remove the supernatant and wash the RNA pellet with 500 µL of 70% ethanol.

3.6 Centrifuge samples at for 5 min 15,400 x g and 4°C.

3.7 Carefully remove the supernatant and air-dry RNA pellets for 15 min, or until all ethanol has evaporated.

3.8 Resuspend RNA pellets in 10 µL of 8 M urea.

3.9 Add 150 U of RNase T1 and incubate at 37 °C overnight.

3.10 Add 2 µL of 6x loading buffer (60% glycerol, 0.1% xylene cyanol).

3.11 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.

3.12 Run gel for 2 h at 25 mA.

3.13 Stop gel and remove from apparatus.

3.14 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.

3.15 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.

3.16 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 Mod524. For example, Mod5 is predicted to modify a subset of tRNAs, which contain the previously described AAA36-38 sequence requirement10, 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 AAA36-38 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 AAA36-38 containing fragment is observed (**Figure 2C**). Interestingly, a partial shift of a 7 nt fragment is observed that does not contain the AAA36-38 (**Figure 2C**). These data suggest that the AAA36-38 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.

**FIGURE LEGENDS:**

**Figure 1: Illustration of isopentenyl transferase assay.** tRNA, internally labeled with 32P-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.

**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 32P-labeled adenosine. Grey highlighted residues are predicted i6A modification sites (*i.e.,* A37). (B) Tyrosine tRNA and (C) serine tRNA were internally labeled with 32P-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 “i6A?”. This figure has been modified from Read *et al.* 24 with permission.

**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 approaches34. 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 effort40. 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 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 RNAs41.

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.

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**DISCLOSURES:**

None to disclose.

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