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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

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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.

7. Please obtain explicit copyright permission to reuse any figures from a previous publication. Explicit permission can be expressed in the form of a letter from the editor or a link to the editorial policy that allows re-prints. Please upload this information as a .doc or .docx file to your Editorial Manager account. The Figure must be cited appropriately in the Figure Legend, i.e. "This figure has been modified from [citation]."
We have included a statement from *FEBS Letters* indicating permission to reuse figures that we have previously published for use in this current *JoVE* manuscript.

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).

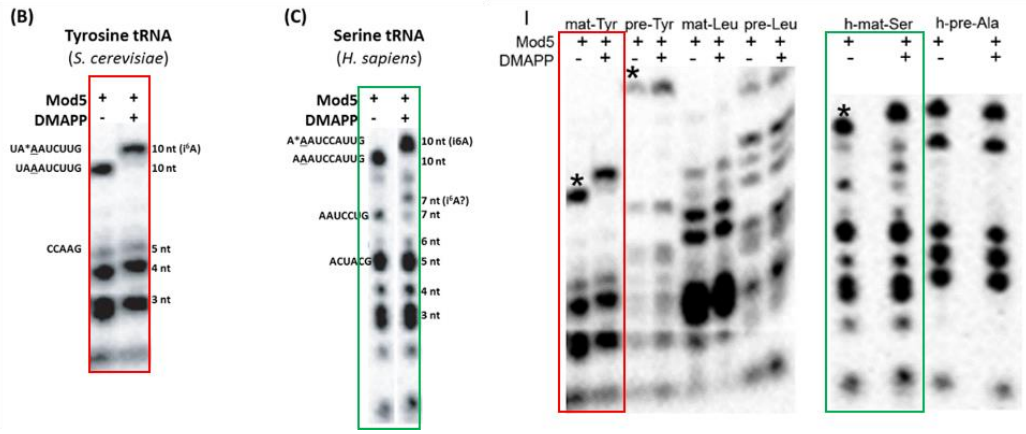
Major Concerns:
1. The observation that i6A-modified RNA fragment migrates slower compared to unmodified sequence is barely convincing in its present state and requires further validation. First, the gel presented in Figure 2a (2B) presents traces of image manipulation at the bottom part and should be 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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