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Post-transcriptional Modification of RNAs by Artificial Box H/ACA and Box C/D RNPs

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Abstract

RNA-guided RNA 2'-O-methylation and pseudouridylation are naturally occurring processes, in which guide RNAs specifically direct modifications to rRNAs or spliceosomal snRNAs in the nucleus of eukaryotic cells. Modifications can profoundly alter the properties of an RNA, thus influencing the contributions of the RNA to the cellular process in which it participates. Recently, it has been shown that, by expressing artificial guide RNAs (derived from naturally occurring guide RNAs), modifications can also be specifically introduced into other RNAs, thus offering an opportunity to study RNAs in vivo. Here, we present strategies for constructing guide RNAs and manipulating RNA modifications in the nucleus.

Keywords

snoRNA; 2'-O-methylation; Pseudouridylation; snRNA; rRNA; mRNA; Telomerase RNA; Box C/D RNA; Box H/ACA RNA

1. Introduction

Present within eukaryotic cells is a large and diverse pool of metabolically stable noncoding RNAs (ncRNAs) (1). Originally identified in eukaryotic cells, small nucleolar RNAs (snoRNAs) and small Cajal body-specific RNAs (scaRNAs) are two such groups of ncRNAs, which primarily localize to subnuclear compartments referred to as the nucleolus and Cajal bodies, respectively (1–3). However, these two groups of RNA are not limited to eukaryotes; in fact, they are also found in abundance in archaea, single-celled organisms lacking nuclei. To date, several hundred of these RNAs have been described in various organisms ranging from archaea to mammals, making these RNAs one of the most abundant groups of ncRNAs (4–17).

Sno/sca RNAs can be further divided into two separate classes of RNAs, referred to, respectively, as Box C/D and Box H/ACA RNAs (Fig. 1) (18). Both Box C/D and Box H/ACA RNAs exist in the cell as ribonucleoproteins (RNPs). The RNPs in each case consist of one sno/scaRNA and four class-specific core proteins. In the case of Box C/D RNPs, the protein components are Nop1p, Nop56p, Nop58p, and Snu13p (19–26), while for Box H/ACA RNPs the protein components are Cbf5p (dyskerin in humans), Gar1p, Nhp2p, and Nop10p (27–33). While some Box C/D and Box H/ACA RNPs are involved in the

nucleolytic processing of rRNA, the majority function as modifying enzymes catalyzing, respectively, the site-specific post-transcriptional 2'-O-methylation and pseudouridylation of cellular RNAs (Fig. 2) (34–38). Nop1p and Cbf5p are the catalytic components of their respective RNPs (39, 40). Site-specificity is dictated by complementary basepairing interactions between the RNA component of the RNP and the substrate RNA. Given the strong evidence for RNP trafficking among subnuclear compartments, perhaps including the nucleoplasm, we believe that Box C/D and Box H/ACA RNPs are not confined within the nucleolus or Cajal bodies in eukaryotic cells. Consequently, Box C/D and Box H/ACA RNPs may be able to direct modifications to other cellular RNAs residing in various subnuclear compartments. Thus, by constructing novel Box C/D or Box H/ACA guide RNAs, we should be able to introduce 2'-O-methylation and pseudouridylation site-specifically into any RNAs in the nucleus.

Post-transcriptional modification of ribonucleotides represents a way to increase the chemical diversity of RNA molecules beyond the four canonical bases (adenine, guanine, cytosine, and uracil) (41). While 2'-O-methylation is a sugar-ring modification, pseudouridylation is a uridine-specific modification. It is well established that both modifications alter the chemical properties of the nucleotide. These distinct chemical properties have the potential to impact numerous aspects of the RNA, including structure, thermal stability, and interactions (41). In each case, the effect of the modifications on the RNA depends on the structural context and can extend beyond the site of modification. In this regard, conformation stabilization appears to be an inherent property of pseudouridine, and is mediated by both an increase in base stacking and the ability to coordinate a water molecule through the extra hydrogen bond present (41–43). Furthermore, pseudouridine is slightly more polar than uridine (41). Similarly, data also suggest that 2'-O-methylation is capable of stabilizing RNA conformations. For instance, 2'-O-methylation blocks sugar edge interactions through the alteration of the hydration sphere around the oxygen (44–46). In addition, 2'-O-methylation inhibits the ability of the ribose to hydrogen bond with bases (41). Furthermore, it also plays a role in protecting the RNA from hydrolysis by alkaline substances and nucleases.

It is anticipated that, by introducing modified nucleotides into an RNA chain, we will be able to manipulate the properties of the RNA, thus offering an opportunity to study the structure of the RNA and its functions and mechanisms of action in the process in which it participates. Through the construction and expression of artificial guide RNAs, we have directed modifications to various cellular RNAs and have manipulated their ability to function as well as participate in various processes. For instance, the expression of an artificial C/D RNA capable of directing 2'-O-methylation to the branch point adenosine of *ACT1* pre-mRNA resulted in the modification of the residue and the subsequent inhibition of pre-mRNA splicing (47). Thus far, we have successfully targeted messenger RNAs (mRNA) ((47), Karijovich and Yu, unpublished data), spliceosomal small nuclear RNAs (snRNA) (Stephenson and Yu, Wu and Yu, unpublished data), ribosomal RNAs (rRNA) (Fig. 3), and telomerase RNA (Fig. 4, Huang and Yu, unpublished data). Here we present detailed strategies for both the construction and expression of a Box C/D RNA targeting rRNA and the mapping of novel 2'-O-methylation sites.

2. Materials

2.1. Construction of an snR52 Box C/D snoRNA Expression Cassette

1. Oligodeoxynucleotide primers: snR52-F1 5'-ACGTCGACA TAAATGATCT ACTATGATGAATGACATTATGCGCGC C T G C T T C T G ATA C A A A AT C G A A A G AT T T TA G GATTAGAA-3', snR52-R1 5'-ATCTGCAGAAAAAATA AA TTTTCTGAGGCGCGCATAAGTTTTTCTAATCCTAAAATC-3' (IDT) (see Note 1).
2. 10× *Taq* DNA polymerase buffer (Fermentas).
3. 10 mM dNTPs (Fermentas).
4. *Pst*I restriction endonuclease (Fermentas).
5. *Sal*I restriction endonuclease (Fermentas).
6. 10× Orange restriction digestion buffer (Fermentas).
7. *Taq* DNA polymerase (5 U/μL) (Fermentas).
8. T4 DNA ligase (1 U/μL) (Fermentas).
9. 5× T4 ligase buffer (Fermentas).
10. DH5α competent cells (Stratagene).
11. LB liquid medium: 10 g NaCl, 10 g peptone, 5 g yeast extract, fill to 1 L with ddH₂O and autoclave.
12. LB-ampicillin solid medium; 20 g Agar, 10 g NaCl, 10 g peptone, 5 g yeast extract, fill to 1 L with ddH₂O and autoclave. Allow to cool and add 1 mL of 100 mg/mL ampicillin before pouring plates.
13. pSEC: snoRNA expression cassette (Fig. 5).

2.2. Transformation of *Saccharomyces cerevisiae* with snR52 Box C/D snoRNA Expression Cassette

1. One-Step-Transformation buffer: 100 mM lithium acetate, 50% (w/v) PEG-3350 solution.
2. YPD liquid medium: 10 g yeast extract, 20 g peptone, and 20 g dextrose, fill with ddH₂O to 1 L and autoclave.
3. SD-LEU liquid medium: 7.5 g Synthetic Leucine Drop Out Powder from Table 1, 20 g dextrose, fill to 1 L with ddH₂O and autoclave.

¹Artificial guide RNAs can be derived from any known Box C/D or Box H/ACA RNAs. Here we chose snR52 because it has two guide sequences available that can be altered to target two unique sites of interest. We have also tested snR50 which has only one guide sequence available, and it also proved to be suitable for delivering artificial modification. The Box H/ACA RNA snR81 has been used for targeting mRNA and snRNA.

4. SD-LEU solid medium: 7.5 g Synthetic Leucine Drop Out Powder from Table 1, 20 g Agar, 20 g dextrose, fill to 1 L with ddH₂O and autoclave.

2.3. Total RNA Extraction from *Saccharomyces cerevisiae*

1. Trizol reagent (Invitrogen).
2. 0.5 mm acid washed glass beads (BioSpec).
3. Chloroform.
4. 10 mg/mL glycogen (Sigma).
5. 3 M Sodium acetate, pH 5.0.
6. Isopropanol.

2.4. Labeling and Purification of snR52-PXT Primer

1. T4 Polynucleotide Kinase (10 U/μL) (Fermentas).
2. snR52-PXT oligonucleotide 5'-GTTATTTATTGTCACTACCTCCCTG-3' (IDT) (see Note 2).
3. 10× T4 Polynucleotide Kinase Buffer A (Fermentas).
4. G50 Buffer: 20 mM Tris-HCl, 300 mM Sodium Acetate, 2 mM EDTA, 0.2% SDS, pH 7.5.
5. [γ -³²P] ATP (adenosine-5'-triphosphate. 6,000 Ci/mmol).
6. PCA: (phenol/chloroform/isoamyl alcohol = 25/24/1 [v/v/v]) saturated with 20 mM Tris-HCl, pH 8.0.
7. 40% Acrylamide:Bis (19:1).
8. 10% Ammonium persulfate (APS).
9. TEMED.
10. 5× TBE buffer: 445 mM Tris-HCl, 445 mM boric acid, 16 mM EDTA.
11. 2× Loading dye: 90% deionized formamide, 10 mM EDTA, 0.1% (w/v) bromophenol blue, 0.1% (w/v) xylene cyanol FF.

2.5. Detection of 2'-O-Methylation by Primer Extension

1. G50 Buffer: 20 mM Tris-HCl, 300 mM sodium acetate, 2 mM EDTA, 0.2% SDS, pH 7.5.
2. 2× annealing buffer: 500 mM KCl, 20 mM Tris-HCl, pH 8.3.
3. 2.5× dNTP 4 mM (High): 1 mM dATP, 1 mM dTTP, 1 mM dGTP, 1 mM dCTP, 8 mM DTT, 16 mM MgCl₂, 24 mM Tris-HCl, pH 8.3.

²We usually use 22–25 nt long oligodeoxynucleotide primers which have a G or C at the 3' end to ensure efficient extension in the reverse transcription reaction. If possible, design the primer such that it hybridizes approximately 40 nt downstream of the expected modification site. This will allow for the best resolution of the band representing the modified nucleotide.

4. 2.5× dNTP 0.04 mM (Low): 0.01 mM dATP, 0.01 mM dTTP, 0.01 mM dGTP, 0.01 mM dCTP, 8 mM DTT, 16 mM MgCl₂, 24 mM Tris-HCl, pH 8.3.
5. Avian myeloblastosis virus (AMV) reverse transcriptase (10 U/μL) (Promega).
6. PCA: (phenol/chloroform/isoamyl alcohol = 25/24/1 [v/v/v]) saturated with 20 mM Tris-HCl, pH 8.0.
7. Ethanol.
8. 70% ethanol.
9. 2× Loading dye: 90% deionized formamide, 10 mM EDTA, 0.1% (w/v) bromophenol blue, 0.1% (w/v) xylene cyanol FF.

2.6. Sequencing Reactions

1. 5× sequencing buffer: 250 mM Tris-HCl, pH 9.0, 10 mM MgCl₂.
2. d/ddATP mixture: 350 μM ddATP, 80 μM dNTP.
3. d/ddCTP mixture: 200 μM ddCTP, 80 μM dNTP.
4. d/ddGTP mixture: 30 μM ddGTP, 80 μM dNTP.
5. d/ddTTP mixture: 600 μM ddTTP, 80 μM dNTP.
6. *Taq* DNA polymerase 5 U/μL (Promega).
7. p18SrRNA: plasmid-containing cDNA of yeast 18S rRNA corresponding to nucleotides 1–500.

3. Methods

3.1. Construction of an snR52 Box C/D snoRNA Expression Cassette

1. In a 0.2 mL PCR tube mix 5 μL of 10× *Taq* DNA polymerase buffer, 2 μL of 10 mM dNTPs, 2 μL of 10 μM snR52-F1 primer, 2 μL of 10 μM snR52-R1 primer, 1 μL (5 U/μL) of *Taq* DNA polymerase, and 38 μL of ddH₂O (see Fig. 6).
2. Perform PCR cycles as follows:
 - Step 1: 95°C 2 min (1 cycle)
 - Step 2: 95°C 30 s
30°C 30 s
72°C 30 s (repeat step 2 35 times)
 - Step 3: 72°C 120 s (1 cycle)
4°C (indefinitely)
3. Transfer PCR reaction to a 1.5-mL Eppendorf tube. Add 450 μL of G50 buffer and 500 μL of PCA. Vortex the mix for 30 s. Spin the mixture for 3 min at 13,000 rpm in a bench-top centrifuge.

4. Collect top aqueous phase and transfer to a clean 1.5-mL Eppendorf tube. Add 1 μ L of 10 mg/mL glycogen and 1 mL of 100% ethanol.
5. Centrifuge sample at 13,000 rpm for 10 min in a bench-top centrifuge. Remove supernatant and allow the pellet to air dry for 5 min. Resuspend the pellet in 10 μ L ddH₂O. Quantify the PCR product by U.V. spectroscopy.
6. In a 1.5-mL Eppendorf tube, add 5 μ g of PCR products, 5 μ L of 10 $\times$ Orange digestion buffer, 2.5 μ L of *SalI* (10 U/ μ L) and 2.5 μ L of *PstI* (10 U/ μ L), and bring the total volume to 50 μ L with ddH₂O. Incubate in a 37°C water bath overnight.
7. In a second 1.5 mL Eppendorf tube, add 1 μ g of pSEC, 5 μ L of 10 $\times$ Orange digestion buffer, 2.5 μ L of *SalI* (10 U/ μ L) and 2.5 μ L of *PstI* (10 U/ μ L), and bring the total volume to 50 μ L with ddH₂O. Incubate in a 37°C water bath overnight.
8. To both tubes add 450 μ L of G50 buffer, 500 μ L of PCA, and vortex for 30 s. Centrifuge at 13,000 rpm for 5 min in a bench-top centrifuge.
9. Transfer the aqueous phase to new 1.5-mL Eppendorf tubes and add 1 mL of 100% ethanol, centrifuge at 13,000 rpm for 10 min in a bench-top centrifuge.
10. Remove supernatant and allow the pellet to air dry for 5 min. Dissolve both pellets in 10 μ L of ddH₂O.
11. In a new 1.5-mL Eppendorf tube, add 5 μ L of digested PCR product, 1 μ L of digested plasmid, 2 μ L of 5 $\times$ T4 DNA ligase buffer, 2 μ L of T4 DNA ligase (1 U/ μ L), and 10 μ L of ddH₂O.
12. Incubate ligation mix in a 16°C water bath overnight.
13. Mix 10 μ L ligation mix with 100 μ L of DH5 α competent cells, and put on ice for 10 min. Heat shock at 42°C for 45 s. Put on ice for 2 min.
14. Add 900 μ L LB liquid medium and shake at 200 rpm for 45 min.
15. Centrifuge at 4,000 rpm for 5 min in a bench-top centrifuge.
16. Remove supernatant and resuspend cell pellet in 100 μ L of LB liquid medium. Spread cells on LB-ampicillin solid medium plate.
17. Incubate in a 37°C incubator overnight to observe colonies.

3.2. Transformation of *Saccharomyces cerevisiae* with snR52 Box C/D snoRNA Expression Cassette

1. Inoculate single yeast colony into 5 mL YPD liquid medium and shake at 200 rpm overnight at 30°C.
2. Dilute cells in 5 mL fresh YPD medium to an OD_{600nm} of 0.5.
3. When the optical density reaches 2 at OD_{600nm}, collect cells at 3,000 rpm using SH3000 rotor in a Sorvall RC-5C Plus centrifuge for 5 min at 4°C. Remove YPD medium completely (see Note 3).

4. Resuspend cell pellet in 200 μ L of One-Step-Transformation Buffer to the concentration of 50 OD_{600nm} cells/mL.
5. Aliquot 50 μ L of Buffer/Cell mix to a new sterile 1.5 mL Eppendorf tube, add 1–5 μ g of artificial snR52-containing pSEC and mix thoroughly.
6. Incubate mixture in 42°C water bath for 30 min.
7. Spin down cells at 3,000 rpm for 3 min at RT in a bench-top centrifuge, and remove supernatant.
8. Resuspend cells in 100 μ L of ddH₂O and evenly spread on the surface of SD-LEU solid medium.
9. Incubate at 30°C for 2–3 days to observe colonies.

3.3. Total RNA Extraction from *Saccharomyces cerevisiae*

1. Inoculate single yeast colony (already transformed with artificial snR52-containing pSEC) in 20 mL of SD-LEU liquid medium. Shake at 200 rpm overnight at 30°C.
2. Collect the cells at 3,000 rpm for 5 min at 4°C using SH3000 rotor in a Sorvall RC-5C plus centrifuge.
3. Resuspend cell pellet in 1 mL of cold ddH₂O and transfer to a 2-mL screw-cap tube (see Note 4). Collect cells at 3,000 rpm for 5 min at 4°C using a chilled bench-top centrifuge.
4. Remove the ddH₂O and resuspend cells in 500 μ L of Trizol reagent. Add 400 μ L of acid washed glass beads.
5. Vigorously vortex the mixture for 1 min in a bench-top vortex. Place on ice for 1 min. Repeat 5 times.
6. Centrifuge at 13,000 rpm for 5 min at RT in a bench-top centrifuge. Transfer aqueous layer to a new 1.5-mL Eppendorf tube.
7. Add 100 μ L of chloroform, vortex briefly and then centrifuge at 13,000 rpm for 5 min at RT in a bench-top centrifuge.
8. Transfer upper aqueous phase to a new 1.5 mL Eppendorf tube. Add 1 μ L of 10 mg/mL glycogen and 500 μ L of isopropanol and vortex briefly. Centrifuge at 13,000 rpm for 10 min at RT in a bench-top centrifuge.
9. Remove the supernatant and allow the pellet to air dry for 5 min. Resuspend the pellet in 20 μ L of ddH₂O.
10. Measure RNA concentration by U.V./VIS spectroscopy. A good RNA preparation should give an A260/280 ratio of 1.8–2.0.

³We have experienced that if trace amounts of YPD medium are present in the transformation buffer the efficiency of transformation may be reduced. Washing the cell pellet with sterile ddH₂O before adding transformation buffer will increase the efficiency of transformation.

⁴Washing yeast cells with sterile ddH₂O is necessary when using cells from saturation phase. Cell pellets can be stored at –70°C for a month without losing the integrity of total RNA.

3.4. Labeling and Purification of snR52-PXT Primer

1. Mix 3 μ L of 10 μ M snR52-PXT primer with 1 μ L of 10 $\times$ PNK buffer A, 1 μ L of [γ - 32 P] ATP (10 μ Ci/ μ L), 1 μ L of (10 U/ μ L) T4 PNK, and 4 μ L of ddH₂O.
2. Incubate at 37°C for 30 min. Terminate reaction by adding 400 μ L of G50 buffer and 400 μ L of PCA.
3. Briefly vortex mixture. Centrifuge at 13,000 rpm for 5 min at RT in a bench-top centrifuge.
4. Transfer aqueous phase to a new 1.5-mL Eppendorf tube and add 1 μ L of 10 mg/mL glycogen and 1 mL of cold ethanol.
5. Centrifuge at 13,000 rpm for 10 min in a bench-top centrifuge. Remove supernatant and allow the pellet to air dry for 5 min.
6. Dissolve pellet in 2 μ L of ddH₂O and 2 μ L of 2 $\times$ Loading Dye. The sample is ready to be loaded.
7. Prepare an 8% polyacrylamide-8 M urea sequencing gel (16 $\times$ 30 cm, 0.4 mm spacer, 1 cm comb). For 60 mL of 8% polyacrylamide-8 M urea gel, dissolve 28.8 g urea in 12 mL 40% acrylamid/bisacrylamide (19:1) solution. Add 6 mL 5 $\times$ TBE buffer and bring the final volume to 60 mL using ddH₂O. Add 360 μ L 10% ammonium persulfate and 36 μ L TEMED. Cast the gel immediately.
8. Pre-run the gel at 16 W of constant power for 30 min.
9. Denature the labeled primer at 95°C for 1 min and immediately load onto the gel.
10. Run the gel at 16 W of constant power. Run the gel until the bromophenol blue dye is approximately half way through the gel (see Note 5).
11. After removing the upper plate, wrap the gel in Saran Wrap plastic film, tape marker strips, and expose the gel to phosphorimager screen for 1 min (see Note 6).
12. Localize and cut the radiolabeled snR52-PXT primer from the gel. Place the gel slice into a 1.5-mL Eppendorf tube and add 500 μ L of G50 buffer. Leave at RT overnight (see Note 7).
13. Add 500 μ L PCA to G50 buffer/gel slice mix and vortex briefly. Centrifuge at 13,000 rpm for 5 min at RT in a bench-top centrifuge. Transfer the aqueous phase to a new 1.5-mL Eppendorf tube (see Note 8).

⁵When the bromophenol blue dye has ran approximately half way through the gel, the unreacted [γ - 32 P] ATP and some radioactive free phosphate are still retained in the gel keeping the lower buffer chamber free of radioactivity and easing the cleaning of the gel box apparatus.

⁶Depending on the intensity of [γ - 32 P] ATP, it is recommended to titrate the exposure time from 30 s to 5 min.

⁷Cutting the gel slice into a 5 $\times$ 5 mm square is ideal for downstream manipulation. Very small slices are difficult to be removed from the aqueous layer and can potentially hinder the precipitation of radiolabeled primers in ethanol.

⁸The purpose of adding PCA is to keep the aqueous layer free of contamination by any trace amounts of protein, as well as keep the gel slice at the bottom of aqueous phase.

14. Precipitate snR52-PXT by adding 1 mL of cold ethanol and 1 μ L of 10 mg/mL glycogen and vortex briefly. Centrifuge at 13,000 rpm for 10 min at RT in a bench-top centrifuge.
15. Remove the supernatant and add 1 mL of 70% ethanol. Centrifuge at 13,000 rpm for 10 min at RT in a bench-top centrifuge.
16. Remove the ethanol and allow the pellet to air dry for 5 min. Dissolve the pellet in 20 μ L of ddH₂O and store at -20°C until use.

3.5. Detection of 2'-O-Methylation by Primer Extension

Perform two primer extension reactions for each RNA sample of interest. These two reactions are almost identical except for the amount of dNTP added. In the high dNTP concentration reaction, AMV can bypass sites of 2'-O-methylation site. However, reverse transcription is inhibited by the presence of a 2'-O-methylated residue when carried out at a low dNTP concentration. Specifically, the presence of a 2'-O-methylated residue will cause the AMV to stop one nucleotide before the modification site, which will appear as a premature stop when the reaction is resolved on a denaturing sequencing gel.

1. In a 1.5-mL Eppendorf tube mix 2 μ L of 2 $\times$ annealing buffer, 1 μ L of labeled snR52-PXT, 5–20 μ g of test RNA in a total of 4 μ L volume, label as Mixture-High. Prepare a second tube exactly as above, but labeled as Mixture-Low (see Note 9).
2. Heat both Mixtures at 95°C for 1 min, and gradually chill at RT for 10 min.
3. In the meantime, prepare two different extension mixes. For Extension-High (High concentration dNTP), mix 16 μ L of 2.5 $\times$ dNTP 4 mM, 3 μ L of ddH₂O, 1 μ L of AMV (10 U). For Extension-Low (Low concentration dNTP), mix 16 μ L of 2.5 $\times$ dNTP 0.04 mM, 3 μ L of ddH₂O, 1 μ L of AMV (10 U) (see Note 10).
4. Add 4 μ L of Extension-High to Mixture-High, and 4 μ L of Extension-Low to Mixture-Low. Mix them well.
5. Incubate in a 42°C water bath for 30 min. Stop the reaction by adding 400 μ L of G50 buffer. Add 400 μ L PCA and vortex briefly. Centrifuge at 13,000 rpm for 5 min at RT in a bench-top centrifuge.
6. Transfer the upper aqueous phase into a new 1.5-mL Eppendorf tube and add 1 μ L of 10 mg/mL glycogen, and 1 mL of cold ethanol.
7. Centrifuge at 13,000 rpm for 10 min at RT in a bench-top centrifuge.
8. Add 1 mL 70% cold ethanol and vortex briefly. Centrifuge at 13,000 rpm for 10 min at RT in a bench-top centrifuge.

⁹The minimum amount of RNA template needed for the reaction depends on the relative abundance of the RNA of interest. For example, 1 μ g of total RNA is sufficient when determining the modification status of 18S ribosomal RNA. However, approximately 20 μ g of total RNA is required when analyzing telomerase RNA.

¹⁰Each 20 μ L Extension mix is enough for four primer extension reactions (considering pipetting error). When dealing with a different number of reactions the mix can be scaled up or down.

9. Remove the ethanol and allow the pellet to air dry for 5 min. Dissolve the pellet in 3 μ L of ddH₂O and 3 μ L of 2 $\times$ loading dye. Samples can be stored at -20°C for a couple of days (see Note 11).

3.6. Sequencing Reactions

To determine the position of 2'-O-methylation, a dideoxy-sequencing ladder should be run next to the primer-extension products from the modification mapping assay. In the sequencing reactions, a recombinant plasmid carrying the cDNA of the RNA of interest is used as a template (for both 18S rRNA and TLC1 RNA).

1. Mix 5 μ L of 5 $\times$ sequencing buffer, 1 μ g of p18SrRNA, 2 μ L of radiolabeled snR52-PXT, 1 μ L of *Taq* DNA polymerase (5 U), and bring the final volume to 17 μ L using ddH₂O. Label as Master Mix.
2. Label four PCR tubes as G, A, T, and C, respectively, and add 2 μ L of the corresponding d/ddNTP mix to each PCR tube.
3. Distribute 4 μ L of Master Mix to each PCR tube.
4. Perform PCR reaction as follows:
Step 1: 94°C 2 min (1 cycle)
Step 2: 94°C 30 s
 42°C 30 s
 72°C 30 s (repeat step 2 25 times)
Step 3: 4°C (indefinitely)
5. Add 6 μ L of 2 $\times$ loading dye to each tube and heat at 95°C for 2 min. Samples can be stored at -20°C for a couple of days.

3.7. Polyacrylamide Gel Electrophoresis

1. To analyze the primer-extension products and sequencing reaction products, prepare an 8% polyacrylamide-8 M urea sequencing gel (30 $\times$ 40 cm, 0.4 mm spacer, 0.5 cm comb). For an 80 mL of 8% polyacrylamide-8 M urea gel, dissolve 38.4 g urea in 16 mL of 40% acrylamide/bisacrylamide (19:1) solution. Add 8 mL 5 $\times$ TBE buffer and bring the final volume up to 80 mL using ddH₂O. Add 480 μ L of 10% ammonium persulfate and 48 μ L of TEMED.
2. Pre-run the gel at 40 W of constant power for 30 min (see Note 12).
3. Denature primer extension and sequencing samples (see above) by heating at 95°C for 1 min immediately prior to loading.

¹¹It is recommended to load the primer-extension products onto the gel immediately after reactions. Although the remaining samples can be loaded and resolved on the gel later, we often experience that the immediate loading of the reactions results in the best looking gels.

¹²When pre-running, one can load 3 μ L of 2 $\times$ loading dye into each well to ensure each lane is free of undesirable air bubbles.

4. Load 3 μ L of primer extension reactions and 3 μ L of each sequencing reaction on the gel. The remaining samples can be stored at -20°C .
5. Run the gel at 40 W of constant power until the bromophenol blue dye is approximately 4 cm from the bottom of the gel.
6. Separate the plates and transfer the gel onto a sheet of Whatman filter paper. Cover the gel side with Saran Wrap plastic film.
7. Dry gel in a BioRad Gel Dryer with the filter paper side facing the vacuum for 60 min at 90°C .
8. Expose dried gel to a phosphorimager screen overnight then visualize sequencing ladders and primer-extension products (see Note 13).

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¹³In order to achieve best result, expose the gel for several days before scanning.

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Fig. 1.

(a) Schematic structure of a Box C/D RNA. The 5' and 3' end of the RNA adopts a terminal stem structure. The RNA has four conserved regions, from 5' to 3' termed Box C, Box D', Box C', and Box D. Between Box C and Box D', and between Box C' and Box D are guide sequences that are capable of basepairing with the complementary sequences within the substrate RNAs. The nucleotide which is basepaired to the fifth nucleotide upstream of Box D and/or Box D' is the target for 2'-O-methylation. The thick black line represents the substrate RNA (including rRNA and snRNA). (b) Schematic representation of a Box H/ACA RNA. Box H/ACA RNAs adopt a hairpin-hinge-hairpin-tail structure. The Box H is located within the hinge region, while the Box ACA is typically located three nucleotides upstream of the 3' end. The internal loop is capable of basepairing with complementary sequences within the substrate RNA. The uridine residue targeted for pseudouridylation, as well the adjacent downstream nucleotide, are positioned at the base of the upper stem

approximately 14–16 nucleotides upstream of either Box H or Box ACA and are left unpaired so as to remain accessible for isomerization.

Fig. 2. The conversion of uridine to pseudouridine (*upper diagram*) and 2'-OH ribose to 2'-O-methylated ribose (*lower diagram*).

Fig. 3.

Detection of 2'-O-methylation in yeast 18S rRNA by Primer Extension. Total RNA extracted from yeast harboring a plasmid targeting nucleotide A428 of 18S rRNA was assayed for novel 2'-O-methylation by primer extension. The Am420 and Am436 are naturally occurring 2'-O-methylation sites which are introduced by endogenous snR52 and endogenous snR87, respectively. A sequencing reaction was run in parallel to verify the modified nucleotide's identity.

Fig. 4.

Detection of 2'-O-methylation in yeast telomerase RNA by primer extension. Total RNA extracted from yeast harboring a plasmid targeting nucleotide A806 and U809 of TLC1 RNA was assayed for novel 2'-O-methylation by primer extension. A sequencing reaction was run in parallel to verify the modified nucleotide's identity. The two bands designated by *arrows* are A806 and U809 according to the sequence ladder.

Fig. 5.

Schematic representation of pSEC (containing artificial snR52). The artificial snR52 gene is flanked by two snoRNA processing elements (an RNT1 cleavage site and an snR13 terminator), and is expressed under the control of the GPD promoter. The RNT1 cleavage site represents the 65 nt long sequence in the 3' ETS region of rRNA that is recognized and cut by the endonuclease Rnt1p (5'-TTT TTA TTT CTT TCT AAG TGG GTA CTG GCA GGA GTC GGG GCC TAG TTT AGA GAG AAG TAG ACT CA-3'); the snR13 terminator represents the 55 nt long sequence downstream of snR13 which is responsible for transcription termination (5'-AGT AAT CCT TCT TAC ATT GTA TCG TAG CGC TGC ATA TAT AAT GCG TAA AAT TTT C-3'). The restriction sites between each element are listed above every junction. pSEC is an *E. coli*-yeast shuttle plasmid. 2 Designates an origin of replication, and LEU2 is the auxotroph selective marker in yeast; pUC ori is the replication origin, and ampR is an ampicillin resistant marker in *E. coli*.

Fig. 6. snR52 PCR amplification strategy. Two long oligodeoxynucleotides were designed: one with the sense strand sequence of snR52 and the other with the antisense strand sequence of snR52. The guide sequences which are depicted as *gray boxes* were altered to basepair with the target sequences in the RNAs of interest. The two long oligodeoxynucleotidedeoxy-oligonucleotides share a 15 nt complementary sequence at each 3' end, which, when utilized in a PCR reaction, allows them to extend using each other as a template to generate an artificial snR52 mini-gene with specific restriction sites at each end (*SalI* and *PstI* in this case).

Table 1

Synthetic leucine drop out powder

Mixed powder	Amount for 15 L
Yeast nitrogen base	25.1 g
Ammonium sulfate	75.4 g
Isoleucine	450 mg
Valine	2.25 g
Adenine	300 mg
Arginine	300 mg
Histidine	300 mg
Leucine (dropped)	0 mg
Lysine	450 mg
Methionine	300 mg
Phenylalanine	750 mg
Tryptophan	300 mg
Tyrosine	450 mg
Uracil	300 mg