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TITLE:

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

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KEYWORDS:

RNA modification, tRNA, isopentenyl transferase, MOD5, TRIT1, i^6A, i^6A37, DMAPP

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:

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^{6}A 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 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^{6}A37) [3,5] on a subset of both cytoplasmic and mitochondrial tRNAs. i^{6}A37 improves translation fidelity and efficiency by increasing the tRNA's affinity for the ribosome[4,6] and i^{6}A37 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[24-26]. 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^{6}A 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^{6}A RNA modifications, including *in vitro* isopentenylation assays, positive hybridization in the absence of i^{6}A (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^{6}A 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^{6}A 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 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[32-34]. 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[37-39]. 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

1.1 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].

1.1.1. 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 °C[24].

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 $MgCl_2$, 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.

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 Mod5[24]. For example, Mod5 is predicted to modify a subset of tRNAs, which contain the previously described $AAA_{36\text{-}38}$ 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 $AAA_{36\text{-}38}$ 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 $AAA_{36\text{-}38}$ containing fragment is observed (**Figure 2C**). Interestingly, a partial shift of a 7 nt fragment is observed that does not contain the $AAA_{36\text{-}38}$ (**Figure 2C**). These data suggest that the $AAA_{36\text{-}38}$ 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 ^{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.

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^6A 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.

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 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.

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DISCLOSURES:

None to disclose.

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Figure 1

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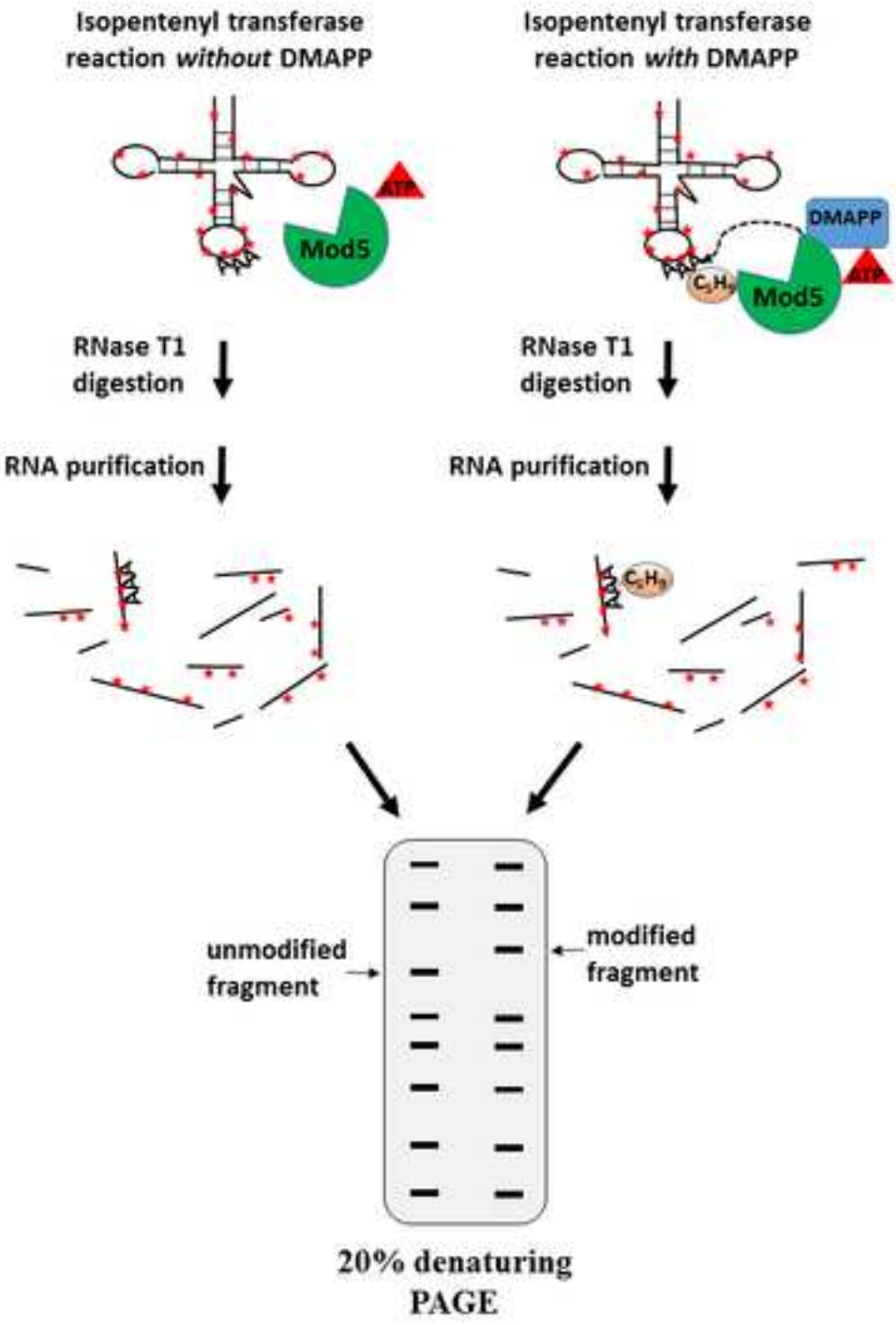
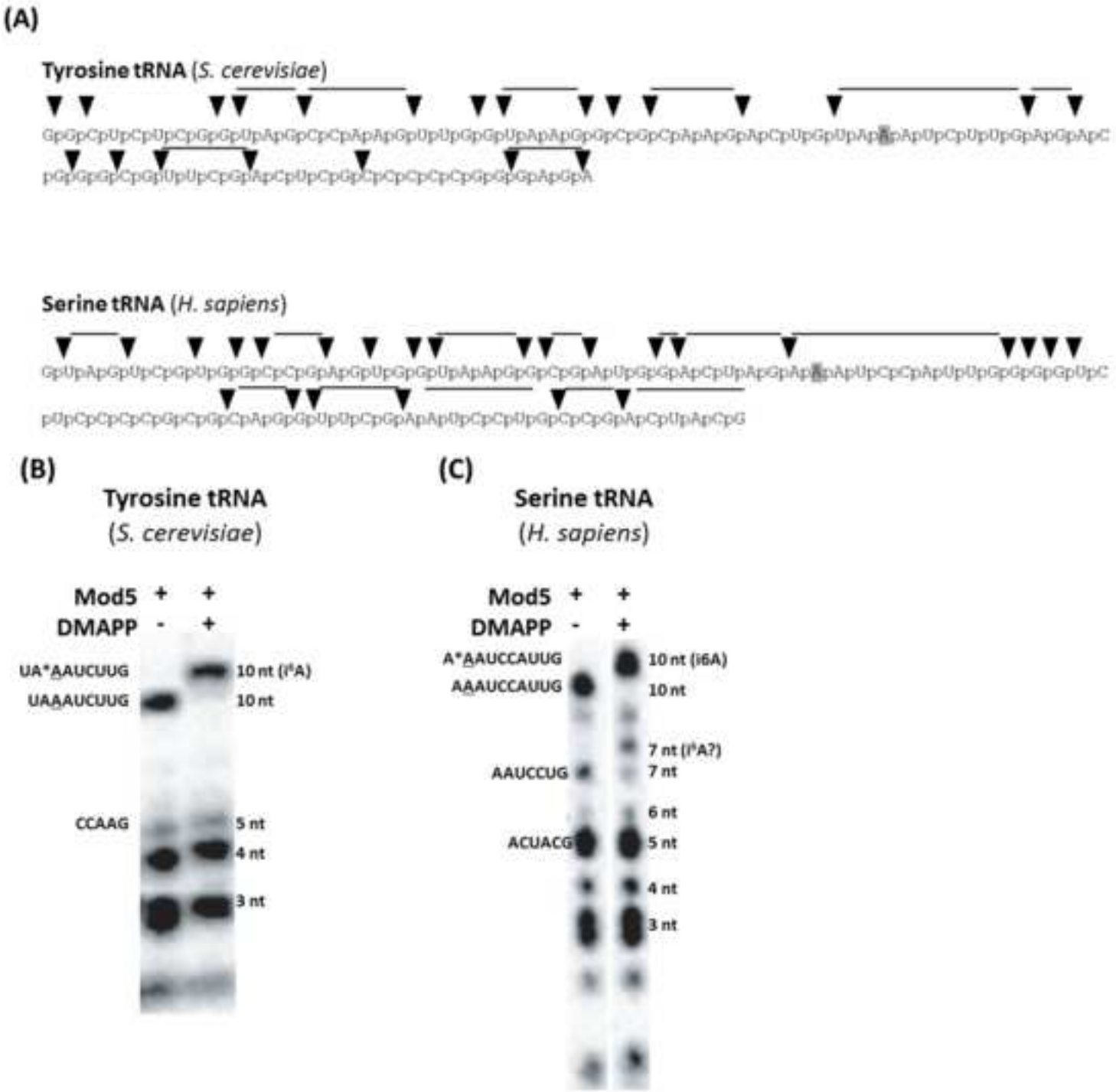


Figure 2



Materials Table

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Name of Material/ Equipment	Company	Catalog Number	Comments/Description
Reagents			
UreaGel Concentrate	National Diagnostics	EC-833	As part of a kit
UreaGel Diluent	National Diagnostics	EC-833	As part of a kit
UreaGel Buffer	National Diagnostics	EC-833	As part of a kit
10x TBE	National Diagnostics	EC-833	As part of a kit
Ammonium persulfate (APS)	Sigma-Aldrich	7727-54-0	
N,N,N′,N′-Tetramethylethylenediamine (TMED)	Sigma-Aldrich	T9281	
Tris base	Sigma-Aldrich	T1503	
Boric acid	Sigma-Aldrich	10043-35-3	
EDTA	Sigma-Aldrich	60-00-4	
ATP	Sigma-Aldrich	34369-07-8	
$MgCl_2$	Sigma-Aldrich	7786-30-3	
DMAPP	Caymen Chemical	1186-30-7	
Super RNaseIN	ThermoFisher Scientific	AM2694	
2-mercaptoethanol	Sigma-Aldrich	60-24-2	
Ethanol	Sigma-Aldrich	64-17-5	
Sodiume acetate	Sigma-Aldrich	127-09-3	
Rnase T1	ThermoFisher Scientific	EN0541	
Glycerol	Sigma-Aldrich	56-81-5	
Xylene cyanol	Sigma-Aldrich	2650-17-1	
Equipment and Supplies			
Short glass plates (20-40 cm W x 40 cm L)	The Gel Company		
Long glass plates (20-40 cm W x 40 cm L)	The Gel Company		

Vertical gel apparatus	The Gel Company	S2-3040
50 mL disposable syringe	Fisher Scientific	03-377-26
Stainless steel binder clips	Idea Scientific	1066
Phosphoscreen	Sigma-Aldrich	28-9564-74
Plastic wrap	(local grocery store)	

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Title of Article: A sensitive and direct in vitro assay to characterize [illegible] activity

Author(s): Chambers, AE, Richardson, AE, Reid, DF, Walker, TJ, [illegible], Smalldridge, TJ

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CORRESPONDING AUTHOR:

Name: Philip Smaldino

Department: Biology

Institution: Ball State University

Article Title: A sensitive and direct immunoassay to characterize tRNA-isopentenyl transferase activity,

Signature: Philip Smaldino Date: 3/1/18

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April 13, 2018
Journal of Visualized Experiments

Ms. Ref. No.: JoVE58100

Dear Dr. DSouza,

Please find included in our resubmission a revised version of our manuscript "A simple and direct *in vitro* assay to detect tRNA-isopentenyl transferase activity." The reviewer comments have enabled us to greatly improve the quality of our manuscript. In the following pages are our point-by-point responses to each of the editorial comments. (Editor/reviewer comments are *italicized* with author responses to each comment are in **bold**).

We thank you for your time and consideration, and look forward to receiving your comments.

Sincerely,

Philip J. Smaldino, PhD

Assistant Professor
Department of Biology
Ball State University
Muncie, IN 47306

RESPONSE TO REVIEWER COMMENTS:

Editorial comments:
Changes to be made by the Author(s):
1. Please take this opportunity to thoroughly proofread the manuscript to ensure that there are no spelling or grammar issues. The JoVE editor will not copy-edit your manuscript and any errors in the submitted revision may be present in the published version.
We have carefully edited and proofread the manuscript.

2. Please combine all panels of one figure into a single image file.
We have combined panels 2B and 2C into one file.

3. Please rephrase the Short Abstract to clearly describe the protocol and its applications in complete sentences between 10-50 words: "Here, we present a protocol to …"
We have made these changes.

4. Please revise the text to avoid the use of any personal pronouns (e.g., "we", "you", "our" etc.).
We have made these changes.

5. Please ensure that all text in the protocol section is written in the imperative tense as if telling someone how to do the technique (e.g., "Do this," "Ensure that," etc.). The actions should be described in the imperative tense in complete sentences wherever possible.
We have made these edits wherever possible.

6. Please specify all volumes and concentrations added for the reactions.
We have made these edits.

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8. Please do not abbreviate journal titles.

Reviewers' comments:
Reviewer #1: Manuscript Summary: The authors describe a protocol for a high-resolution, low-cost assay that detects tRNA modification by Mod5. First, RNA is isopentenylated by incubation with Mod5 and DMAPP and radioactively labeled with P-32. RNAs are subsequently digested with RNase T1 and subjected to denaturing electrophoresis. Phosphor imaging reveals shifts in band size among isopentenylated RNA fragments, compared to DMAPP-lacking control reactions. The authors used this technique to successfully detect Mod5-mediated isopentenyl modification of tyrosine (S cerevisiae) and serine (H. sapiens) tRNAs. A major advantage of this protocol is its adaptability for the study of different enzymes and RNAs. The authors nicely review additional techniques for the detection of RNA modifications.

Major Concerns: None

Minor Concerns:
(1) The authors refer to a 7M urea denaturing gel in Step 3.11. However, the urea gel is 7.5M urea, according to Section 2.
We have edited this typo so that 7.5 M Urea is consistently stated throughout.

(2) Mod5 is an S. cerevisiae enzyme. Does the H. sapiens origin of the serine tRNA inform the finding of a novel i6A modification site and, if so, how?
This is an interesting question, and we cannot be sure of the answer without additional experiments that confirm the identity of the novel modification, which we are presuming to be i6A. We have added the following statement to address this consideration:

"Interestingly, we also observe a partial shift of a 7 nt fragment that does not contain the AAA_{36-38} sequence (Figure 2C). These data suggest that the AAA_{36-38} sequence and structure is not required for Mod5 *in vitro* activity, however, future studies using LC-MS or other methods are required to confirm the exact chemical nature of the modification."

Reviewer #2: Manuscript Summary:

I suggest to eliminate the last sentence since it depends on the effects produced by the modification on Endonuclease accessibility
We have removed the last sentence.

Major Concerns:
The article shows a method for the study of specific enzymes that catalyze the isopentenylation, specifically the protocol addresses de activity of the Mod5 enzyme, which modifies position A37 of specific tRNAs. The authors declare that the method presented is sensitive and allow the study of determinants for the activity of this type of transferase enzymes. However, the sensitivity of this method is not commented or compared with other methods and experiments were not done to specifically show this. Radioactive signal is sensitive per se, but quantitation or estimation of the changes in the amount or pattern in the signal produced by different conditions or variations in the presence of determinants in the tRNAs are not documented. Moreover, although different profiles of i6A-modified fragments are produced from tRNAs from yeast and human, no direct assay was done to show how changes in other modifications present in this specific tRNAs (yeast or human) could influence Mod5 transferase activity. This would be very useful in order to evidence the sensitivity and capability of the method to address what is state in the manuscript.
A specific problem of the protocol is related with the way that the amount of the enzyme used is presented. It appears that is not commercial and the amount is presented as a concentration, therefore with this assay is not possible to estimate the sensitivity of this specific method. Using this same method one could calculate the Units of activity of Mod5 present in the concentration used in the assay proposed.

We acknowledge the limitation brought up by Reviewer 2 regarding quantitation and claims of sensitivity. Although we agree that developing a quantitative protocol to measure i6A modification would be useful, we believe that the extensive characterization that this would require is beyond the scope of this current *JoVE* protocol. The revised version of this manuscript is edited to present this method as a simple, direct, and *qualitative* assay useful in detecting isopentenylation of tRNAs. Claims of sensitivity and quantitative language throughout the manuscript have been edited to reflect these changes, including in the title and elsewhere. We have also added the following statement to the Discussion:

"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."

Minor Concerns: **[none]**

Reviewer #3: Manuscript Summary: *The paper discussed current methods for tRNA-isopentenyl transferase activity assay and described the details of an in vitro assay method.*
Minor Concerns:

1) The method to label RNAs with P32 needs to be described.

The method to radioactively-label the RNAs was referenced in the original text of the manuscript. We have retained this reference in the resubmitted manuscript and have added the following text:
"Briefly, RNAs were *in vitro*-transcribed using T7 RNA polymerase in the presence of unlabeled ATP, UTP, CTP, GTP and 10 µCi of α-ATP, gel-purified, ethanol-precipitated, resuspended in 1x TE (10 mM Tris-HCl pH 7.5, 0.1 mM EDTA), and stored at -80°C"

2) Figure 2C showed an unexpected modification of the 7nt fragment. Is there a limitation of this method that other types of modification would show similar results as i6A modification?

This is an interesting idea. It is possible that Mod5 could potentially modify the RNA in a way which is distinct from its well-characterized isopentenyl transferase activities. Given the well-known function of Mod5 as an isopentenyl transferase, and given that the shift requires DMAPP, strongly suggests that the shift represents a non-canonical i6A modification. Despite, these data, we acknowledge the lack of direct evidence for this claim and therefore we agree with the reviewer that we cannot completely rule out another type of modification. Therefore, we have adjusted the text to reflect this consideration:

"Interestingly, we also observe a partial shift of a 7 nt fragment that does not contain the $AAA_{36\text{-}38}$ sequence (Figure 2C). These data suggest that the $AAA_{36\text{-}38}$ sequence and structure is not required for Mod5 *in vitro* activity, however, future studies using LC-MS or other methods are required to confirm the exact chemical nature of the modification."

Reviewer #4: Manuscript Summary:
The manuscript by Chambers et al describes the original method for detection and characterization of RNA i6A-modifying enzymatic activity. Other methods exist for this purpose, but current version provides not only detection of isopentenyl group incorporation but also gives the position of modification, which is an extra value compared to other methods. The method uses internally 32P labelled RNA transcript and non-labelled DMAPP. After incubation with the enzyme, the transcript is completely digested by RNAse T1 and the resulting fragments are separated by denaturing 20% gel. The fragment with extra i6-group migrates slower compared to unmodified counterpart, allowing identification of

modification position. The manuscript is clearly written, even if some improvements can be suggested (see below).

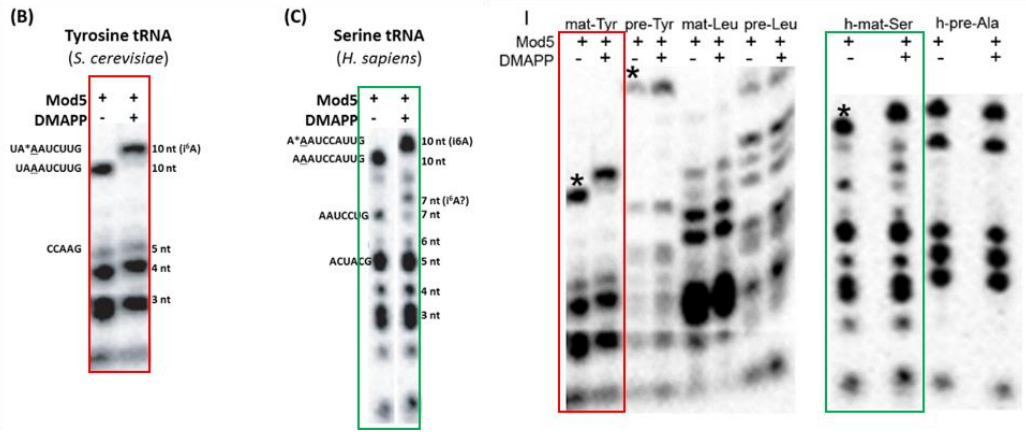
Major Concerns:
1. The observation that i6A-modified RNA fragment migrates slower compared to unmodified sequence is barely convincing in its present state and requires further validation. First, the gel presented in Figure 2a (2B) presents traces of image manipulation at the bottom part and should be replaced by the version where both samples migrate together on the SAME gel in parallel. The same applies also to Figure 2b (2C), where two lanes are just assembled from different gels or gel parts.

We believe that the Reviewer meant Figures 2B and 2C, since there gel images are not present in Figure 2A. The reactions shown in Figures 2B and 2C are run on different gels and as such, are presented as separate panels. Secondly, we do not draw comparisons between the images in Figures 2B and 2C. The only comparisons that we make are between the lanes containing the same RNA's with and without DMAPP. For these comparisons, we agree with the reviewer in that the samples should be on the same gel and adjacent to each other on that gel; this is how the experiments presented in this manuscript were performed. Samples in Figure 2B were run on a single gel and samples in Figure 2 C were run on a single gel. We have included the original images for each of these gels. We cropped Figures 2B and 2C for purely aesthetic reasons (see image below). The degree of spacing between lanes for each gels was slightly different, therefore we cropped out the extra space in Figure 2C. We left a gap between the lanes to disclose that the image had been cropped. The cropping does not alter the result; in the presence of DMAPP we observe a shifted band for both tRNAs. We have added the following statement to the Figure 2 legend:

"Cropped images are indicated by white separation."

Other than image cropping as described above, there were no other "image manipulations" to Figure 2B or 2C. The reviewer may be referring to a horizontal line across the bottom of the image in 2B. This line continues across the width of the original image as well, and is simply an imaging artifact and not an image manipulation.

Figure 2 included in JoVE submission **Original gel image**



2. Size ladder is missing on the gels, thus one can only guess the fragments' sizes.

The sequence of each *in vitro*-transcribed RNA used in these experiments is known beforehand, therefore the band sizes and RNase digest patterns are predicable (see Figure 2A). For each RNA, we observed a banding pattern consistent with the predicted patterns. Therefore, although an RNA size ladder might be helpful, it is not essential enough to warrant repeating these experiments in their entirety in order to obtain an image with a size marker included. Furthermore, the suggested experiments would be practically impossible to complete within the given revision time (~3 weeks).
We have added the following suggestion for the reader:
"Radiolabeled RNA size ladders may be included to serve as an additional mobility marker."

3. It is not clear which internal 32P-labelling was used in the experiments. I strongly recommend repeating the assay using transcripts separately labelled by all four 32P-rNTP and compare obtained profiles.

This submission of this current article is an effort to publish a previously published method in a video-format. Therefore, the authors suggest that the above described experiments are beyond the scope of this *JoVE* submission. Furthermore, our protocol describes a method that specifically detects isopentenylation of adenosine (A), therefore radiolabeled-A is used, allowing for identification of A-containing fragments. Although, repeating this experiment with all 4 radiolabeled nucleotides would increase the resolution of this assay, it would also add a substantial amount of costs, labor, and time, and greatly reduce the practicality of this protocol. The suggested experiments would also require far more time than has been allotted for revision (~3 weeks)

4. Analysis of RNAse T1 fragments should be completed by RNAse P1 and T2 digestions made on the same samples and 2D TLC plates confirming i6A formation upon incubation.

The submission of this current article is an effort to publish a previously published method in a video-format [Read, D. F. *et al.* Aggregation of Mod5 is affected by tRNA binding with implications for tRNA gene-mediated silencing. *FEBS Lett.* 591 (11), (2017)]. Therefore we feel that the above described experiments are beyond the scope of this *JoVE* submission.
However, we have added the following comment in the Discussion to address the reviewer's concern:
"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 the assay."

5. The observation that human tRNASer is modified at the non-conventional site is contradictory to all previous observations, and thus should be confirmed by independent approaches, like LC-MS or similar onthe authors' choice.

Given the well-known function of Mod5 as an isopentenyl transferase enzyme and given that the modification is DMAPP-dependent, this strongly suggests that the shift represents a non-canonical i6A modification. Despite these data, we acknowledge the lack of direct evidence for this claim and we cannot completely rule out another type of modification.

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We have included the following statement as a suggestion for the readers:
"Interestingly, we also observe a partial shift of a 7 nt fragment that does not contain the AAA_{36-38} sequence (Figure 2C). This suggest that the AAA_{36-38} sequence and structure is not required for Mod5 *in vitro* activity, however, future studies using LC-MS or other methods are required to confirm the exact chemical nature of the modification."

Minor Concerns:
Both short and long abstract are not informative since mostly describe i6A biology and not the method suggested.
We have edited the short and long abstracts to place more emphasis and focus on the method.

Part of the introduction on deep-sequencing approaches is not relevant since these methods are not described for i6A for the moment. Instead, authors should describe more in details existing protocols for i6A detection, in order to compare with current protocol proposed in the manuscript.
We have provided details for 5 different existing protocols for detecting i6A in 3 full paragraphs within the Introduction. We respectively disagree with the Reviewer's suggestion to remove discussion of deep-sequencing approaches in the Introduction. Although it is true that i6A has not yet be characterized by deep-sequencing techniques, it does not follow that the researcher would not have this option when choosing a protocol. Therefore, we think that mention of all feasible methods to characterize i6A, including yet be done deep sequencing-based approaches, should be included.

Limitations of the approach should be clearly defined in discussion.
We have added additional limitations of this protocol to the Discussion section, including discussion of the qualitative nature of this protocol.

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Date: 5/16/17

Contributor name: Philip J Smaldino

Contributor address: Ball State Univ. Riverside Ave, Muncie, IN 47306

Manuscript number: FEB212627

Re: Manuscript entitled Aggregation of Mod5 is affected by tRNA binding with implications for tRNA gene-mediated silencing. (the "Contribution")

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