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TITLE:

DNAzyme-Dependent Analysis of rRNA 2'-O-Methylation

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SUMMARY:

Here we present a protocol for DNAzyme-dependent cleavage of RNA. This enables fast and site-dependent analysis of RNA 2'-O-methylation. This approach can be used for the preliminary or major assessment of snoRNA activity.

ABSTRACT:

Guide box C/D small nucleolar RNAs (snoRNAs) catalyze 2'-O-methylation of ribosomal and small nuclear RNA. However, a large number of snoRNA in higher eukaryotes may promiscuously recognize other RNA species and 2'-O-methylate multiple targets. Here, we provide step-by-step guide for the fast and non-expensive analysis of the site-specific 2'-O-methylation using a well-established method employing short DNA oligonucleotides called DNAzymes. These DNA fragments contain catalytic sequences which cleave RNA at specific consensus positions, as well as variable homology arms directing DNAzyme to its RNA targets. DNAzyme activity is inhibited by 2-'O-methylation of the nucleotide adjacent to the cleavage site in the RNA. Thus, DNAzymes, limited only by the consensus of the cleaved sequence, are perfect tools for the quick analysis of snoRNA-mediated RNA 2'-O-methylation. We analyzed snoRNA snR13- and snR47-guided 2'-O-methylation of 25S ribosomal RNA in *Saccharomyces cerevisiae* to demonstrate the simplicity of the technique and to provide a detailed protocol for the DNAzyme-dependent assay.

INTRODUCTION:

RNA modifications play important roles in the regulation of gene expression. RNA 2'-O-methylation and pseudouridylation, which are guided by box C/D and box H/ACA small nucleolar RNAs (snoRNAs) respectively, protect RNA from degradation and stabilize their higher-order structures[1-3]. SnoRNA targets have been identified mainly in ribosomal RNAs (rRNA) and small nuclear RNAs (snRNAs). However, in higher eukaryotes, there are potentially hundreds of snoRNA with no assigned functions and some of them may recognize multiple RNAs[1,4-7]. Therefore, methods which allow for the identification and analysis of snoRNA-guided modifications are

important tools in uncovering mechanisms governing cellular processes.

A box C/D snoRNA-guided putative 2'-O-methylation site can be identified bioinformatically and confirmed experimentally by many techniques, including RNase H-directed cleavage, or site-specific and genome-wide methods, which employ reverse transcription in low nucleotides (dNTPs) concentration approach[8-11]. These techniques are very sensitive but also laborious and expensive, therefore, may not be suitable for the initial or quick testing. One of the simplest and low-cost methods to identify 2'-O-methylation sites is DNAzyme-dependent RNA cleavage[12]. DNAzymes are short, single-stranded and catalytically active DNA molecules capable of endonucleolytic cleavage of RNA at specific positions. They consist of a conserved and catalytically active core sequence and 5' and 3' binding arms composed of variable sequences designed to hybridize by Watson-Crick base-pairing to the RNA target (**Figure 1**). Thus, the 5' and 3' arms deliver the catalytic sequence to the specific RNA site. DNAzyme-dependent cleavage is inhibited by 2'-O-methylation of the nucleotide positioned directly upstream of the cleavage site[12,13]. This makes DNAzymes very practical tools for the analysis of putative or known RNA 2'-O-methylation sites.

Two types of DNAzymes are used for RNA modifications analyses[12]. The active sequence of 10-23 DNAzyme (**Figure 1A**) consists of 15 nucleotides (5'RGGCTAGCTACAACGA3') which form a loop around the targeted RNA purine-pyrimidine (RY) dinucleotide and catalyze the cleavage between these two nucleotides. The RNA purine (R) is not base-paired with the DNAzyme and the 2'-O-methylation presents on the DNAzyme inhibits the cleavage. The binding arms of 10-23 DNAzymes are usually 10-15 nucleotides long. The second DNAzyme class, 8-17 DNAzymes (**Figure 1B**) contain 14-nucleotide catalytic sequence (5'TCCGAGCCGGACGA3'). Nucleotides C_2, C_3 and G_4 pair with C_9 G_{10} and G_{11} forming a short stem-loop structure. 8-17 DNAzymes cleave RNA upstream of any guanine that is imperfectly paired with the first thymine from the DNAzyme active sequence. The RNA nucleotide upstream of the guanine is not base-paired with DNAzyme and its 2'-O-methylation impairs the cleavage. 8-17 DNAzymes require longer homology arms of around 20 nucleotides to direct DNAzyme to its specific sequence.

Here, we provide a step-by-step protocol for the analysis of 2'-O-methylation of rRNA in *Saccharomyces cerevisiae* using 10-23 and 8-17 DNAzyme-dependent approaches[12,13] (**Figure 1C**). This protocol can be easily adapted for other organisms and RNA species and employed for the fast, preliminary or major analyses of site-specific RNA 2'-O-methylation.

PROTOCOL:

1. Strains, media and buffer recipes

1.1. Prepare yeast (*S. cerevisiae*) media as detailed here: 1% w/v yeast extract, 2% w/v bacteriological peptone and, glucose and galactose stocks at 20% w/v.

1.2. Prepare sodium acetate (NaAc)-EDTA (AE) buffer by adding 50 mM NaAc pH 5.3 and 10 mM EDTA.

1.3. Prepare 10-23 DNAzyme 4x Incubation Buffer as detailed here: 24 mM Tris pH 8, 60 mM NaCl and 10-23 DNAzyme 4x Reaction Buffer: 200 mM Tris pH 8 and 600 mM NaCl.

1.4. Prepare 8-17 DNAzyme 2x Reaction Buffer as detailed here: 200 mM KCl, 800 mM NaCl, 100 mM HEPES pH 7.0, 15 mM $MgCl_2$, and 15 mM $MnCl_2$.

1.5. Prepare 10x MOPS Buffer as detailed here: 200 mM MOPS, 50 mM NaAc, 1 mM EDTA; pH 7.0 and 1.5x Sample Denaturing Buffer: 50% v/v formamide, 20% v/v formaldehyde, 1.5x MOPS buffer.

1.6. Obtain *S. cerevisiae* strains, BY4741 (*MATa his3Δ1 leu2Δ0 met15Δ0 ura3Δ0*); *GAL1::SNR13* (as BY4741 but *GAL1::SNR13:KANmX*); *GAL1::SNR47* (as BY4741 but *GAL1::SNR47:HIS3mX*). Any other yeast strain can be used for this analysis.

2. DNAzyme design

2.1. Find RNA sequence of interest or putative methylation site using an appropriate database. For *S. cerevisiae* snoRNA targets, use yeast snoRNA database: http://people.biochem.umass.edu/fournierlab/snornadb/mastertable.php[14]

2.2. To find the methylation site of interest, e.g., snR13-dependent site, select "snR13" and make a note of the position of the modified nucleotide (e.g., snR13-guided A_{2281} in 25S rRNA).

2.3. Find sequences upstream and downstream of the modified nucleotide using appropriate database. For *S. cerevisiae,* use the Saccharomyces Genome Database: https://www.yeastgenome.org/

2.4. Search for the target gene name e.g., RDN25 (coding 25S rRNA).

2.5. Select 10-15 nucleotides upstream and downstream of the methylation site when using a 10-23 DNAzyme assay and 20 nucleotides upstream and downstream of the methylation site for the 8-17 DNAzyme.

2.6. Flank 10-23 or 8-17 DNAzyme catalytic sequence with a 5' arm and a 3' arm.

2.7. Order DNAzyme as a normal DNA oligonucleotide from the supplier.

3. *S. cerevisiae* growth conditions

NOTE: *S. cerevisiae* BY4741 strain derivatives were used, in which the expression of either *SNR13* or *SNR47* snoRNA is driven from the inducible *GAL1* promoter. In order to induce or inhibit their synthesis, grow cells on medium containing galactose (*GAL1*-dependent transcription on) or glucose (*GAL1*-dependent transcription off). As a control, use the wild type strain (BY4741) grown

either on galactose or glucose.

3.1. Grow yeast strains in an appropriate medium and conditions. To analyze *GAL1::SNR13* and *GAL1::SNR47* strains as well as the isogenic wild type strain, grow cells in 50 mL of YP media with either 2% glucose (YPD) or galactose (YPGal) at 30 °C to the middle exponential phase.

3.2. Centrifuge cells at 1,000 *x g*, for 3 min at 4 °C and keep the pellets.

3.3. Remove and discard the supernatant.

3.4. Freeze cell pellets in liquid nitrogen and store them at -80 °C.

CAUTION: Liquid nitrogen may cause severe cryogenic burns. Always wear protective clothing and exercise safety precautions.

NOTE: Cell pellets can be stored at -80 °C up to 1 month. The protocol can be paused here if needed.

4. RNA isolation[15]

NOTE: Use the most appropriate method to isolate RNA. For yeast *S. cerevisiae*, hot-phenol RNA extraction can be used.

4.1. Add 1 mL of ice-cold water, resuspend the pellets and transfer resuspended cells to 1.5 ml microtubes.

4.2. Centrifuge at 20,000 *x g* for 10 s at 4 °C and remove the supernatant.

4.3. Add 400 µL of AE buffer and resuspend the cells.

NOTE: Steps 4.4 - 4.15 are performed at room temperature unless stated otherwise.

4.4. Add 40 µL of 10% SDS and 400 µL of acid phenol (pH 4.5).

CAUTION: Phenol is toxic and should be handled under a fume hood. Always wear a lab coat, protective gloves, and glasses when working with phenol. Dispose of the waste according to the institutional regulations.

4.5. Mix well by vortexing for 20 s.

4.6. Incubate at 65 °C for 10 min. Every 2 min, gently open and close the tube to release the pressure and flip the tube 2-3 times to mix the phases.

4.7. Transfer the tubes to -80 °C and incubate for 10 min.

4.8. Defrost the tubes on the bench and centrifuge at 20, 000 *x g*, for 5 min at room temperature.

4.9. Transfer the upper phase to a new tube containing 400 μL acid phenol:chloroform:isoamyl alcohol (25:24:1). Do not disrupt the interphase.

CAUTION: Chloroform is toxic and should be handled under a fume hood. Always wear a lab coat, protective gloves, and glasses when working with chloroform. Dispose of the waste according to institutional regulations.

4.10. Mix well by vortexing for 30 s and centrifuge at 20, 000 *x g* for 10 min at room temperature.

4.11. Transfer the upper phase (~400 μL) to a new tube containing 400 μL chloroform.

4.12. Mix well by vortexing for 30 s and centrifuge at 20,000 *x g* for 5 min at room temperature.

4.13. Transfer the upper phase (~300-350 μL) to a new tube containing 1 mL of EtOH and 40 μL of 7.5 M ammonium acetate (NH_4AC). Mix by flipping the tube a few times.

4.14. Incubate at -80 °C for 2 h or overnight at -20 °C.

NOTE: The procedure can be paused here.

4.15. Centrifuge at 20, 000 *x g*, for 10 min at 4 °C. A small, white RNA pellet will become visible on the bottom of the tube.

4.16. Remove EtOH by pipetting to avoid disturbing the pellet.

4.17. Add 1 mL of 70% EtOH and centrifuge at 20, 000 *x g* for 5 min at room temperature.

4.18. Remove 70% EtOH by pipetting.

4.19. Centrifuge at 20, 000 *x g* for 15 s and remove the remaining EtOH with 2-20 μL pipette.

4.20. Leave the tube open on the bench for 5 min to dry the RNA pellet.

NOTE: RNA pellet changes its color from white to transparent when dry.

4.21. Resuspend the RNA pellet in 30 μL of RNase/DNase-free H_2O, transfer the tube immediately on the ice and measure RNA concentration on microspectrophotometer.

4.22. Freeze the samples at -20 °C.

NOTE: RNA can be stored at -20 °C up to 1 month and at -80 °C up to 1 year. The procedure can

be paused here or proceeded directly to the next step.

5. DNAzyme digestion

5.1. 10-23 DNAzyme digestion

5.1.1. In 1.5 mL tubes prepare an incubation mix by combining 5 µg of RNA, 200 pmol of 10-23 DNAzyme (2 µL of 100 µM stock solution) and 2.5 µL of 4x 10-23 Incubation Buffer in a total volume of 10 µL. Keep the tubes on ice.

5.1.2. Transfer the tubes to a dry heat block set at 95 °C and incubate for 3 min.

5.1.3. Transfer the tubes immediately on ice and incubate for 5 min.

5.1.4. Spin down briefly and put the tubes back on the ice.

5.1.5. Add 20 U of RNase inhibitor (e.g., 0.5 µL RiboLock RNase inhibitor).

5.1.6. Place the tubes in a dry heat block set for 25 °C and incubate for 10 min.

5.1.7. In the meantime, prepare a reaction mixture in a 1.5 mL tube by combining 5 µL of 4x 10-23 Reaction Buffer with 4 µL of 300 mM $MgCl_2$ and 1 µL H_2O. Place the tube in a dry block set to 37 °C.

5.1.8. Transfer the incubation mix to a dry heat block set for 37 °C and add 10 µL of pre-warmed reaction mix.

5.1.9. Incubate the reaction at 37 °C for 1 h.

5.1.10. Transfer the tubes on ice and proceed to step 5.3.1.

5.2. 8-17 DNAzyme digestion

5.2.1. Prepare a 1.5 mL microtube with 5 µg of RNA in a total volume of 6 µL. Keep the tube on ice.

5.2.2. Prepare a 1.5 mL microtube with 400 pmol of 8-17 DNAzyme (4 µL out of 100 µM stock). Keep the tube on ice.

5.2.3. Transfer the tubes to a dry heat block set for 95 °C and incubate for 2 min.

5.2.4. Move the RNA sample on ice.

5.2.5. Spin down the tube with DNAzyme for 5 s and incubate at 25 °C for 10 min.

5.2.6. At the same time, prepare a 1.5 mL tube with 10 μL of 2x 8-17 Reaction Buffer and incubate at 25 °C.

5.2.7. Prepare a reaction mixture by adding 10 μL of pre-warmed 2x Reaction Buffer to the tube with DNAzyme.

5.2.8. Transfer 14 μL of the reaction mix to the tube with RNA and add 20 U of RNase inhibitor.

5.2.9. Incubate the reaction at 25 °C for 2h.

5.2.10. Transfer the tube on ice and proceed to RNA purification (step 5.3.1).

5.3. RNA purification

5.3.1. Add 350 μL of water and 400 μL of chloroform to the reaction tube, mix well by vortexing for 30 s and centrifuge at 20,000 *x g* for 5 min at room temperature.

5.3.2. Transfer the upper phase (~300-350 μL) to a new tube containing 1 mL of EtOH, 40 μL of 7.5 M NH_4AC and 1 μL of glycogen (10 μg/μL). Mix by flipping the tube a few times.

5.3.3. Incubate at -80 °C for 2 h or overnight at -20 °C.

NOTE: The procedure can be paused here.

5.3.4. Repeat steps from 4.15 to 4.21.

5.3.5. Resuspend the RNA pellet in 10 μL of RNase/DNase-free H_2O and transfer the tubes immediately on ice.

5.3.6. Freeze the samples at -20 °C.

NOTE: RNA can be stored at -20 °C up to a month and at -80 °C up to 1 year. The procedure can be paused here or proceed to RNA electrophoresis.

6. RNA electrophoresis

6.1. Spray the electrophoresis equipment (tank, tray, comb) with 1% SDS, leave for 15 min and rinse with plenty of ddH_2O.

6.2. Dissolve 1.5 g of agarose in 127.5 mL of ddH_2O by heating it in the microwave.

6.3. Add 15 mL of 10x MOPS and 7.5 mL of 37% formaldehyde to the agarose solution (total volume is 150 mL).

CAUTION: Formaldehyde is toxic and should be handled under a fume hood. Always wear a lab coat, protective gloves, and glasses when working with formaldehyde. Dispose of the waste according to institutional regulations.

6.4. Add an appropriate amount of a gel stain of choice to the agarose solution (e.g., 15 μL of SYBR Safe DNA gel stain). Mix well and pour the agarose to the tray.

6.5. Insert a comb in the gel immediately.

6.6. Leave it for 45 min under the fume hood. Cover the tray with aluminum foil when using a light-sensitive gel stain.

6.7. Prepare 600 mL of 1x MOPS buffer.

6.8. RNA sample preparation

6.8.1. In a 1.5 mL tube, combine 10 μL of the digested and purified RNA sample, 5 μL of Sample Denaturing Buffer and 0.5 μL of 6x Loading Dye.

CAUTION: Formamide is toxic and should be handled under a fume hood. Always wear a lab coat, protective gloves, and glasses when working with formamide. Dispose of the waste according to institutional regulations.

6.8.2. Incubate RNA samples at 70 °C for 5 min. Transfer the samples on ice. Incubate for 5 min.

6.8.3. Spin down briefly before loading on the gel.

6.9. Put the gel in the electrophoresis tank and fill with 1x MOPS buffer. Load the entire volume of each sample (15 μL) on the gel. Run at 80 V until bromophenol blue reaches 2/3 of the gel length.

6.10. Image the gel using an imager appropriate to detect the chosen gel stain (e.g., UV transilluminator).

REPRESENTATIVE RESULTS:

The utility of the DNAzyme-dependent cleavage in the analysis of rRNA modifications has been shown recently in the context of snoRNAs maturation[13]. The DNAzyme-dependent assay was used to show that lack of 5'-end pre-snoRNA processing affects 2'-O-methylation levels of 25S and 18S rRNA in *S. cerevisiae*[13].

Here, we used an inducible snoRNA transcription system to demonstrate the effectiveness and simplicity of the technique. Box C/D snR13 guides methylation at two positions in 25S rRNA, including adenine 2281 (**Figure 2A**). This nucleotide is followed by uracil, which constitutes the consensus dinucleotide (RY) cleavable by a 10-23 DNAzyme. Box C/D snR47 also guides methylation of two nucleotides in 25S rRNA (**Figure 2B**). The adenine in position 2220 is followed by a guanine residue and this dinucleotide can be cleaved by an 8-17 DNAzyme. In order to induce or inhibit synthesis of either snR13 or snR47 snoRNA, we inserted the inducible *GAL1* promoter upstream of either *SNR13* or *SNR47* genes and cultivated cells in medium containing galactose (*GAL1*-dependent transcription on) or glucose (*GAL1*-dependent transcription off). Next, RNA isolated from *GAL1::SNR13* cells were incubated with 10-23 DNAzyme designed to cleave 25S rRNA at snR13-dependent site, in between nucleotides 2281 and 2282 (**Figure 2C**). RNA from *GAL1::SNR47* strain was treated with 8-17 DNAzyme targeting snR47-dependent site in between nucleotides 2220 and 2221 (**Figure 2D**). As a control, RNA from the wild-type BY4741 strain growing on either galactose or glucose was incubated with both DNAzymes. Electrophoresis of the DNAzyme-treated RNA revealed that 25S rRNA extracted from *GAL1::SNR13* and *GAL1::SNR47* strains growing on galactose (GAL) remained intact (**Figure 3A**,**B;** lanes 3). In contrast, RNA isolated from *GAL1::SNR13* and *GAL1::SNR47* cells growing on glucose (GLC) was digested by respective DNAzymes (**Figure 3A,B**; lanes 4). In both cases, the 25S rRNA band decreased and 5' and 3' cut-off cleavage products (A and B) were observed. This indicates that in *GAL1::SNR13* and *GAL1::SNR47* strains, 25S rRNA was 2'-O-methylated at snR13- or snR47-guided sites when galactose was used as a carbon source and these snoRNA were expressed. The lack of 25S rRNA methylation when snR13 or snR47 expression was shut off on glucose allowed for DNAzyme-dependent cleavage. No RNA digestion was observed for wild-type samples (**Figure 3A**,**B;** lanes 1 and 2), as the expression of snR13 and snR47 is galactose/glucose-independent in this strain. Therefore, rRNA was normally methylated and so resistant to DNAzymes activity.

Overall, our experiment shows that the cleavage activity of 10-23 (**Figure 3A**) and 8-17 (**Figure 3B**) DNAzymes correlated with the absence of box C/D snR13 or snR47, clearly indicating that these snoRNA are responsible for 25S rRNA 2'-O-methylation at particular sites.

FIGURE AND TABLE LEGENDS:

Figure 1: DNAzymes and their RNA substrates. (**A**) 10-23 DNAzymes cleave a purine-pyrimidine (RY) RNA dinucleotide. R in the RNA is not paired with DNAzyme, while Y is complementary to the R base in the DNAzyme. Methylation of the purine (R) in RNA suppresses DNAzyme-dependent cleavage. (**B**) 8-17 DNAzymes cleave RNA upstream of guanine that is imperfectly paired with the first thymine in the DNAzyme catalytic sequence. The nucleotide preceding guanine is not paired and its methylation protects from DNAzyme-dependent cleavage. RNA is shown in grey (apart from the methylation site), DNAzyme is shown in purple. N – any nucleotide, R – purine: adenine or guanine, Y – pyrimidine: cytosine or uracil; CH3- denotes RNA methylation. Base pairing within the DNAzyme active sequences is marked by dotted lines. A blue lightning bolt marks the cleavage site. (**C**) A flowchart showing the steps of a DNAzyme-dependent analysis.

Figure 2: DNAzymes targeting snR13- and snR47-dependent methylation sites in 25S rRNA
(**A, B**) Browser screenshots showing snR13-dependent (**A**) and snR47-dependent (**B**) methylation sites in 25S rRNA (**C**) 25S rRNA sequence surrounding snR13-dependent methylation site (A_{2281}) and 10-23 DNAzyme (shown in purple) designed to cleave RNA between A_{2281} and U_{2282}. A_{2281} is not paired with the DNAzyme while U_{2282} forms a pair with the first nucleotide from the DNAzyme active sequence (marked by a blue line). A blue lightning bolt marks the cleavage. (**D**) 25S rRNA sequence surrounding snR47-dependent methylation site (A_{2220}) and 8-17 DNAzyme (shown in purple) designed to cleave RNA between A_{2220} and G_{2221}. A_{2220} is not hybridized with the DNAzyme while G_{2221} is imperfectly paired with thymine (denoted with a dashed line). A blue lightning bolt marks the cleavage site.

Figure 3: Analysis of site-specific 2'-O-methylation of 25S rRNA using 10-23 and 8-17 DNAzyme-dependent assay. (**A**) Analysis of snR13-dependent 25S rRNA methylation using 10-23 DNAzyme. (**B**) Analysis of snR47-dependent 25S rRNA methylation using 8-17 DNAzyme. RNA was visualized staining in a denaturing agarose gel. Cleavage products A and B are marked by red arrows. WT – wild-type strain; GAL – galactose, GLC – glucose.

DISCUSSION:

DNAzyme-dependent digestion can be used as a simple and quick method to analyze site-specific RNA 2'-O-methylation[12,13]. DNAzymes cleave RNA if the nucleotide upstream of the cleavage site is not methylated. In contrast to other approaches, including RNase H-directed digestion, alkaline degradation or reverse transcription in low nucleotides concentration followed by quantitative PCR or sequencing[8,10,11,16], DNAzyme approach requires a simple DNA oligonucleotide and basic reagents that are present in any molecular biology laboratory. Moreover, DNAzymes may be used in a similar way to analyze RNA pseudouridylation mediated by box H/ACA snoRNA[12], which makes them versatile tools in studying snoRNA targets.

DNAzyme-dependent approaches are limited only by cleavage site consensus sequences[17]. 10-23 DNAzymes can be used to analyze 2'-O-methylation only at position R of the RY dinucleotide, while 8-17 DNAzymes recognize the modification of the nucleotide located upstream of guanine. As a result, modifications like 2'-O-methylation of the first nucleotide in the dinucleotides guanine-adenine (GA), adenine-adenine (AA), pyrimidine-adenine (YA) and pyrimidine-pyrimidine (YY) cannot be analyzed. Moreover, the low efficiency of DNAzyme-dependent cleavage[12] should be considered. Although some DNAzymes cleave RNA almost completely (**Figure 3B**), many DNAzymes only partially digest their targets (**Figure 3B**). The efficiency may depend on the sequence surrounding the cleavage site. For example, RNA regions with stretches of the same nucleotide may affect the correct positioning of the DNAzyme active sequence. Furthermore, RNA regions forming strong secondary structure may re-hybridize and suppress DNAzyme binding to the target sequence. To overcome these issues, cycles of heating and cooling of the 10-23 DNAzyme and its RNA substrate can be applied[18].

We used the DNAzyme approach to investigate 2'-O-methylation of rRNA. One can also use this technique to analyze other RNA modifications, such as N^6-methyladenosine[19]. Ribosomal RNA, due to its abundance, can be analyzed by electrophoresis and the cleavage products can be

visualized under the UV light. However, this is not applicable for less abundant RNAs like RNA Polymerase II-generated coding RNAs (mRNA) and non-coding RNAs (ncRNA). These RNAs cannot usually be detected directly by RNA staining in agarose or polyacrylamide gels. In such cases, DNAzyme-dependent cleavage can be visualized by Northern blotting, indirectly detected by PCR/quantitative PCR or analyzed by quantitative PCR with polymerases (e.g., KlenTaq DNA Polymerase) capable of discriminating 2'-O-methylated RNA from unmethylated RNA[20,21].

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DISCLOSURES:

The authors have nothing to disclose.

REFERENCES:

1 Dieci, G., Preti, M., Montanini, B. Eukaryotic snoRNAs: a paradigm for gene expression flexibility. *Genomics.* **94** (2), 83-88, (2009).

2 Watkins, N. J., Bohnsack, M. T. The box C/D and H/ACA snoRNPs: key players in the modification, processing and the dynamic folding of ribosomal RNA. *Wiley Interdisciplinary Review RNA.* **3** (3), 397-414, (2012).

3 Kufel, J., Grzechnik, P. Small Nucleolar RNAs Tell a Different Tale. *Trends in Genetics.* 10.1016/j.tig.2018.11.005, (2018).

4 Li, T., Zhou, X., Wang, X., Zhu, D., Zhang, Y. Identification and characterization of human snoRNA core promoters. *Genomics.* **96** (1), 50-56, (2010).

5 Jorjani, H. et al. An updated human snoRNAome. *Nucleic Acids Research.* **44** (11), 5068-5082, (2016).

6 Hubbard, T. J. et al. Ensembl 2009. *Nucleic Acids Research.* **37** (Database issue), D690-697, (2009).

7 Makarova, J. A., Kramerov, D. A. SNOntology: Myriads of novel snoRNAs or just a mirage? *BMC Genomics.* **12** 543, (2011).

8 Yu, Y. T., Shu, M. D.,Steitz, J. A. A new method for detecting sites of 2'-O-methylation in RNA molecules. *RNA.* **3** (3), 324-331, (1997).

9 Decatur, W. A., Liang, X. H., Piekna-Przybylska, D., Fournier, M. J. Identifying effects of snoRNA-guided modifications on the synthesis and function of the yeast ribosome. *Methods in Enzymology.* **425** 283-316, (2007).

10 Dong, Z. W. et al. RTL-P: a sensitive approach for detecting sites of 2'-O-methylation in RNA molecules. *Nucleic Acids Research.* **40** (20), e157, (2012).

11 Birkedal, U. et al. Profiling of ribose methylations in RNA by high-throughput sequencing. *Angewandte Chemie International Edition, England.* **54** (2), 451-455, (2015).

12 Buchhaupt, M., Peifer, C., Entian, K. D. Analysis of 2'-O-methylated nucleosides and pseudouridines in ribosomal RNAs using DNAzymes. *Analytical Biochemistry.* **361** (1), 102-108, (2007).

13 Grzechnik, P. et al. Nuclear fate of yeast snoRNA is determined by co-transcriptional Rnt1 cleavage. *Nature Communication.* **9** (1), 1783, (2018).
14 Piekna-Przybylska, D., Decatur, W. A., Fournier, M. J. New bioinformatic tools for analysis of nucleotide modifications in eukaryotic rRNA. *RNA.* **13** (3), 305-312, (2007).
15 Schmitt, M. E., Brown, T. A.,Trumpower, B. L. A rapid and simple method for preparation of RNA from Saccharomyces cerevisiae. *Nucleic Acids Research.* **18** (10), 3091-3092, (1990).
16 Maden, B. E. Mapping 2'-O-methyl groups in ribosomal RNA. *Methods.* **25** (3), 374-382, (2001).
17 Santoro, S. W., Joyce, G. F. A general purpose RNA-cleaving DNA enzyme. *Proceedings of the National Academy of Science, U. S. A.* **94** (9), 4262-4266, (1997).
18 Hengesbach, M., Meusburger, M., Lyko, F., Helm, M. Use of DNAzymes for site-specific analysis of ribonucleotide modifications. *RNA.* **14** (1), 180-187, (2008).
19 Sednev, M. V. et al. N(6) -Methyladenosine-Sensitive RNA-Cleaving Deoxyribozymes. *Angewandte Chemie International Edition, England.* **57** (46), 15117-15121, (2018).
20 Aschenbrenner, J., Marx, A. Direct and site-specific quantification of RNA 2'-O-methylation by PCR with an engineered DNA polymerase. *Nucleic Acids Research.* **44** (8), 3495-3502, (2016).
21 Lee, K. W., Bogenhagen, D. F. Assignment of 2'-O-methyltransferases to modification sites on the mammalian mitochondrial large subunit 16 S ribosomal RNA (rRNA). *Journal of Biological Chemistry.* **289** (36), 24936-24942, (2014).

Figure 1

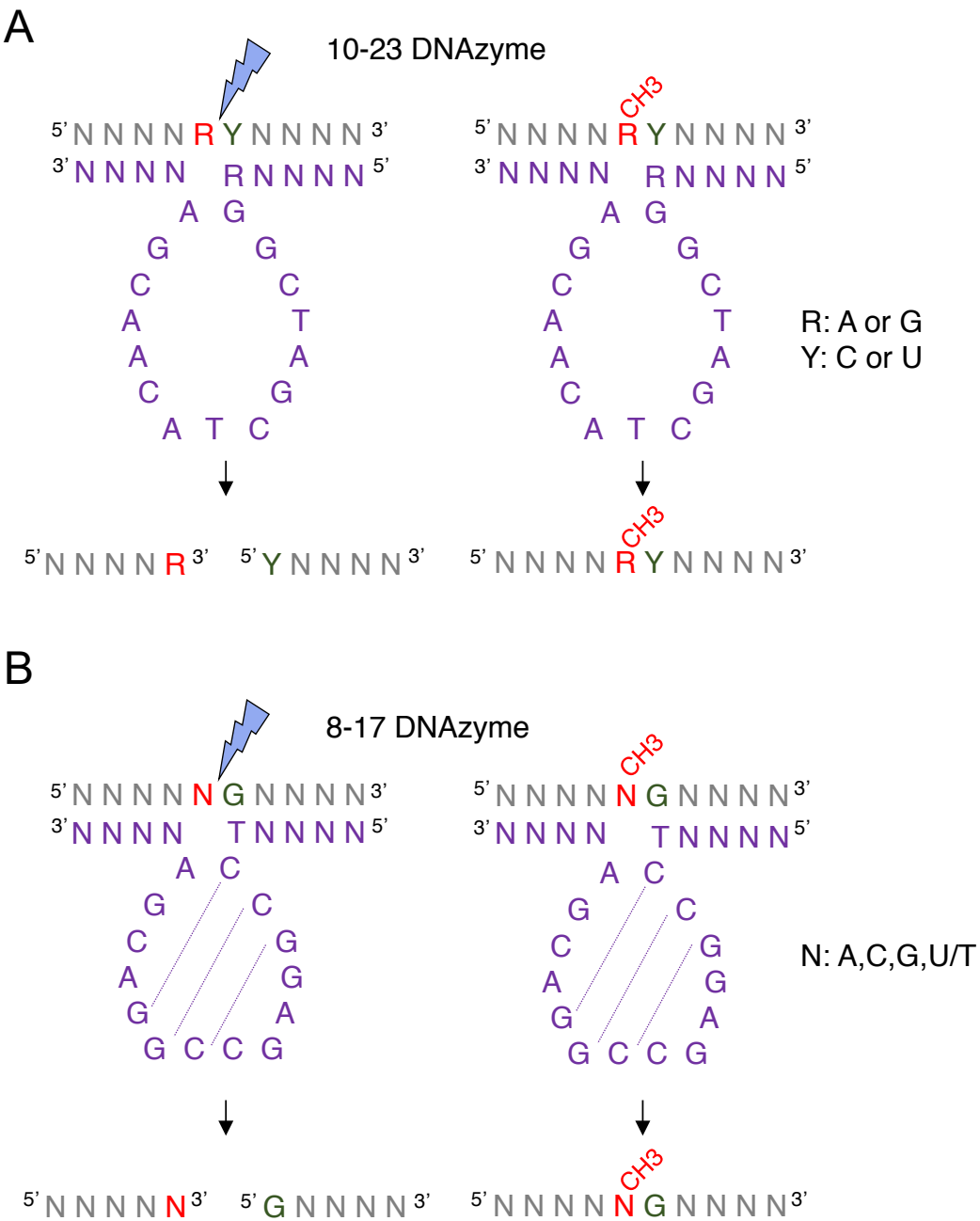
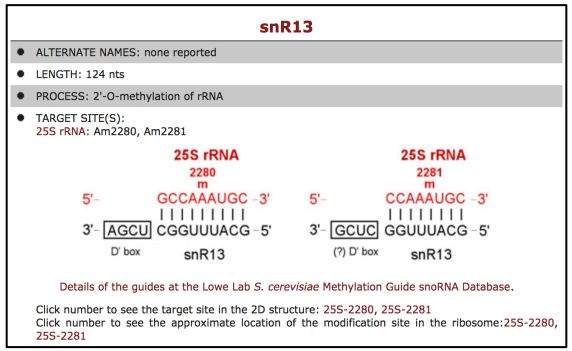
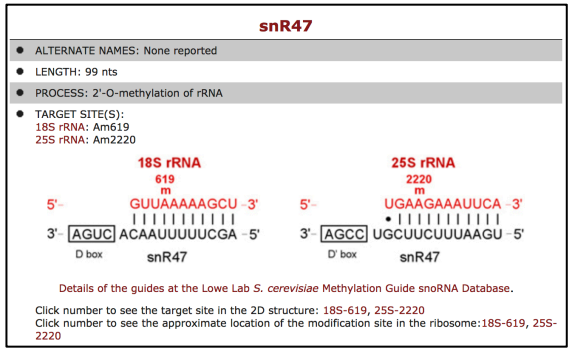


Figure 2

A

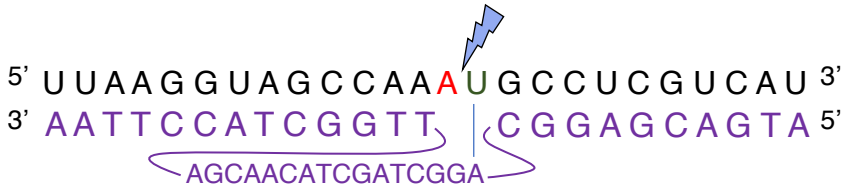


B



C

10-23 DNAzyme targeting snR13-dependent methylation



D

8-17 DNAzyme targeting snR47-dependent methylation

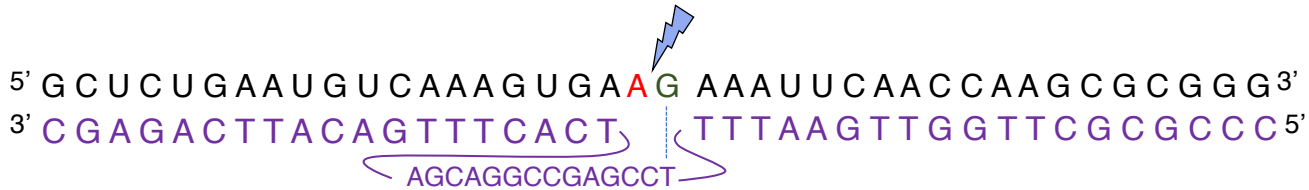
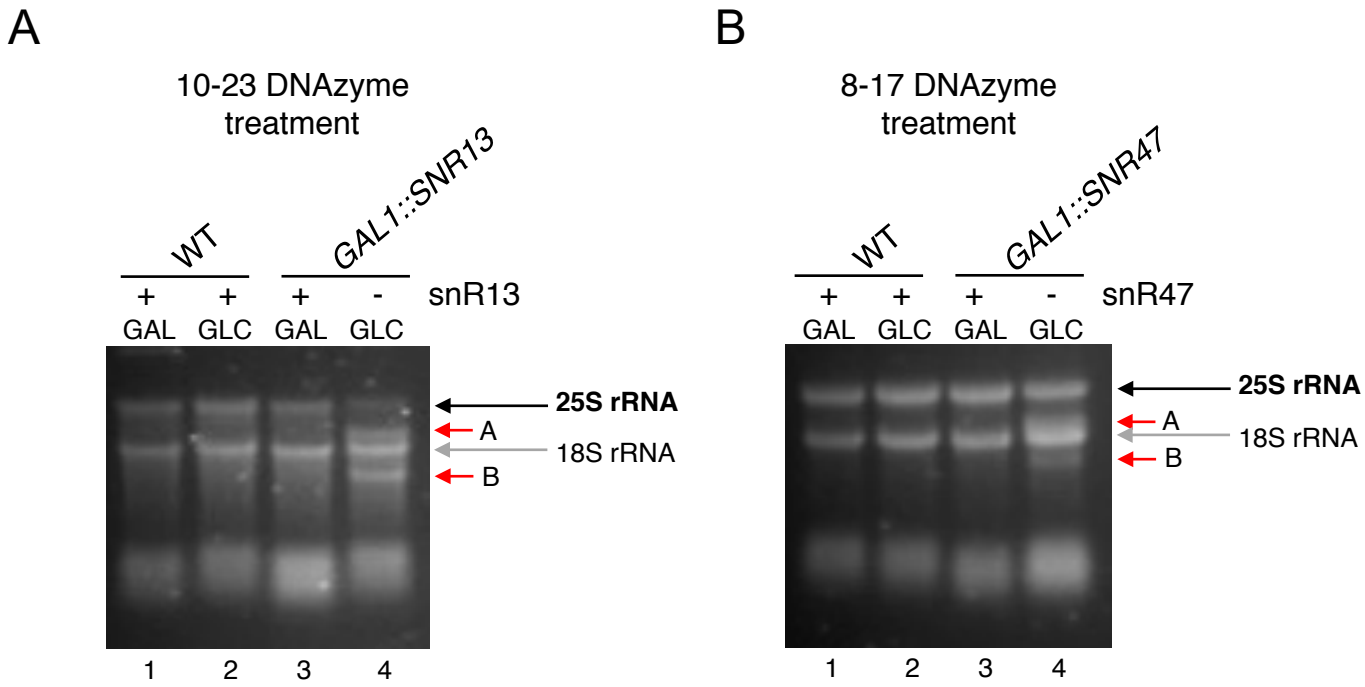


Figure 3



Chemicals	Company	Catalog Number	Comments/Description
Acid phenol	SIGMA	P4682	
Agarose	VWR	A2114	
Ammonium acetate	SIGMA	A1542	
Chlorophorm	Fisher scientific	10293850	
DNase/RNase free water	Fischer Scientific	10526945	
DNAzyme	Integrated DNA Technology	Custom oligo DNA	
EDTA	SIGMA	E9884	
Ethanol Absolute	Fisher scientific	10437341	
Formaldehyde	Sigma	F8775	
Formamide	sigma	F9037	
Galactose	SIGMA	G0750	
Gel Loading Dye	Thermo Fisher Scientific	R0611	
Glucose	SIGMA	G7021	
Glycogen	Thermo Fisher Scientific	R0561	
HEPES	SIGMA	H3375	
Isoamyl	SIGMA	W205702	
KCl	SIGMA	P9333	
MgCl2	SIGMA	M8266	
MnCl2	SIGMA	244589	
MOPS	SIGMA	M1254	
NaCl	SIGMA	S7653	
Oxoid Peptone Bacteriological	Thermo Fisher Scientific	LP0037	
Oxoid Yeast Extract Powder	Thermo Fisher Scientific	LP0021	
RiboLock RNase Inhibitor (40 U/µL)	Thermo Fisher Scientific	EO0382	
SDS	SIGMA	74255	
Sodium acetate trihydrate	SIGMA	S8625	
SYBR Safe DNA Gel Stain	Thermo Fisher Scientific	S33102	

Tris base	SIGMA	TRIS-RO

Equipment	Company
1.5 mL microtubes	Sarstedt
152VR5C01M -80°C freezer	Thermo Fisher Scientific
250 mL Erlenmeyer flasks	Cole-Parmer
50 mL conical tubes	Sarstedt
Combicup VX200 vortex	Appleton Woods
DS-11 microspectrophotometer	Denovix
Electrophoresis chamber (20 cm tray)	SIGMA
FiveEasy F20 pH meter	Appleton Woods
Gel documentation system	Syngene
Heraeus Fresco 21 micro centrifuge	Fisher Scientific
Megafuge 8R centrifuge with rotator suitable for 50 mL conical tubes	Fisher Scientific
Mini Fuge Plus mini centrifuge	Starlab
Mixer HC thermal block	Starlab
OLS26 Shaking Water Bath	Grant
PowerPac power supplier	BioRad

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Author(s):	Kinga Winczura, Pawel Grzechnik

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CORRESPONDING AUTHOR

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Department:	School of Biosciences
Institution:	University of Birmingham
Title:	Dr, PI
Signature:	
Date:	03.04.2019

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Birmingham, 3rd April 2019

The Review Editor
Journal of Visualized Experiments

Dear Vineeta,

Thank you for your reply and for the helpful comments from the reviewers. We have worked hard to address the comments and suggestions of the editor and the reviewers, placing particular emphasis on the clarity of the protocol.
Substantial improvement has been done regarding composition of the essential steps of the protocol for the video and clarity of text. We have added requested references and figures as well as we have discussed weaknesses and strengths of the technique.
Overall, we believe the manuscript is now very much improved and hope it is suitable for publication.
Below we give responses to the reviewers' individual comments.

Yours sincerely,

Pawel Grzechnik

Dr Pawel Grzechnik
Sir Henry Dale Fellow

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.
A: Done

2. Please sort the Materials Table alphabetically by the name of the material.
A: Sorted.

3. Please revise the title to be more concise: "Quick Protocol for" can be removed.
A: Corrected.

4. 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. Avoid usage of phrases such as "could be," "should be," and "would be" throughout the Protocol. Any text that cannot be written in the imperative tense may be added as a "Note." However, notes should be concise and used sparingly. Please include all safety procedures and use of hoods, etc.
A: Corrected.

5. Please add more details to your protocol steps. Please ensure you answer the "how" question, i.e., how is the step performed? Alternatively, add references to published material specifying how to perform the protocol action.
A: Corrected.

6. 3.2: What happens after centrifugation?
A: Explained.

7. Please highlight 2.75 pages or less of the Protocol (including headings and spacing) that identifies the essential steps of the protocol for the video, i.e., the steps that should be visualized to tell the most cohesive story of the Protocol. Remember that non-highlighted Protocol steps will remain in the manuscript, and therefore will still be available to the reader.
A: Done.

8. Please ensure that the highlighted steps form a cohesive narrative with a logical flow from one highlighted step to the next. Please highlight complete sentences (not parts of sentences). Please ensure that the highlighted part of the step includes at least one action that is written in imperative tense.
A: Done.

9. Please do not abbreviate journal titles in the References.
A: We downloaded and used JoVE Endnote style. The titles in the references appear according to the style indicated by JoVE's "guidelines for authors".

Reviewers' comments:

Please note that novelty is not a requirement for publication and reviewer comments questioning the novelty of the article can be disregarded.
A: We are not willing to disregard any comment as this is disrespectful for the reviewers. We recommend to advise reviewers about the character and mission of the journal.

Reviewer #1:
Manuscript Summary:
The manuscript outlines a protocol for detection of ribose-methylation in ribosomal RNA. The detection is based on the inhibitory effect of ribose-methylation on custom-designed DNAzymes, resulting in absence of cleavage if the targeted RNA nucleotide is ribose-methylated. DNAzyme cleaving activity on ribosomal RNA is assayed by denaturing agarose gel electrophoresis.
The manuscript is generally well written, and the protocol is easy to follow with only a few minor points that should be clarified (see below). The protocol essentially only requires standard lab equipment, but it is debatable if analysis is "quick" as stated in the Abstract, since design and production of DNAzymes is required for each new target.
A: We were advised by the journal to remove “quick protocol” from the title. However, we would like to point out that design, purchase and delivery of the oligo does not take longer than 3 working days.

Major Concerns:
A main concern with the method is the extremely low "catalytic" efficiency of the DNAzymes. The protocol uses several hundred-fold excess of DNAzyme to substrate, and still only a minor fraction of the substrate rRNA is cleaved. Given this very low efficiency, absence of cleavage may not mean methylation of the target nucleotide, but merely that the amount of cleaved substrate is below detection level. Many biological set-ups will not have the option for positive or negative controls as used in this work. This aspect must be included in the discussion as a minimum.
Secondly, the protocol described here is very close to a protocol publishes in "Analytical Biochemistry" in 2007 (reference 12 in this manuscript). It is not obvious that the current manuscript offers improvements or "tricks" that cannot be found in the 2007 publication.
A: In the revised version of the manuscript we discussed low efficiency issue and possible ways to tackle this problem.
Indeed, we followed established protocol in our studies. We raised the issue of the lack of novelty of the protocol when we had been initially approached by the editor. We were informed that the scope of JoVE is to provide a video guidance, not to publish a new or an improved method.

Minor Concerns:
-The title should be modified to make it clear that the protocol is essentially only applicable to ribosomal RNA due to its abundance.
A: We modified the title. We also discuss that the method can be used for less abundant RNA and the presence of the cleavage can be detected by more sensitive methods.

-Step 4.13: NH4AC is not an obvious abbreviation - define. Is the pH relevant here - acidic salts are often preferred for RNA precipitation
A: Corrected. We do not adjust pH of this solution.

-Step 4.22: A gel check for RNA quality should be introduced. Partially degraded RNA will make subsequent DNAzyme cleavage nearly impossible to assess.
A: RNA degradation usually manifests in equal disappearance of bands and smearing. It is very unlikely that degradation would be only endonucelolytic and would appear as a single band which corresponds to the size of the cleavage product.

-Step 5.3.4: Is there a need for glycogen as precipitation carrier?
A: Glycogen facilitates visualization and handling of the precipitated RNA pellet.

-Step 6.2: I guess that dissolving of agarose in water requires boiling!?
A: Yes. Information added.

Reviewer #2:
Manuscript Summary:
Using DNAzymes for RNA modification analysis is an attractive low cost approach that might allow targeted RNA modification analysis in low-throughput format and which should be accessible to a broader audience. The manuscript by Winczura and Grzechnik represents an easy-to-use protocol for detecting 2'-O-Me, which might be a good addition to the experimental repertoire of any lab interested in performing some targeted analysis of specific transcripts before embarking on a genome-wide hunt for additional ribose-methylation sites. This reviewer supports publication after the minor concerns I state below have been addressed.

Minor Concerns:
The introductory section should contain a paragraph that informs the interested reader that the use of DNAzymes for the detection of RNA modifications could and does extend beyond testing for 2'-O-Me.

- Z. Zaborowska, J. P. Fürste, V. A. Erdmann, J. Kurreck, J. Biol. Chem. 2002, 43, 40617.
- S. Schubert, D. C. Gül, H.-P. Grunert, H. Zeichhardt, V. A. Erdmann, J. Kurreck, Nucleic Acids Res. 2003, 31, 5982.
- m6A in RNA (DOI: 10.1002/ange.201808745) should be mentioned and referenced.
- 2'-O-Me analysis (PMID: 25074936) should be mentioned and referenced.
A: We added references *Sednev et al., 2018* as well as *Lee and Bogenhagen, 2014*. *Zaborowska et al.* and *Schubert et al.* describe optimization of the technique and therefore are not suitable in this context.

Figure 3 should be inverted (black/white) like it was done in Grzechnik, P. et al. Nuclear fate of yeast snoRNA is determined by co-transcriptional Rnt1 cleavage. Nat Commun. 9 1783, (2018). This would allow to see the weak banding pattern in Figure 3b to believe that an agarose gel is indeed a readout for the technique.
A: We compare both options and, in our opinion, white and black is more informative.

The discussion contains a list of potential drawbacks of the method. One is: "The second drawback is low efficiency of DNAzyme-dependent cleavage". As other protocols using DNAzymes used cycles of heating/annealing and cutting to improve the efficiency, it would be good if the authors would mention such possibility in their workflow or the discussion with the appropriate reference such as PMID: 17998290).

A: We added this information to the manuscript.

Reviewer #3:
In this manuscript Winzcura and Grzechnik describe the method allowing to check the 2'-O-methylation status of certain rRNA nucleotides, using specific RNA-cleaving DNAzymes. In the presence of 2'-O-methylation the cleavage is compromised, allowing to distinguish fully methylated and unmethylated sites. In principle the method is somehow useful and sometimes used in the RNA methylation field, however the reviewer has serious doubts on the proposed manuscript.
My major concerns are both total lack of novelty and also limited efficiency/usefulness for the proposed protocol. Original idea comes from Dr. D.Entian's lab (ref 12 in the manuscript) and the protocol used here was already published >10 years ago. As far as I can see, in the current manuscript the protocol remains essentially the same, so, except additional visual illustration of routine RNA biology manipulations, the manuscript does not bring anything new nor really helps new users in the field to apply it efficiently to other projects.
This is related to the intrinsic weakness of the method itself, by my own experience I know that the success of this approach heavily relies on the design of DNAzymes used for cleavage and some sites in rRNA are even not amenable to testing due to unfavourable sequence context, and most probably strong secondary rRNA structure around. Regrettably, authors provide only some very broad and imprecise guidelines on the DNAzyme design and illustrate the success with only one example of cleavage for both DNAzyme types used in this study.
To be really useful for the field, such method development study should be completed by much more extensive repertoire of "testable" sites (even for yeast rRNA, one should indicate which sites can be tested and with which DNAzyme, followed by experimental validation) and more serious optimization of the length and sequence of DNAzyme regions complementary to target rRNA.
In conclusion, in its current state, the manuscript brings very little new insight into the proposed analytical approach, and more experimental work and optimizations are required to warrant publication.
A: As the reviewer noticed this is established method which has been known and used for many years. We raised the issue of the lack of novelty of the protocol when we had been initially approached by the journal. The editor informed us that the scope of JoVE is to provide a video guidance for researchers and students not to publish a new or an improved method. Therefore, the above comment should be directed to the editor, not to the authors.

Reviewer #4:
Manuscript Summary:
This is a methods article to determine site specific 2'-O-Methylation of 25S ribosomal RNA in Saccharomyces cerevisiae by snoRNA snR13 and snR47. The authors utilize DNAzyme, short DNA oligonucleotides, for quick and cheap analysis of RNA methylation via snoRNA as determined by RNA electrophoresis. However, there are some suggested changes that needs to be addressed.

Major Concerns:
1. This is a methods paper, so it would be helpful for the readers to have a flowchart or a diagrammatic representation of the methods added to the figures.
A: A flow chart has been added.

2. Please incorporate the following paper in your citation:
"Direct and site-specific quantification of RNA 2′-O-methylation by PCR with an engineered DNA polymerase" by Joos Aschenbrenner and Andreas Marx and was published in NAR in 2016. This paper described the detection of 2'-O-methylation via a thermostable KlenTaq DNA polymerase variant. Perhaps this can be incorporated in the last paragraph of page 9 where the different methods for detecting 2'-O-methylation of RNA is discussed.
A: The citation has been added.

3. Please elaborate the last paragraph of page 9. The discussion for detecting 2'-O-methylation of RNA by various different methods should be more detailed.
A: We improved the discussion.

Minor Concerns:
1. Please discuss in a bit more detail the known functions of snoRNAs in the first paragraph of your introduction section (page1).
A: We think that discussing snoRNA functions is above the scope of the manuscript.

2. Please check the sentence structure of line 62 in page 1.
A: Corrected.

3. Line 97: provide full form for AE buffer.
A: Corrected.

4. The suggestion as provided in line 170 and others should start with "Note:" or "Helpful Suggestion:". I suggest changing "CAUTION" (line 174 etc.) to "Note" or "Helpful Suggestion" as well.
A: We feel that “Caution” is an adequate word when giving precautionary measures in order to be safe. We introduced “Note” in other parts of the manuscript.

5. Please mention an approximate days/months for the duration of which the sample can be stored (line 159, 206, 228, 298, 307)
A: Information added.

6. Please add citations for line 359 "The utility of the DNAzyme-dependent... of snoRNAs maturation." and line line 436 "The major disadvantage... consensus sequences."
A: The citation has been added.