Published in final edited form as:

Methods. 2016 July 1; 103: 68–76. doi:10.1016/j.ymeth.2016.02.003.

Detection and quantification of RNA 2'-O-methylation and pseudouridylation

Chao Huang^a, John Karijovich^b, and Yi-Tao Yu^c

Chao Huang: Chao.Huang@bms.com; Yi-Tao Yu: yitao_yu@urmc.rochester.edu

^aDepartment of Process Development, Bristol-Myers Squibb Company, 6000 Thompson Road, East Syracuse, New York 13027, USA

^bDepartment of Plant and Microbial Biology, University of California, 565 Li Ka Shing Center #3370, Berkeley, California 94720-337, USA

^cDepartment of Biochemistry and Biophysics, University of Rochester Medical Center, School of Medicine and Dentistry, 601 Elmwood Avenue, Box 712 Rochester, New York 14642, USA

Abstract

RNA-guided RNA modification is a naturally occurring process that introduces 2'-O-methylation and pseudouridylation into rRNA, spliceosomal snRNA and several other types of RNA. The Box C/D ribonucleoproteins (RNP) and Box H/ACA RNP, each containing one unique guide RNA (Box C/D RNA or Box H/ACA RNA) and a set of core proteins, are responsible for 2'-O-methylation and pseudouridylation respectively. Box C/D RNA and Box H/ACA RNA provide the modification specificity through base pairing with their RNA substrate. These post-transcriptional modifications could profoundly alter the properties and functions of substrate RNAs. Thus it is desirable to establish reliable and standardized modification methods to study biological functions of modified nucleotides in RNAs. Here, we present several sensitive and efficient methods and protocols for detecting and quantifying post-transcriptional 2'-O-methylation and pseudouridylation.

Keywords

Post-transcriptional modification; RNA; 2'-O-methylation; Pseudouridylation; Box C/D guide RNA; Box H/ACA guide RNA

(1) Introduction

Although more than 100 biochemically distinct RNA modifications have been described to date[1], the two most abundant internal modifications are 2'-O-methylation and pseudouridylation[2] (Figure 1). RNA 2'-O-methylation is a sugar-ring modification in

Correspondence to: Chao Huang, Chao.Huang@bms.com; Yi-Tao Yu, yitao_yu@urmc.rochester.edu.

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which the 2'OH of the ribose is methylated, while pseudouridylation is base-specific modification that results from an internal trans-glycosylation of a uridine residue. Both modifications substantially change the biophysical and biochemical properties of the modified nucleotide relative to the unmodified nucleotide. For instance, 2'-O-methylation alters the hydration sphere around the oxygen and impacts sugar edge interactions and sugar pucker conformation. 2'-O-methylation also increases the stability of RNA protecting against alkaline hydrolysis [3-5]. In comparison, pseudouridylation increases the conformation stability of RNA by increasing base stacking [6] and by coordinating a water molecule through the extra hydrogen bond provided by pseudouridylation [7]. In addition, pseudouridine (Ψ) is more polar than uridine [8].

2'-O-methylation and pseudouridylation can be catalyzed by two distinct mechanisms, namely protein-only and RNA-dependent mechanisms. In the protein-only mechanism, a single polypeptide protein or protein complex carries out both substrate recognition and catalysis. On the other hand, RNA-dependent RNA modifications are catalyzed by Box C/D and Box H/ACA ribonucleoprotein (RNP) complexes [9]. Box C/D RNPs consists of one single Box C/D guide RNA (Figure 2a) and four core proteins, including Nop1 (the catalytic component, also known as fibrillarin in human), Nop56, Nop58, and Snu13. Similarly, Box H/ACA RNPs consists of one single Box H/ACA guide RNA (Figure 2b) and four core proteins, including Cbf5 (the catalytic component, also known as dyskerin/NAP57 in human), Gar1, Nhp2, and Nop10. In contrast to the protein-only mechanism, the RNA-dependent modification machinery uses the noncoding RNA component of the RNP for substrate recognition via complementary base-pair interactions, while the catalytic activity, i.e. modification activity, is provided by a protein component of the RNP.

There are numerous 2'-O-methylation and pseudouridylation that have been found in rRNAs, spliceosomal snRNAs, and tRNAs. Additionally, recent “omic” strategies have revealed a complex modification landscape of mRNAs. With regard to the stable noncoding RNAs, i.e. rRNA, snRNA, and tRNA, the modifications are often clustered in functionally important regions and are conserved from species to species. Together, the abundance, the conservation, and the strategic location of these modifications strongly suggest that they contribute to RNA function. In fact, functional analyses have demonstrated that the post-transcriptional modifications in rRNAs and spliceosomal snRNAs play important roles in protein translation and pre-mRNA splicing, respectively.

Here, we describe various methods for the detection of 2'-O-methylated and pseudouridylated nucleotides. The methods described here utilize techniques that can be performed in any well-equipped molecular biology laboratory, and can be used to both identify novel modified nucleotides as well as for quantification. For instance, the primer extension-based methods described in detail below are useful tools for quickly identifying novel sites of modification, while the thin layer chromatography and ligation-based methods provide a quantifiable measurement.

(2) Protocol

2.1. Detect RNA 2'-O-methylation

2.1.1. Primer extension method—It is well established that 2'-O-methylated nucleotides induce reverse transcription stops/pauses at low concentrations of dNTPs, while at high dNTP concentrations reverse transcriptase can bypass 2'-O-methylated sites. It was proposed that the 2'-O-methyl group may present as a conformational “bump” to the passage of reverse transcriptase, whose effect is minimized at high dNTP concentrations [10]. Here, we describe the use of primer extension carried out at varying concentrations of dNTPs to detect 2'-O-methylation of yeast telomerase RNA (TLC1). It should be noted that the 2'-O-methylation within TLC1 that we are detecting was artificially induced *in vivo* via the expression of a Box C/D guide RNA targeting TLC1 for 2'-O-methylation [11].

(1) Isolation of yeast total RNA: Inoculate a single yeast colony in a 50-mL falcon tube containing 20 mL of SD-LEU liquid medium (mixing 7.5 g of synthetic leucine dropout powder from Table 1 and 20 g of dextrose; fill to 1 liter with ddH₂O and autoclave) and shake at 200 rpm for 16 h at 30 °C. Collect the cells by centrifuging the tube in a SH3000 rotor at 3,000 rpm (1864 × g) for 5 min at 4 °C using a Sorvall RC-5C plus centrifuge. Re-suspend cell pellet in 1 mL of cold ddH₂O and transfer the slurry to a 2-mL screw-cap tube. Spin the tube at 3,000 rpm (735 × g) for 5 min at room temperature in a bench-top centrifuge. Remove the supernatant (ddH₂O) and re-suspend cell pellet in 500 µL of TRIzol reagent (Thermo Fisher Scientific). Add 400 µL of acid washed glass beads and vigorously vortex the mixture for 1 min in a bench-top vortex. Repeat vortex 5 times and place mixture on ice for 1 min in between each vortex. Centrifuge the tube at 13,000 rpm (13799 × g) for 5 min at room temperature in a bench-top centrifuge. Transfer the aqueous layer to a new 1.5-mL Eppendorf tube and add 100 µL of chloroform. Vortex briefly and then centrifuge at 13,000 rpm for 5 min at room temperature in a bench-top centrifuge. Transfer upper aqueous phase to a new 1.5-mL Eppendorf tube, then add 1 µL of 10 mg/mL glycogen (Sigma) and 500 µL of isopropanol and vortex briefly. Centrifuge at 13,000 rpm for 10 min at room temperature in a bench-top centrifuge. Remove the supernatant and allow the pellet to air dry for 5 min. Re-suspend the pellet in 20 µL of ddH₂O. Measure yeast total RNA concentration and adjust the concentration to 5 µg/µL by diluting with ddH₂O.

(2) Designing and radiolabeling of DNA primer: In order to detect 2'-O-methylation in a substrate RNA, a DNA primer (with T_m 55 °C to 65°C) should be designed to a region complementary to the RNA approximately 30 to 50 nt downstream of the potential modification site. Following this rule of thumb, to detect U809 site of TLC1 RNA, a 25 nt (nucleotide) long DNA primer (5'-TTCTCTGTCACATCGTTCGATGTAC-3', T_m=61.2 °C), complementary to 849-873 nt (40 nt downstream of U809) of TLC1 RNA was designed. Order 100 nmole of DNA primer with a 5'-OH group (Integrated DNA Technologies) and dissolve in ddH₂O to make 10 µM stock. Mix 3 µL of 10 µM DNA primer with 1 µL of 10× PNK buffer A (Thermo Fisher Scientific), 1 µL of [γ-³²P] ATP (10 µCi/µL from Perkin Elmer), 1 µL of T4 PNK (10 U/µL) (Thermo Fisher Scientific), and 4 µL of ddH₂O. Incubate the mixture at 37 °C for 30 min and terminate reaction by adding 400 µL of G50 buffer (20 mM Tris-HCl, 300 mM sodium acetate, 2 mM EDTA, 0.2 % (w/v) SDS, pH 7.5) and 400 µL

of PCA (phenol/chloroform/isoamyl alcohol = 25/24/1[v/v/v], saturated with 20 mM Tris-HCl, pH 8.0). Briefly vortex the mixture and centrifuge at 13,000 rpm for 5 min in a bench-top centrifuge. 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 100 % ethanol. Centrifuge at 13,000 rpm for 10 min at room temperature in a bench-top centrifuge. Remove supernatant and allow the pellet to air dry for 5 min. Re-suspend the pellet in 2 μ L of ddH₂O and 2 μ L of 2 $\times$ Loading Dye (90 % deionized formamide, 10 mM EDTA, 0.1 % (w/v) bromophenol blue, 0.1 % (w/v) xylene cyanol FF). Prepare an 8 % polyacrylamide-8 M urea gel (16 cm $\times$ 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 % acrylamide/bisacrylamide (19:1) solution. Add 6 mL 5 $\times$ TBE buffer (445 mM Tris-HCl, 445 mM boric acid, 16 mM EDTA, pH 8.0) and bring the final volume to 60 mL using ddH₂O. Just before casting the gel, add 360 μ L of 10 % ammonium persulfate and 36 μ L of TEMED (sigma) and cast the gel immediately. Pre-run the gel at 16 W for 30 min. Heat the labeled primer at 95 $^{\circ}$ C for 1 min and immediately load onto the gel. Run the gel at 16 W until the bromophenol blue dye is approximately half way through the gel. After removing the upper plate, wrap the gel in Saran Wrap plastic film, and tape marker strips. Expose the gel to phosphorimager screen (Molecular Dynamic) for 0.5-1 min. Localize and cut the radiolabeled DNA primer from the gel and place the gel slice into a 1.5-mL Eppendorf tube. Add 500 μ L of G50 buffer to the gel slice, place it in dry ice for 5 min, and then leave it at room temperature for overnight. After overnight soaking, add 500 μ L of PCA to the tube and vortex briefly. Centrifuge at 13,000 rpm for 5 min at room temperature in a bench-top centrifuge. Transfer the aqueous phase to a new 1.5-mL Eppendorf tube and add 1 mL of 100 % ethanol and 1 μ L of 10 mg/mL glycogen. Vortex briefly and centrifuge at 13,000 rpm for 10 min at room temperature in a bench-top centrifuge. Remove the supernatant. Add 1 mL of 70 % ethanol to wash the pellet and centrifuge again at 13,000 rpm for 10 min at room temperature in a bench-top centrifuge. Remove the ethanol and allow the pellet to air dry for 5 min. Re-suspend the pellet (DNA primer) in 20 μ L of ddH₂O and store at -20 $^{\circ}$ C until use.

(3) Primer extension analysis: In a 1.5-mL Eppendorf tube mix 2 μ L of 2 $\times$ annealing buffer (500 mM KCl, 20 mM Tris-HCl, pH 8.3), 1 μ L of labeled DNA primer, 1 μ L of yeast total RNA (5 μ g/ μ L) in a total of 4 μ L volume, and label the tube as Annealing-mix-1. Prepare a duplicate tube with above recipe and label it as Annealing-mix-2. Heat both mixtures at 95 $^{\circ}$ C for 2 min, and gradually chill at room temperature for 10 min. In the meantime, prepare two different extension mixtures. For High-dNTP-mix, mix 3.2 μ L of 4 mM dNTP (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) and 0.8 μ L of AMV reverse transcriptase (2.5 U/ μ L) (Promega). For Low-dNTP-mix, mix 3.2 μ L of 0.04 mM dNTP (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) and 0.8 μ L of AMV reverse transcriptase (2.5 U/ μ L). Add 4 μ L of High-dNTP-mix to Annealing-mix-1, and 4 μ L of Low-dNTP-mix to Annealing-mix-2. Incubate in a 42 $^{\circ}$ C water bath for 30 min. Terminate the reaction by adding 400 μ L of G50 buffer and 400 μ L of PCA. Vortex briefly and centrifuge at 13,000 rpm for 5 min at room temperature in a bench-top centrifuge. 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 100 % ethanol. Centrifuge at 13,000 rpm for 10

min at room temperature in a bench-top centrifuge. Remove the supernatant. Add 1 mL 70 % ethanol to wash the pellet and centrifuge again at 13,000 rpm for 10 min in a bench-top centrifuge. Remove the ethanol and allow the pellet to air dry for 5 min. Re-suspend the pellet in 3 μ L of ddH₂O and 3 μ L of 2 $\times$ loading dye. Samples are ready for electrophoresis (can be stored at -20 °C for a week).

(4) Sequencing ladder preparation: To determine the position of 2'-O-methylation, a dideoxy sequencing ladder should be electrophoresed next to the primer extension products. In the sequencing reactions, a recombinant plasmid carrying the cDNA of the TLC1 RNA is used as a template. Prepare a Master-mix by mixing 5 μ L of 5 $\times$ sequencing buffer (250 mM Tris-HCl, 10 mM MgCl₂, pH 9.0), 1 μ L of (1 μ g/ μ L) plasmid, 2 μ L of radiolabeled DNA primer (same primer used in the 2.1.1 (3) primer extension analysis), 1 μ L of (5 U/ μ L) Taq DNA polymerase (Promega), and 8 μ L of ddH₂O. Label four PCR tubes as G, A, T, and C, respectively, and add 2 μ L of the corresponding d/ddNTP mix: d/ddATP (350 μ M ddATP, 80 μ M dNTP), d/ddCTP (200 μ M ddCTP, 80 μ M dNTP), d/ddGTP (30 μ M ddGTP, 80 μ M dNTP), and d/ddTTP (600 μ M ddTTP, 80 μ M dNTP) (Promega) to each PCR tube (The ddNTP concentrations are universal for DNA template with various GC content). Aliquot 4 μ L of Master-mix to each PCR tube. Perform PCR reaction as follows:

Step 1 94 °C for 2 min (1 cycle)

Step 2 92 °C for 30 s

42 °C for 30 s

72 °C for 30 s (repeat Step 2 for 25 times)

Step 3 4 °C (indefinitely)

Transfer each PCR reaction to 1.5-mL Eppendorf tube containing 400 μ L of G50 buffer. Add 400 μ L of PCA and vortex briefly and centrifuge at 13,000 rpm for 5 min at room temperature in a bench-top centrifuge. 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 100 % ethanol. Centrifuge at 13,000 rpm for 10 min at room temperature in a bench-top centrifuge. Remove the supernatant. Add 1 mL 70 % ethanol to wash the pellet and centrifuge again at 13,000 rpm for 10 min in a bench-top centrifuge. Remove the ethanol and allow the pellet to air dry for 5 min. Re-suspend the pellet in 3 μ L of ddH₂O and 3 μ L of 2 $\times$ loading dye. Samples are ready for electrophoresis (can be stored at -20 °C for a week).

(5) Visualization of modification by autoradiography: To analyze the primer extension products and sequencing reaction products, prepare an 8 % polyacrylamide-8 M urea sequencing gel as described in Section 2.1.1 (2). Denature primer extension and sequencing samples (from steps (3) and (4), respectively) by heating at 95 °C for 1 min immediately prior to loading. 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. Run the gel at 40 W of constant power until the bromophenol blue dye is approximately 4 cm from the bottom of the gel. Separate the plates and transfer the gel onto a sheet of Whatman filter paper. Cover the gel side with Saran Wrap plastic film. Dry the gel in a BioRad Gel Dryer with the filter paper side facing the vacuum for 60 min at 90 °C. Expose dried gel to a phosphorimager

screen overnight to visualize sequencing ladders and primer-extension products. An expected result is shown in Figure 3.

2.1.2. Ligation-based method—Originally developed in 2006 by Pan and Colleagues, the ligation-based method provides a straightforward method to quantitatively measure 2'-O-methylation in RNA substrate [12]. To quantify the 2'-O-methylation at U809 site of TLC1 RNA, two parallel ligation reactions (called Discriminating and Non-discriminating reactions) are performed. In the Discriminating reaction, the first pair of DNA primers (Discriminating or D primer pair), upon hybridization, leaves the ligation junction [13] 3' to the test nucleotide (U809) in the RNA substrate. While in the Non-discriminating reaction, the second pair of DNA primers (Non-discriminating or N primer pair) leaves the ligation junction [13] 5' to the test nucleotide (U809) in the RNA substrate. If the test nucleotide is 2'-O-methylated, the D primer pair will not be ligated while the N primer pair will. If the test nucleotide is not 2'-O-methylated, both D and N primer pairs will be quantitatively ligated (Figure 4). The ligation efficiency correlate well with the modification level at the test site. The yeast TLC1 RNA that is used in this section bears artificially introduced 2'-O-methylation at U809 site. This TLC1 RNA is extracted from the yeast strain that transformed with an artificial Box C/D RNA targeting TLC1 U809 for 2'-O-methylation [11].

(1) Designing and radiolabeling of DNA primers: For testing U809 site of TLC1 RNA, the D primer pair are DF-809: 5'-AATTCATCAGTAAGTTCAGTGAAT-3', and DA-809: 5'-AGATTTTTTATTTTACCTTTTTTGTA-3'. The N primer pair are NF-809: 5'-AATTCATCAGTAAGTTCAGTGAATA-3', and NA-809: 5'-GATTTTTTATTTTACCTTTTTTGTA-3'. In all ligation reactions, another pair of DNA primers (NF-1145: 5'-TTCCAAAATTATCTAAA-3'; NA-1145: 5'-TGCATCGAAGGCATTAGGAGAAGTA-3'), which placed the ligation junction 5' of U1145 (a non-modified site) of TLC1 RNA, are used as a loading control. The DA-809, NA-809, and NA-1145 are radiolabeled at their 5' end and gel purified according to the protocol described in 2.1.1 (2).

(2) Isolation of TLC1 RNA: To increase the signal to noise ratio, the TLC1 RNA is further purified from yeast total RNA using biotinylated DNA primer (5'-biotin-TCAATCCGAAATCCGACACTATCTC-3', complementary to 163-187 nt in the TLC1 RNA) and biotin-streptavidin affinity chromatography [14-15]. Briefly, in an 1.5-mL Eppendorf tube, prepare RNA/biotinylated primer mixture by combining 20 µL of (5 µg/µL) yeast total RNA, 10 µL of 10 µM biotinylated-primer, 20 µL of 5× NET-2-MgCl₂ buffer (250 mM Tris-HCl, 750 mM NaCl, 0.25 % (v/v) NP-40, 10 mM MgCl₂, pH 7.5), and 50 µL of ddH₂O. Heat at 95°C for 2 min, and gradually cool down at room temperature for 10 min. In the meantime, transfer 100 µL of immobilized streptavidin gel (Pierce) to a new 1.5-mL Eppendorf tube and centrifuge for 1 min at 3,000 rpm at room temperature in a bench-top centrifuge. Discard supernatant and wash gel twice by adding 200 µL of 1× NET-2-MgCl₂ buffer (50 mM Tris-HCl, 150 mM NaCl, 0.05 % (v/v) NP-40, 2 mM MgCl₂, pH 7.5). Centrifuge for 1 min at 3,000 rpm at room temperature in a bench-top centrifuge and discard the supernatant. Add the RNA/biotinylated primer mixture to the gel and nutate for 1.5 h at room temperature. Centrifuge for 1 min at 3,000 rpm at room temperature in a bench-top

centrifuge and discard the supernatant. Wash the gel with 200 μ L of 1 $\times$ NET-2-MgCl₂ buffer and centrifuge for 1 min at 3,000 rpm at room temperature in a bench-top centrifuge. Discard the supernatant and repeat wash procedure four more times. After washing, add 200 μ L of dissociation buffer (10 mM Tris-HCl, 0.1 % (w/v) SDS, and 0.5 mM EDTA, pH 7.5) and heat the tube at 95 °C for 5 min before centrifuge for 1 min at 3,000 rpm at room temperature in a bench-top centrifuge. Collect supernatant immediately and repeat the elution one more time. Pool the two supernatants and add 400 μ L of PCA. Vortex briefly and centrifuge at 13,000 rpm for 5 min at room temperature in a bench-top centrifuge. 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 100 % ethanol. Centrifuge at 13,000 rpm for 10 min at room temperature in a bench-top centrifuge. Remove the supernatant. Add 1 mL 70 % ethanol to wash the pellet and centrifuge again at 13,000 rpm for 10 min at room temperature in a bench-top centrifuge. Remove the ethanol and allow the pellet to air dry for 5 min. Re-suspend the pellet in 10 μ L of ddH₂O. Measure RNA concentration using Nanodrop (Thermo Fisher Scientific) and dilute to 0.1 μ g/ μ L with ddH₂O. The purified TLC1 RNA per biotin-streptavidin affinity chromatography is sufficient for 2-3 times of ligation-based reactions.

(3) Ligation-based reaction: In the discriminating reaction, mix 2 μ L of purified (0.1 μ g/ μ L) TLC1 RNA, 0.5 μ L of radiolabeled DA-809, 0.5 μ L of 10 μ M DF-809, 0.5 μ L of radiolabeled NA-1145, 0.5 μ L of 10 μ M NF-1145. In the non-discriminating reaction, mix 2 μ L of purified (0.1 μ g/ μ L) TLC1 RNA, 0.5 μ L of radiolabeled NA-809, 0.5 μ L of 10 μ M NF-809, 0.5 μ L of radiolabeled NA-1145, 0.5 μ L of 10 μ M NF-1145. The mixtures are incubated at 95°C for 2 min, and gradually chilled at room temperature for 10 min. Each mixture is further supplied with 10 μ L of 2 $\times$ ligation buffer (66 mM Tris-HCl, 6.6 mM MgCl₂, 10 mM DTT, 66 mM ATP, 15 % DMSO, pH 7.6) and 1 μ L of (1 U/ μ L) T4 ligase. The ligation reactions are carried out at 37 °C for 30 min, and are terminated by adding 400 μ L of G50 buffer and 400 μ L of PCA. Vortex briefly and centrifuge at 13,000 rpm for 5 min at room temperature in a bench-top centrifuge. 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 100 % ethanol. Centrifuge at 13,000 rpm for 10 min at room temperature in a bench-top centrifuge. Remove the supernatant. Add 1 mL 70 % ethanol to wash the pellet and centrifuge again at 13,000 rpm for 10 min at room temperature in a bench-top centrifuge. Remove the ethanol and allow the pellet to air dry for 5 min. Re-suspend the pellet in 3 μ L of ddH₂O and 3 μ L of 2 $\times$ loading dye. Samples are ready for electrophoresis (can be stored at -20 °C for a week).

(4) Visualization of modification by autoradiography: Prepare 8 % polyacrylamide-8 M urea gel (see section 2.1.1 (2) for detailed description), and load 3 μ L of each reaction. After electrophoresis, dry the gel in a BioRad Gel Dryer (see section 2.2.2 (5)), and expose the dried gel to a phosphorimager screen overnight to visualize the ligation products. An expected result is shown in Figure 5. To quantify the results, the ligated band intensities at the U809 site were normalized by their loading controls, the U1145 site. The normalized U809 ligation band intensity in D lane was then divided by its paired N lane to obtain a D/N ratio. If the D/N ratio of control sample is set to 100% (no modification), the relative D/N

ratio of U809 was calculated as 21.4 %, meaning 78.6 % population of TLC1 RNAs carries 2'-O- methylation at U809 site.

2.2. Detect RNA pseudouridylation

2.2.1. CMCT/reverse transcription method—The CMCT chemical is able to specifically modify Ψ , U and G residues in an RNA substrate. While CMCT on U and G residues can be subsequently removed under alkaline conditions, the CMCT- Ψ modified residue is resistant and will stop primer extension one nucleotide before the CMCT- Ψ site. In this section, we describe the CMCT-reverse transcription method to detect Ψ s in an RNA. We use *S. cerevisiae* U2 snRNA, which contains three Ψ s at positions 35, 42 and 44, as an example.

(1) CMCT modification: Prepare a 1 M CMCT solution in BEU buffer (50mM Bicine, 7 M Urea, 4 mM EDTA, pH 8.5). Prepare two 1.5-mL Eppendorf tubes and label them as CMCT minus and CMCT plus. In the CMCT minus tube, add 100 μ L of BEU buffer, 12 μ L of (5 μ g/ μ L) of yeast total RNA. In the CMCT plus tube, add 80 μ L BEU buffer, 12 μ L of (5 μ g/ μ L) of yeast total RNA, and 20 μ L of 1 M CMCT solution. Incubate the mixtures at 37 °C for 20 min. Add 250 μ L of G50 buffer, 300 μ L of PCA to each tube. Vortex briefly and centrifuge at 13,000 rpm for 5 min at room temperature in a bench-top centrifuge. Transfer the upper aqueous phase to a new 1.5-mL Eppendorf tube and add the following solutions in the order of: 12 μ L of 3 M sodium acetate pH 5.2, and 1 μ L of (10 mg/mL) glycogen, and 1 mL of 100 % ethanol. Vortex for 30 s and centrifuge at 13,000 rpm for 10 min at room temperature in a bench-top centrifuge. Remove the supernatant. Wash the pellet with 1 mL of 70 % ethanol and centrifuge again at 13,000 rpm for 10 min at room temperature in a bench-top centrifuge. Remove the ethanol and allow the pellet to air dry for 5 min. Re-suspend the pellet in each tube with 50 μ L of Na₂CO₃ Solution (50 mM Na₂CO₃, 2 mM EDTA, pH 11.5). To completely remove CMCT from U and G residues, the re-suspended RNA is incubated at 37 °C for 110 min. The reaction is terminated by addition of 350 μ L of G50 buffer, 200 μ L of PCA, and 5 μ L of 3 M sodium acetate pH 5.2. After a brief vortexing, centrifuge at 13,000 rpm for 10 min at room temperature in a bench-top centrifuge. Transfer upper phase to new tubes. Add 1 mL of 100 % ethanol and 1 μ L of (10 mg/mL) glycogen and centrifuge at 13,000 rpm for 10 min at room temperature in a bench-top centrifuge. Remove the supernatant. Wash the pellet with 1 mL of 70 % ethanol and centrifuge again at 13,000 rpm for 10 min at room temperature in a bench-top centrifuge. Air dry the pellet, and re-suspend it in 10 μ L of ddH₂O. The sample is ready for primer extension analysis.

(2) Designing and radiolabeling of DNA primer: In order to detect the pseudouridylation in a substrate RNA, a DNA primer (with T_m 50 °C to 60°C) should be designed to a region complementary to the RNA approximately 50 to 70 nt downstream of the potential modification site. Following this rule of thumb, a 23 nt long DNA primer (5'-TGGGTGCCAAAAAATGTGTATTG-3', complementary to 104-126 nt of yeast U2 snRNA) is designed to detect Ψ 35, Ψ 42 and Ψ 44 in yeast U2 snRNA. The primer is radiolabeled at its 5' end and gel purified according to the protocol described in 2.1.1 (2).

(3) Primer extension analysis: Prepare annealing-mix by combining 1 μ L of radiolabeled DNA primer, 4 μ L of 2 $\times$ annealing buffer, 3 μ L of CMCT treated RNA. Heat the mixture for 5 min at 95 $^{\circ}$ C and gradually cool down the sample at room temperature for 10 min. Add 0.8 μ L of 10 mM dNTP mix (2.5 mM dATP, 2.5 mM dTTP, 2.5 mM dGTP, 2.5 mM dCTP, 8 mM DTT, 16 mM $MgCl_2$, 24 mM Tris-HCl, pH 8.3), 1 μ L of (2 U/ μ L) AMV reverse transcriptase, and 7 μ L of ddH₂O to each tube. Incubate at 42 $^{\circ}$ C for 30 min. Terminate the reaction by adding 400 μ L of G50 buffer and 400 μ L of PCA. Vortex briefly and centrifuge at 13,000 rpm for 5 min at room temperature in a bench-top centrifuge. 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 100 % ethanol. Centrifuge at 13,000 rpm for 10 min at room temperature in a bench-top centrifuge. Remove the supernatant. Add 1 mL 70 % ethanol to wash the pellet and centrifuge again at 13,000 rpm for 10 min in a bench-top centrifuge. Remove the ethanol and allow the pellet to air dry for 5 min. Re-suspend the pellet in 3 μ L of ddH₂O and add 3 μ L of 2 $\times$ loading dye. Samples are ready for electrophoresis (and can be stored at -20 $^{\circ}$ C for a week).

(4) Sequencing ladder preparation: To determine the position of Ψ , a dideoxy sequencing ladder should be electrophoresed in parallel with the primer extension products. In the sequencing reactions, a recombinant plasmid carrying the cDNA of the U2 snRNA is used as a template. Prepare a Master-mix by mixing 5 μ L of 5 $\times$ sequencing buffer (250 mM Tris-HCl, 10 mM $MgCl_2$, pH 9.0), 1 μ L of (1 μ g/ μ L) plasmid, 2 μ L of radiolabeled DNA primer (same primer used in the 2.2.1 (3) primer extension analysis), 1 μ L of (5 U/ μ L) Taq DNA polymerase (Promega), and 8 μ L of ddH₂O. Label four PCR tubes as G, A, T, and C, respectively, and add 2 μ L of the corresponding d/ddNTP mix: d/ddATP (350 μ M ddATP, 80 μ M dNTP), d/ddCTP (200 μ M ddCTP, 80 μ M dNTP), d/ddGTP (30 μ M ddGTP, 80 μ M dNTP), and d/ddTTP (600 μ M ddTTP, 80 μ M dNTP) (Promega) to each PCR tube. Aliquot 4 μ L of Master-mix to each PCR tube. Perform PCR reaction as follows:

Step 1 94 $^{\circ}$ C for 2 min (1 cycle)

Step 2 94 $^{\circ}$ C for 30 s

42 $^{\circ}$ C for 30 s

72 $^{\circ}$ C for 30 s (repeat Step 2 for 25 times)

Step 3 72 $^{\circ}$ C (indefinitely)

Transfer each PCR reaction to 1.5-mL Eppendorf tube containing 400 μ L of G50 buffer and 400 μ L of PCA. Vortex briefly and centrifuge at 13,000 rpm for 5 min at room temperature in a bench-top centrifuge. 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 100 % ethanol. Centrifuge at 13,000 rpm for 10 min at room temperature in a bench-top centrifuge. Remove the supernatant. Add 1 mL 70 % ethanol to wash the pellet and centrifuge again at 13,000 rpm for 10 min in a bench-top centrifuge. Remove the ethanol and allow the pellet to air dry for 5 min. Re-suspend the pellet in 3 μ L of ddH₂O and 3 μ L of 2 $\times$ loading dye. Samples are ready for electrophoresis (can be stored at -20 $^{\circ}$ C for a week).

(5) Visualization of modification by autoradiography: To analyze the primer extension products and sequencing reaction products, prepare an 8 % polyacrylamide-8 M urea sequencing gel as described in Section 2.1.1 (2). Denature primer extension and sequencing samples (from step (3) and (4), respectively) by heating at 95 °C for 1 min immediately prior to loading. Load 3 µL of primer extension reactions and 3 µL of each sequencing reaction on the gel. The remaining samples can be stored at -20 °C. Run the gel at 40 W of constant power until the bromophenol blue dye is approximately 4 cm from the bottom of the gel. After electrophoresis, dry the gel in a BioRad Gel Dryer (see section 2.2.2 (5)), and expose the dried gel to a phosphorimager screen overnight to visualize the ligation products. An expected result is shown in Figure 6.

2.2.2. TLC-based method—The TLC-based (Thin Layer Chromatography-based) method is used to quantify RNA pseudouridylation at single nucleotide resolution. The substrate RNA is first site-specifically cleaved with RNase H at the site 5' of the nucleotide of interest. The 3'-fragment is then radiolabeled with ³²P. After nuclease P1 digestion, the released radiolabeled nucleotide is analyzed by 2D-TLC and quantitated by phosphorimager [16]. In this section, we describe the use of 2D-TLC for detection and quantification of Ψ35 of *S. cerevisiae* U2 snRNA [17].

(1) Isolation of U2 snRNA: Mix 20 µL of yeast total RNA (5 µg/µL) with 10 µL of 10 µM 20-nt long biotinylated primer (5'-Biotin-TAGCGGAAAACAACCTTGCTT-3', which is complementary to 158-177 nt of yeast U2 snRNA), 20 µL of 5× NET-2-MgCl₂ buffer (250 mM Tris-HCl, 750 mM NaCl, 0.25 % (v/v) NP- 40, 10 mM MgCl₂, pH7.5), and 50 µL of ddH₂O. After heating at 95 °C for 2 min, the sample is gradually cooled down for 10 min at room temperature. In the meanwhile, transfer 100 µL of immobilized streptavidin gel (Pierce) to an 1.5-mL Eppendorf tube and centrifuge for 1 min at 3,000 rpm at room temperature in a bench-top centrifuge. Discard supernatant and wash gel twice by adding 200 µL of 1× NET-2-MgCl₂ buffer (50 mM Tris-HCl, 150 mM NaCl, 0.05 % (v/v) NP- 40, 2 mM MgCl₂, pH 7.5). Centrifuge for 1 min at 3,000 rpm at room temperature in a bench-top centrifuge and discard the supernatant. Add the RNA-biotinylated primer mixture to the gel and nutate for 1.5 h at room temperature. Centrifuge for 1 min at 3,000 rpm at room temperature in a bench-top centrifuge and discard the supernatant. Wash the gel with 200 µL of 1× NET-2-MgCl₂ buffer and centrifuge for 1 min at 3,000 rpm at room temperature in a bench-top centrifuge. Discard the supernatant and repeat wash procedure four times. Add 200 µL of dissociation buffer (10 mM Tris-HCl, 0.1 % (w/v) SDS, and 0.5 mM EDTA, pH 7.5) and heat the sample at 95 °C for 5 min before centrifuging for 1 min at 3,000 rpm at room temperature in a bench-top centrifuge. Immediately collect supernatant and repeat the elution one more time. Pool the supernatants and add 400 µL of PCA. Vortex briefly and centrifuge at 13,000 rpm for 5 min at room temperature in a bench-top centrifuge. Transfer the upper aqueous phase into a new 1.5-mL Eppendorf tube and add 1 µL of glycogen (10 mg/mL) and 1 mL of 100 % ethanol. Centrifuge at 13,000 rpm for 10 min at room temperature in a bench-top centrifuge. Discard the supernatant, and add 1 mL 70 % ethanol to wash the pellet. Centrifuge again at 13,000 rpm for 10 min at room temperature in a bench-top centrifuge. Remove the ethanol and allow the pellet to air dry for 5 min. Re-suspend the pellet in 10 µL of ddH₂O. Measure RNA concentration using Nanodrop

(Thermo Fisher Scientific) and dilute to 0.1 µg/µL with ddH₂O. The purified U2 snRNA per biotin-streptavidin affinity chromatography is sufficient for 5-10 times of RNase H cleavage reactions.

(2) RNase H cleavage: The RNase H cleavage specificity is provided by a 2'-O-methyl RNA-DNA chimera, which has the following features: it is usually 17 to 18 nt long and consists of three 2'-O-methyl RNA nucleotides at the 5' end, four deoxynucleotides in the middle, and eleven to twelve 2'-O-methyl RNA nucleotides at the 3' end. The cleavage site is the phosphodiester bond 3' of the substrate nucleotide that base pairs with the 5'-most deoxynucleotide of the chimera. The 2'-O-methyl RNA-DNA chimera that direct the RNase H digestion between positions 34 and 35 of yeast U2 snRNA is 5'-UmAmdCdAdCdTUmGmAmUmCmUmAmAmGmCmCm-3'. RNase H cleavage generates 3'-OH on the 5'-fragment and a 5'-phosphate on the 3'-fragment.

In a new 1.5 mL-Eppendorf tube, mix 1 µL of 0.1 µg/µL purified yeast U2 snRNA, 1 µL of 10 µM of 2'-O-methyl RNA-DNA chimera, and 2 µL of ddH₂O. Heat the mixture at 95 °C for 2 min and gradually cool down at room temperature for 10 min. Add 2 µL of 5× Digestion buffer (100 mM Tris-HCl, 50 mM MgCl₂, 500 mM KCl, 125 mM DTT, 25 % sucrose pH 7.5), 2 µL of (1 U/µL) RNasin (Thermo Fisher Scientific), and 2 µL of (1 U/µL) RNase H (Amersham). Incubate the tube at 37 °C for 1 h. Terminate the reaction by adding 400 µL of G50 buffer and 400 µL of PCA. Vortex briefly and centrifuge at 13,000 rpm for 5 min at room temperature in a bench-top centrifuge. Transfer the upper aqueous phase into a new 1.5-mL Eppendorf tube and add 1 µL of 10 mg/mL glycogen and 1 mL of 100 % ethanol. Centrifuge at 13,000 rpm for 10 min at room temperature in a bench-top centrifuge. Remove the supernatant. Add 1 mL 70 % ethanol to wash the pellet and centrifuge again at 13,000 rpm for 10 min in a bench-top centrifuge. Remove the ethanol and allow the pellet to air dry for 5 min. Dissolve the pellet in 3 µL of ddH₂O and 3 µL of 2× loading dye. Prepare 8 % polyacrylamide-8 M urea gel (see section 2.1.1 (2) for detailed description), load the cleaved U2 sample and an RNA size marker (Thermo Fisher Scientific) next to each other, and carry out electrophoresis. With the help of the size marker, locate the 3'-half U2 snRNA on the gel by UV-shadowing, and excise the band from the gel. Place the excised gel slice into a 1.5-mL Eppendorf tube. Add 500 µL of G50 buffer to the gel slice, freeze it on dry ice for 5 min and then transfer it to room temperature for overnight. After overnight soaking, add 500 µL of PCA to the tube and vortex briefly. Centrifuge at 13,000 rpm for 5 min at room temperature in a bench-top centrifuge. Transfer the aqueous phase to a new 1.5-mL Eppendorf tube and add 1 mL of 100 % ethanol and 1 µL of 10 mg/mL glycogen. Vortex briefly and centrifuge at 13,000 rpm for 10 min at room temperature in a bench-top centrifuge. Remove the supernatant. Add 1 mL of 70 % ethanol to wash the pellet (3'-half U2 snRNA) and centrifuge again at 13,000 rpm for 10 min at room temperature in a bench-top centrifuge. Remove the ethanol and allow the pellet to air dry for 5 min. Re-suspend the pellet in 5 µL of ddH₂O.

(3) Dephosphorylation: In a new 1.5 mL-Eppendorf tube, mix 5 µL of 3'-half U2 snRNA (from step (2) RNase H cleavage), 1 µL of 10× dephosphorylation buffer (Thermo Fisher Scientific), 3 µL of ddH₂O, and 1 µL of Calf Intestinal Alkaline Phosphatase (1 U/µL)

(Thermo Fisher Scientific). Incubate at 50 °C for 45 min. Terminate the reaction by adding 400 μ L of G50 buffer and 400 μ L of PCA. Vortex briefly and centrifuge at 13,000 rpm for 5 min at room temperature in a bench-top centrifuge. 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 100 % ethanol. Centrifuge at 13,000 rpm for 10 min at room temperature in a bench-top centrifuge. Remove the supernatant. Add 1 mL 70 % ethanol to wash the pellet and centrifuge again at 13,000 rpm for 10 min in a bench-top centrifuge. Remove the ethanol and allow the pellet to air dry for 5 min. Re-suspend the pellet (de-phosphorylated 3'-half U2) in 5 μ L of ddH₂O.

(4) Rephosphorylation: In a new 1.5 mL-Eppendorf tube, mix 5 μ L of de-phosphorylated 3'-half U2 snRNA (from step (3) Dephosphorylation) with 1 μ L of 10 $\times$ PNK buffer (Thermo Fisher Scientific), 1 μ L of [γ -³²P]ATP (6000 Ci/mmol), 2 μ L of ddH₂O, and 1 μ L of (10 U/ μ L) T4 PNK (Thermo Fisher Scientific). Incubate at 37 °C for 30 min. Terminate the reaction by adding 400 μ L of G50 buffer and 400 μ L of PCA. Vortex briefly and centrifuge at 13,000 rpm for 5 min at room temperature in a bench-top centrifuge. 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 100 % ethanol. Centrifuge at 13,000 rpm for 10 min at room temperature in a bench-top centrifuge. Discard the supernatant, and add 1 mL 70 % ethanol to wash the pellet. Centrifuge again at 13,000 rpm for 10 min in a bench-top centrifuge. Remove the ethanol and allow the pellet to air dry for 5 min. Re-suspend the pellet in 3 μ L of ddH₂O and 3 μ L of 2 $\times$ loading dye. Prepare an 8 % polyacrylamide-8 M urea gel (see section 2.1.1 (2) for detailed description), load the sample, and carry out electrophoresis. Dry the gel in a BioRad Gel Dryer (see section 2.2.2 (5)), and expose the dried gel to phosphorimager screen (Molecular Dynamic) for 0.5-1 min. Locate the 3'-half U2 snRNA (5' radiolabeled), and excise the band from the gel. Place the excised gel slice into a 1.5-mL Eppendorf tube. Add 500 μ L of G50 buffer to the gel slice, freeze it on dry ice for 5 min, and elute the RNA overnight at room temperature. After overnight soaking, add 500 μ L of PCA to tube and vortex briefly. Centrifuge at 13,000 rpm for 5 min at room temperature in a bench-top centrifuge. Transfer the aqueous phase to a new 1.5-mL Eppendorf tube. Add 1 mL of 100 % ethanol and 1 μ L of 10 mg/mL glycogen. Vortex briefly and centrifuge at 13,000 rpm for 10 min at room temperature in a bench-top centrifuge. Remove the supernatant. Add 1 mL of 70 % ethanol to wash the pellet ([³²P]-labeled 3'-half U2 snRNA) and centrifuge again at 13,000 rpm for 10 min at room temperature in a bench-top centrifuge. Remove the ethanol and allow the pellet to air dry for 5 min. Re-suspend the pellet in 20 μ L of ddH₂O.

(5) Nuclease P1 digestion: In a new 1.5 mL-Eppendorf tube, mix 1 μ L of radiolabeled 3'-half U2 snRNA (from step (4) Rephosphorylation) with 3 μ L of (1 U/ μ L) nuclease P1 (Sigma) (dissolved in 100 mM sodium acetate, pH 5.2). Incubate at 37 °C for 1 h.

(6) 2D-TLC analysis: Spot 3 μ L of the nuclease P1 digestion reaction (from step (5)) on the lower left corner (~1.5cm away from both edge) of a cellulose TLC PEI plate (20 $\times$ 20 cm) (EM Science). After the spotted sample has completely dried, develop the TLC plate with the first dimension solvent (Isobutyric acid/ ammonium hydroxide/ddH₂O (v/v/v=66/1/33)) in a clean glass tank with cover [18]. After approximately 20 h of development (solvent has reached the top of the plate), remove the TLC plate and let it air dry in the hood

for more than 5 h. Rotate the TLC plate 90° counter-clockwise and place it in a tank containing the second dimension solvent (Isopropanol/HCl/ddH₂O (v/v/v=68/18/14)). Develop the TLC in the second dimension solvent until the solvent has reached the top of the plate. Remove the TLC plate and let it air dry in the hood for more than 3 h. Wrap the plate in Saran Wrap plastic film, and expose it to phosphorimager screen (Molecular Dynamic) for overnight to visualize the digested nucleotides (U versus Ψ). An expected result is shown in Figure 7.

3. Discussion

Recent years have seen an immense surge in the interest in to the biological function of RNA modifications. Inherent to the analysis of their function is the ability to identify and quantify the modifications present. Here, we have described four distinct methods for the identification and quantification of RNA modifications, and cite specific examples for their use in detecting RNA 2'-O-methylation and pseudouridylation. While we only describe the use of a specific assay for one modification, i.e. 2'-O- methylation or pseudouridylation, indeed, the methods described here are interchangeable and can also be used to analyze a wide range of other modifications [12, 16, 19-21]. For example, in the case of the ligation-based detection method, modification of the D primer with a N6 -phenanthren-dA at its 5' end allows the ligation-based analysis of Ψ [22].

It should be noted that these methods do have some limitations when dealing with low abundance RNA and low percentage (less than 50%) modifications at the site of interest. However, there is an increasing trend in biology for high-throughput studies and indeed the analysis of RNA modifications has proved to be no different. For instance, recently four groups have provided the first transcriptome-wide maps of RNA pseudouridylation in both yeast and humans [23]. While these studies have greatly expanded our view of RNA pseudouridylation, all of the high-throughput approaches have the roots in the CMCT-primer extension method described here. It is our belief, that with the development of novel methods for modification detection and quantification, as well as the development of high-throughput analyses based on current detection strategies, that our understanding of the roles of RNA modification in biology will continue to expand.

Acknowledgments

We thank members of the Yu laboratory for valuable discussions and comments. The work presented in this paper is supported by grants GM104077 and AG39559 from the National Institute of Health.

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Fig. 1.

The conversion of Uridine to Pseudouridine (upper diagram) and 2'-hydroxyl ribose to 2'-O-methylated ribose (lower diagram).

Fig 2a.

The 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, there lie the guide sequences that can base pair with the complementary sequences in the targeted RNAs (including rRNA and snRNA), which is depicted in thick black line. The guide sequences serve as a ruler that precisely locate the 2'-O-methylation sites 5 nucleotide upstream Box D' and Box D.

Fig 2b.

The schematic representation of a Box H/ACA RNA. Box H/ACA RNAs adopt a hairpin-hinge-haipin-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 3.

2'-O-methylation at U809 of TLC1 RNA is detected by primer extension analysis. The primer extension is carried out in the presence of high (H; lanes 1 and 3) or low (L; lanes 2 and 4) dNTP concentrations. Lanes 1 and 2 are from cells expressing a Box C/D guide RNA with random guide sequence (Con.), and lanes 3 and 4 are from cells expressing a Box C/D guide RNA targeting TLC1 at U809 site for 2'-O-methylation. A TLC1 sequencing ladder is electrophoresed in parallel on the left. A signal corresponding to 2'-O-methylated U809 is indicated.

Fig 4.

The strategy of ligation-based 2'-O-methylation assay. The thick lines represent the target RNA substrate, and the thin lines stand for primer pairs (N, Non-discriminating; D, discriminating) used for ligation. X denotes a test nucleotide lacking 2'-O-methylation; X_m represents a test nucleotide that is 2'-O-methylated. Nicks (on the 5' or 3' side of the test nucleotide) are also shown.

Fig 5.

2'-O-methylation at U809 of TLC1 RNA is detected by ligation-based 2'-O-methylation assay. TLC1 RNA is purified from cells expressing a Box C/D guide RNA with random guide sequence (lanes 1 and 2), and a Box C/D guide RNA targeting TLC1 at U809 site for 2'-O-methylation (lanes 3 and 4). D, discriminating primer pair; N, nondiscriminating primer pair. In all lanes, an additional pair of labeled primers is also included for a loading control (U1145). According to the unmodified/ligated fraction, the relative efficiency of 2'-O-methylation relative to controls (Con.) is calculated (shown in parentheses at the top).

Fig 6.

Pseudouridylation at U35, U42 and U44 sites of U2 snRNA are detected by CMCT-primer extension analysis. The primer extension is carried out using U2 snRNA treated with and without CMCT (lane 2 and 1 respectively). An U2 sequencing ladder is electrophoresed in parallel on the right. The signals corresponding to Ψ35, Ψ42 and Ψ44 are indicated.

Fig 7.

Pseudouridylation at U35 site of U2 snRNA is detected by 2D-TLC analysis. The U2 snRNA with 35 site ^{32}P - radiolabelled is digested by nuclease P1 and is spotted on to TLC plate. The sample is also spiked with ^{32}P - radiolabelled Adenosine 5'-monophosphate (pA), cytidine 5'-monophosphate (pC), and guanosine 5'- monophosphate (pG) to locate the relative position of uridine 5'-monophosphate (pU) and pseudouridine 5'- monophosphate (pΨ). The spot origin and run dimensions are also indicated. The percentage of pseudouridylation is calculated as $p\Psi / (p\Psi + pU)$.

Table 1
Synthetic Leucine Drop-out Powder

Component	Amount for 15L
Yeast nitrogen base	25.1 g
Ammonium sulfate	75.4 g
Isoleucine	450 mg
Valine	2.25 g
Adnine	300 mg
Arginine	300 mg
Histidine	300 mg
Leucine (Drop-out)	0 mg
Lysine	450 mg
Methionine	300 mg
Phenylalanine	750 mg
Tryptophan	300 mg
Tyrosine	450 mg
Uracil	300 mg