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Purification and Functional Reconstitution of Box H/ACA Ribonucleoprotein Particles

Chao Huang, Guowei Wu, and Yi-Tao Yu

Abstract

Pseudouridylation is the most abundant and widespread RNA modification, and it plays an important role in modulating the structure and function of RNA. In eukaryotes and archaea, RNA pseudouridylation is catalyzed largely by box H/ACA ribonucleoproteins (RNPs), a distinct group of RNA–protein complexes each consisting of a unique RNA and four common proteins. The RNA component of the complex serves as a guide that base-pairs with its substrate RNA and specifies the target uridine to be modified. In order to systematically study the function and mechanism of pseudouridylation, it is desirable to have a reconstitution system in which biochemically purified/reconstituted box H/ACA RNPs are capable of introducing pseudouridines into an RNA at any target site. Here, we describe a method for the reconstitution of functional box H/ACA RNPs using designer box H/ACA guide RNAs, which in principle can be adopted to reconstitute other RNA–protein complexes as well.

Keywords

Pseudouridylation; RNA modification; Immunoprecipitation; Reconstitution; Box H/ACA RNP

1 Introduction

Pseudouridine (Ψ) is the most abundant post-transcriptionally modified nucleotide, and exhibits chemical properties that are distinct from those of all other known nucleotides [1 – 5]. When compared with uridine, Ψ has an extra hydrogen bond donor and contains, between the base and sugar, a carbon–carbon bond, which is ~10 % longer than the normal nitrogen–carbon glycosidic bond found in all other nucleotides. It has long been known that in all eukaryotic species, tRNAs, rRNAs, and spliceosomal snRNAs are highly enriched with Ψ [6 – 8]. Most recently, Ψ has also been found in other noncoding RNAs, as well as in mRNAs [9, 10]. It has been reported that Ψ s within rRNAs and spliceosomal snRNAs contribute significantly to protein translation and pre-mRNA splicing, respectively [11, 12].

In eukaryotes and archaea, pseudouridylation (U-to- Ψ conversion) is mainly catalyzed by a family of ribonucleoproteins called box H/ACA RNPs, each of which consists of four common core proteins (NAP57/dyskerin/Cbf5, Nhp2, Nop10, and Gar1) and a single unique small RNA (box H/ACA RNA) [13]. In the complex, this RNA component serves as a guide that base-pairs with its substrate RNA, positioning the target uridine at the pseudouridylation pocket (Fig. 1a). The catalytic component (NAP57/dyskerin/Cbf5) of the complex then

catalyzes the pseudouridylation reaction, converting the specified (target) uridine into Ψ . Target specificity is thus determined by base-pairing interactions between the guide sequence of the box H/ACA RNA and the target sequence of the substrate RNA [14]. Based on this guide-substrate base-pairing scheme, one can in theory engineer artificial box H/ACA RNAs (with different guide sequences) to introduce pseudouridines into RNA at novel sites. Indeed, by designing an artificial box H/ACA RNA, Karijovich and Yu were able to introduce a Ψ into a premature termination codon (PTC) of an mRNA molecule, promoting nonsense suppression in vivo [15, 16]. Using the same concept, our lab also demonstrated that box H/ACA RNP complexes could be purified (from yeast cells) and functionally reconstituted in vitro [17, 18]. This in vitro reconstitution system has been very helpful for studying the function and mechanism of RNA-guided RNA pseudouridylation [17, 18].

Here, we describe a protocol for reconstitution of functional box H/ACA RNP in vitro. Starting with a yeast strain where one of the four core proteins is TAP (tandem affinity purification)-tagged [19], we purify, using anti-tag immunoprecipitation, the whole family of box H/ACA RNPs from yeast cell lysate. We then treat the purified box H/ACA RNPs with micrococcal nuclease to remove the endogenous box H/ACA RNAs from the complexes. Finally, upon inactivation of micrococcal nuclease, we add in vitro transcribed designer box H/ACA RNAs to the RNA-depleted box H/ACA RNP complexes, thereby allowing reconstitution of functional box H/ACA RNPs capable of directing RNA pseudouridylation at the desired sites. In this protocol, the designer artificial box H/ACA RNA is based on snR81 [17], as illustrated in Fig. 1a. Using this technique, we have been able to introduce pseudouridines into various RNA substrates. We believe that this technique can in principle be used to reconstitute other RNA–protein complexes as well.

2 Materials

2.1 Yeast Cell Culture

1. SD-URA drop out medium: To make powder for 60 L SX-URA drop out medium (X represents the type of sugar that will be added separately), weigh 100.4 g of yeast nitrogen base, 301.6 g of ammonium sulfate, 1.8 g of isoleucine, 9.0 g of valine, 1.2 g of adenine, 1.2 g of arginine, 1.2 g of histidine, 1.8 g of leucine, 1.8 g of lysine, 1.2 g of methionine, 3.0 g of phenylalanine, 1.2 g of tryptophan, and 1.8 g of tyrosine. Add the mixture into a ball mill grinder and grind the mixture overnight. To make SD-URA liquid medium, weigh 3.7 g of SX-URA powder, 10 g of dextrose, then add 500 mL of ddH₂O, autoclave to sterilize it.
2. SR-URA liquid medium: same as SD-URA liquid medium except that raffinose is used instead of dextrose (*see* Note 1).
3. 3×-YPGal: weigh 30 g of yeast extract, 60 g of peptone, 60 g of galactose and add ddH₂O to 1 L. Autoclave to sterilize it.

¹Raffinose is used to bridge the transition from dextrose to galactose, so that all the dextrose will be removed (dextrose represses the expression of a Gal promoter).

4. TAP-tagged yeast strain: For reconstitution of Box H/ACA RNP, any of the three core protein-tag (Cbf5-TAP, Gar1-TAP, Nhp2-TAP) in wild type yeast strains (BY4741, BY4742, etc.) can be used. Nop10 cannot be TAP-tagged in our experience [17]. The tag used in this protocol contains successive (6×His-HA-3C-Protein A) tags [20], which is a modified version of the original TAP tag [21]. This tag is used in conjunction with a URA marker (*see* Note 2).
5. UV–VIS Spectrophotometer and plastic cuvette: Measure OD_{600nm} of yeast culture to monitor cell growth.
6. Extraction Buffer: 50 mM Tris–HCl pH 7.5, 1 mM EDTA, 4 mM MgCl₂, 10 % glycerol, 1 M NaCl (add β-mercaptoethanol and protease inhibitors immediately before use, *see* below).
7. Sorvall centrifuge: Equipped with a rotor for 500 mL bottles (e.g., SLA-3000) and a rotor for 50 mL conical tubes (e.g., SH-3000).

2.2 Immunoprecipitation

1. IgG Sepharose 6 Fast Flow Bead: purchased from GE Healthcare Life Sciences.
2. IPP150 buffer: 10 mM Tris–HCl pH 8.0, 150 mM NaCl, 0.1 % NP40.
3. Table top centrifuge, maximum speed is at least 13,000 rpm (13800 × *g*).
4. β-mercaptoethanol: Before use, add β-mercaptoethanol to Extraction Buffer to 5 mM final concentration (*see* Note 3).
5. Protease inhibitors: PMSF can be dissolved in ethanol at stock concentration of 100 mM and stored at –20 °C. Pepstatin can be dissolved in ethanol at 2.5 mg/mL and used as 1000× stock solution. Leupeptin can be dissolved in ddH₂O at 1 mg/mL and used as 400× stock solution (*see* Note 4).
6. 3C cleavage buffer: 10 mM Tris–HCl pH 8.0, 150 mM NaCl, 0.1 % NP40, 2 mM β-mercaptoethanol.
7. Bleach: Dilute 1 volume of bleach with 9 volume of ddH₂O (This is for decontaminating and discarding used yeast media). Add diluted bleach to the yeast media, and incubate for 20 min before discarding it down the drain.

2.3 Micrococcal Nuclease Treatment

1. Micrococcal nuclease: Use it at a final concentration of ~1 U/μL.
2. 100 mM CaCl₂: Weigh 111 g of CaCl₂ and dissolve in 1 L of ddH₂O to make 1 M stock solution. Dilute the stock to 100 mM before use.
3. 0.5 M EGTA: dissolve 19.02 g of EGTA in 90 mL of ddH₂O. Use 10 N NaOH to adjust pH to 8.0. Add ddH₂O to 100 mL.

²Yeast TAP-tagged library is available from Open Biosystems of Thermo Scientific [24].

³Pure β-mercaptoethanol has a concentration of 14.6 M.

⁴Protease inhibitors are not stable. PMSF will lose its activity within an hour of use. Prepare protease inhibitors immediately before use and always keep them cold.

2.4 In Vitro Transcription of RNA

1. DNA oligos (template sequence):

T7-snR81-F1 template: 5'-*TAATACGACTCACTATAGGG*
AATGTCTGACTAGGGACTGC NNNNNNN GCGGCGAGG
CAGCCCACATCAAGTGGAACTACAC-3'

snR81-R1 template: 5'-TAGGATTGCTCTTGGGACCG NNNNNNN
GCGACAAGGAAGTCTGTGTAGTTCCACTTGATG-3'

snR81-R2 template: 5'-TCCGTGGACTGTACAGGTTTCAGC
GGGGG NNNNNNNNN TTGCTTGTTAGGATTGCTCTTGG-3'

snR81-R3 template: 5'-AACTGCAGAAGATGTGAAAAAGCG NNNN
CCCCCAAATCATATACTTCTGCACCATCCGTGGACTG TACAG 3'

The italic letters represent the T7 promoter sequence, and the “N” letters represent the guide sequence (it varies depending on the substrate sequence).

Briefly spin the DNA oligo tubes (purchased from IDT) and add ddH₂O to dissolve pellets to desired stock concentration of 100 μM. Stock could be further diluted to 10 μM before use.

2. PCR Thermocycler: If the thermocycler does not have heated lid, add a layer of mineral oil on top of the PCR mixture prior to PCR cycling.
3. 1.5 % Agarose Gel: Weigh 0.75 g of agarose and add it to 50 mL of 1× TBE buffer. Microwave the mixture and boil it for 5 ~ 10 s. Shake gently in-between to avoid local overheating. Cool the solution down to ~50 °C in water bath and add ethidium bromide to a final concentration of 0.5 μg/mL.
4. 10× transcription buffer: 500 mM Tris–HCl pH 7.5, 100 mM MgCl₂, 100 mM DTT, 20 mM spermidine. Aliquot to 1.5 mL tubes and store at –20 °C.
5. DNase I: Typically 1 unit is enough to digest 2 μg of DNA in 20 min.
6. [α-P³²] GTP, or any other [α-P³²] NTP (*see* Note 5).
7. 6 % denaturing gel: To make 100 mL of gel mix, use 20 mL of 30 % acrylamide mix (acrylamide:bis 19:1), 10 mL of 10× TBE, 48 g of urea, and add ddH₂O to 100 mL. Use a stir bar to dissolve and mix the solution. Add 1 mL of 10 % APS, 100 μL of TEMED immediately before casting the gel (*see* Note 6).
8. G50 buffer: 20 mM Tris–HCl pH 7.5, 300 mM NaOAc, 2 mM EDTA, 0.2 % SDS. Store at room temperature (*see* Note 7).
9. PCA: Phenol/chloroform/isoamyl alcohol (25:24:1) (*see* Note 8).

⁵³²P has a short half-life of 14 days. They should be ordered not long before the experiment.

⁶Acrylamide is a neurotoxin. Wear gloves and exercise caution when making the gel. Dissolving urea in water is an endothermic reaction, thus it is desirable to turn on low heat on the stirring device.

⁷Keep G50 buffer above 4 °C, otherwise SDS will precipitate.

⁸Phenol and chloroform are toxic and harmful in contact with skin. Handle them in the hood and wear safety glasses.

10. 70 % Ethanol: Mix 7 volume of 100 % ethanol with 3 volume of ddH₂O.
11. 10× DNA loading dye: Mix 4.9 mL of glycerol, 200 µL 0.5 M EDTA, 0.025 g bromophenol blue, 0.025 g xylene cyanol FF. Bring to a total volume of 10 mL with ddH₂O.
12. 2× RNA loading dye: Mix 9.5 mL formamide, 25 µL 10 % (w/v) SDS, 10 µL 0.5 M EDTA, 0.025 g bromophenol blue, 0.025 g xylene cyanol FF, bring to a total volume of 10 mL with ddH₂O.

2.5 Functional Reconstitution

1. 5× in vitro pseudouridylation buffer: 1 M Tris-HCl at pH 8.0, 1 M ammonium acetate, 50 mM MgCl₂, 2 mM DTT, 0.1 mM EDTA.
2. 2 % Glycogen: Dissolve 0.2 g of glycogen into 10 mL of ddH₂O, aliquot and store at −20 °C.
3. Nuclease P1 digestion buffer: 200 mM acetic acid, 10 mM CaCl₂, pH 6.0.
4. Nuclease P1: 0.1 µg of nuclease P1 is sufficient to digest 10 pmol of RNA substrate, when incubation is carried out at 37 °C for 1 h.
5. TLC PEI cellulose F plate: Purchased from EMD.
6. TLC developing buffer: isopropanol/HCl/H₂O (70:15:15). Make fresh buffer for each experiment. TLC developing chamber should be placed in the chemical hood.

3 Methods

3.1 TAP-Tagged Yeast Cell Culture

1. Thaw glycerol stock (Cbf5-, Gar1-, or Nhp2-TAP-tagged yeast strain) at room temperature (*see Note 2*). Immediately transfer 250 µL into a 50 mL conical tube containing 5 mL of SD-URA medium. Grow the cells by shaking at 200 rpm, 30 °C for overnight (*see Note 22*).
2. Inoculate 5 mL of overnight culture into 50 mL of SR-URA medium (*see Note 9*). Grow by shaking at 200 rpm, 30 °C for 8 h.
3. Measure the OD_{600nm} of the culture. Inoculate the culture into 600 mL of SR-URA medium at a starting OD_{600nm} of 0.02. Grow by shaking at 200 rpm, 30 °C for overnight.
4. When the OD_{600nm} of the overnight culture reached 1.0, add 300 mL of 3×-YPGal to the culture to activate Gal promoter and induce protein expression. Induction is typically carried out for 24 h.

²²All cell culture procedures should be performed under sterile conditions (close to a flame, and on a clean bench top). The operation should be done in a short period of time to avoid possible contamination, especially for operation involving nutrient-rich 3×-YPGal medium.

⁹Use at least 250 mL flask to grow a 50 mL culture. Adequate air space is necessary for efficient aeration and growth of the culture.

5. Harvest cells by centrifugation at $2000 \times g$ for 5 min (SLA-3000 rotor) (*see Note 10*). Remove the supernatant (treat with bleach before discard). Keep the cell pellet.
6. Add 80 mL of ddH₂O to the cell pellet and resuspend the cells. Transfer the cells to two 50 mL tubes in equal volume (~40 mL each).
7. Centrifuge the 50 mL tubes at $2000 \times g$ for 5 min in SH-3000 swing bucket. Discard supernatant and resuspend cell pellets in 30 mL of Extraction Buffer. Spin down the cells at $2000 \times g$ and remove the Extraction Buffer. Freeze the cells in liquid nitrogen and store at -80°C until IgG purification.

3.2 Immunoprecipitation of Box H/ACA RNPs

1. Pre-wash IgG Sepharose beads: Take 200 μL of IgG beads, spin at 5000 rpm ($2040 \times g$) for 1 min to remove supernatant. Add 1 mL of cold IPP150 buffer; invert the tube by hand until all beads are resuspended. Spin at 5000 rpm for 1 min and remove 900 μL of the supernatant (*see Note 11*).
2. Repeat **step 1** three times. After wash, keep beads on ice until use (beads plus sample have a volume of ~200 μL).
3. To the Extraction Buffer, add β -mercaptoethanol to a final concentration of 5 mM, PMSF to 1 mM, pepstatin to 2 $\mu\text{g}/\text{mL}$, and leupeptin to 2 $\mu\text{g}/\text{mL}$. Chill on ice, and dissolve the cell pellet in 15 mL of cold Extraction Buffer. Break yeast cells by running through French Press eight times (18,000 PSI). Keep the sample on ice whenever possible (*see Note 12*).
4. Spin the cell lysate at $12,000 \times g$ for 10 min to remove cell debris.
5. Transfer the supernatant into a cold 50 mL conical tube and measure the volume V. Split the supernatant evenly into two 50 mL conical tubes. To each tube, add 2.83 V volume of IPP150 buffer, 0.005 V volume of 10 % NP40, 100 μL of prewashed IgG beads, and 0.005 V volume of 100 mM PMSF.
6. Rotate the conical tube from end to end at 4°C for 2 h.
7. Spin at $400 \times g$ for 10 min to precipitate the beads.
8. Carefully remove supernatant without disturbing beads. Resuspend the beads in 1 mL of IPP150 buffer. Transfer the bead suspension from the 50 mL conical tubes to 1.5 mL low binding tubes.
9. Spin at 5000 rpm in Table Top Centrifuge at 4°C for 2 min, and remove the supernatant.
10. Repeat **steps 8 and 9** at least two times.

¹⁰Chill the yeast culture, ddH₂O, and Extraction Buffer on ice before harvesting cells. Turn on Sorvall centrifuge and keep rotor and centrifugation bottles at 4°C .

¹¹To facilitate transfer of beads, cut pipette tips at the fine ends with scissors or razor blades to make a wider opening.

¹²To evaluate yeast cell lysis, take 1 μL of the extract and dilute it with 9 μL of ddH₂O. Examine 2 μL of diluted samples under a light microscope. (Lysed yeast cells are dark while un-lysed cells appear to be bright and refractile.)

11. Wash the beads again with 1 mL of 3C cleavage buffer two times.
12. Resuspend the beads in 150 μ L of 3C cleavage buffer. Add 4 μ g of 3C protease. Rotate the tube from end to end at 4 °C overnight.
13. Use a flame-heated needle to poke a small hole at the bottom of the tube, and place it on top of another 1.5 mL tube. Spin at 1000 rpm for 2 min to collect the purified box H/ACA RNPs (*see* Note 13).
14. Add 100 μ L of cold 100 % glycerol to each tube and store the box H/ACA RNPs at –20 °C for short-term use and –80 °C for long-term storage (*see* Note 14).

3.3 Depletion of Endogenous Box H/ACA RNAs

1. To each 250 μ L of immunoprecipitated RNP (Subheading 3.2, **step 14**), add 5 μ L of 100 mM CaCl₂ (final 2 mM) and 300 units of micrococcal nuclease. Incubate at 30 °C for 30 min.
2. Add EGTA to a final concentration of 5 mM to inactivate micrococcal nuclease. The micrococcal nuclease-treated complexes are now ready for functional reconstitution (*see* Subheading 3.5 below)

3.4 In Vitro Synthesis of Designer Box H/ACA RNA

1. According to the substrate sequence information, design and purchase DNA oligos (template sequence) (listed in Subheading 2.4, **step 1**, and illustrated in Fig. 1b). Dilute the DNA oligos to 10 μ M [22].
2. Set up a standard PCR reaction where the final concentrations of the four oligos are: 1 μ M of F1 oligo, 0.1 μ M of R1 oligo, 0.1 μ M of R2 oligo, 1 μ M of R3 oligo. The PCR cycle parameters are: 94 °C for 20 s, 30 cycles of [94 °C for 20 s, 50 °C for 15 s, and 72 °C for 30 s], followed by 72 °C for 3 min.
3. Add 1/10 (v/v) of 10 $\times$ DNA loading dye to the completed PCR reaction. Load and resolve the PCR product on a freshly prepared 1.5 % agarose gel.
4. Excise the band of expected size (PCR product) and gel-purify the product using the Qiagen gel extraction kit (*see* Note 15).
5. Set up a 200- μ L in vitro transcription reaction containing 50 mM Tris–HCl pH 7.5, 10 mM MgCl₂, 10 mM DTT, 2 mM spermidine, 1 mM of each rNTP, 10 pmol DNA template (purified PCR product) and 5 U/ μ L of T7 RNA polymerase [23]. Transfer 10 μ L of the reaction mixture to a new tube containing ~50,000 cpm (input cpm) [α -³²P]GTP (less than ~0.5 μ L). This ~10- μ L reaction is to be used later for locating and quantifying RNA transcript produced (*see* Note 23). Incubate both reactions (190 μ L and 10 μ L) at 37 °C for 2 h (*see* Note 16).

¹³The hole should be small enough to prevent beads from going through. If leaky, the leaked beads can be spun down, and the supernatant recovered.

¹⁴Store the Box H/ACA RNP in small aliquots. Avoid repeated freezing and thawing.

¹⁵In our experience, higher molecular weight nonspecific bands might show up in the gel. It is important to cut the right band using DNA ladder as reference to avoid contamination.

6. Add 5 U of DNase I (RNase free) to the 190- μ L reaction and $\sim 1/4$ U (1 μ L of diluted DNase (1:20 dilution)) to the ~ 10 - μ L reaction, and incubate at 37 °C for 20 min.
7. Add G50 buffer to bring both reactions to 300 μ L. Add 500 μ L of PCA and mix thoroughly. After a brief spin (13,000 rpm, 1–2 min), transfer the aqueous phase to new tubes. Add 750 μ L of 100 % ethanol, mix well by vortexing, and place the tubes on dry ice. 20 min later, precipitate the RNA transcript by centrifugation (bench-top centrifuge, 13,000 rpm, 4 °C, 15 min) (*see* Note 17).
8. Wash the RNA pellet once with 70 % ethanol by spinning at top speed for 5 min. Resulting pellet is dried and resuspended in a small volume of water (<10 μ L, if possible), add an equal volume of 2 $\times$ RNA loading dye, and load both samples (from the 190- μ L and 10- μ L reactions) side-by-side onto a 6 % denaturing polyacrylamide gel (8 M urea).
9. After electrophoresis, excise the bands (containing the RNA transcript) from both lanes, and place them in new tubes. The band in the 10- μ L reaction lane (containing 32 P) is visualized by autoradiography and used as a marker to pinpoint the position of the RNA transcript (unlabeled) in the neighboring lane (the 190- μ L reaction) containing no radioisotope.
10. Add 400 μ L of G50 to both tubes, and place the tubes on dry ice for 10 min. Transfer the tubes to room temperature, and elute the RNA (from the gel slices) overnight.
11. Spin down the gel slices, and transfer the eluent to new tubes. Add 400 μ L of PCA to each tube containing the RNA eluate, mix well, and spin at top speed (13,000 rpm) in a bench-top centrifuge for 3 min.
12. Carefully transfer the aqueous phase to new tubes, and add 10 μ g of glycogen, and then 1 mL of 100 % ethanol. Spin at 13,000 rpm in the bench-top centrifuge at 4 °C for 15 min. Wash the pellet with 250 μ L of 70 % ethanol by spinning in the bench-top centrifuge (13,000 rpm) at 4 °C for 5 min. Remove ethanol and air-dry the pellet.
13. Resuspend the pellet in ddH₂O, and use scintillation counter to obtain cpm (from the 10- μ L reaction). Calculate the percentage of [α - 32 P]GTP-incorporation (incorporated cpm/input cpm; usually ~ 5 %), and determine the amount of RNA transcript recovered (*see* Note 23). Typically, a 190- μ L reaction generates ~ 10 –15 μ g of RNA transcript. Store the RNA transcript at -80 °C until use.

²³If the percentage of [α - 32 P]GTP-incorporation [dividing the incorporated cpm (assume ~ 2500) by the input cpm ($\sim 50,000$)] is ~ 5 %, the amount of RNA generated from the 10 μ L reaction can be calculated as follows: 1 mM (GTP concentration) $\times$ 10 μ L (reaction volume) $\times$ 5 % (percentage of [α - 32 P]GTP-incorporation) $\times$ 4 (assuming the 4 nucleotides, A, U, C, and G, are equally distributed in the RNA molecule) $\times$ 330 (average nucleotide molecular weight) = 660 ng of RNA. Thus, a 190 μ L reaction will generate 660 ng $\times$ 19 = 12,540 ng (or 12.54 μ g) of RNA.

¹⁶A radioactive transcript is essential for locating the position of the RNA transcript on the gel and quantifying the amount of RNA recovered.

¹⁷It is necessary to clean up the RNA transcript by phenol/chloroform extraction and concentrate it by ethanol precipitation followed by resuspension into a small volume of water prior to loading onto the gel (a large volume will not fit in the well).

3.5 Reconstitution of Box H/ACA RNP (Pseudouridylation Assay)

1. Assemble in vitro pseudouridylation reaction: 5 μ L of micrococcal nuclease-treated Box H/ACA RNP (*see* Subheading 3.3), 50 ng of in vitro synthesized box H/ACA RNA, 5000 cpm of singly labeled substrate RNA (*see* Note 18), 40 μ g of yeast tRNA in 100 mM Tris-HCl at pH 8.0, 100 mM ammonium acetate, 5 mM MgCl₂, 2 mM DTT, 0.1 mM EDTA (*see* Note 19). Add ddH₂O to bring to 20 μ L, and incubate at 37 °C for 1 h [18].
2. Add 280 μ L of G50 buffer and 300 μ L of PCA to the reaction, vortex vigorously for 1 min, and spin at 13,000 rpm in the bench-top centrifuge at room temperature for 5 min.
3. Transfer the supernatant to a new tube and add 1 mL of 100 % ethanol, 10 μ g of glycogen. Spin at 13,000 rpm in the bench-top centrifuge at 4 °C for 15 min. Wash RNA pellet using 70 % ethanol and air-dry the pellet (*see* Note 20).
4. Resuspend the RNA pellet in 10 μ L of nuclease P1 digestion buffer. Add 5 U of nuclease P1 and incubate the reaction at 37 °C for 2 h.
5. Spot 1.5 μ L of the digestion products onto TLC PEI cellulose plate with 1 cm space between dots. Develop the plate in TLC Developing Buffer for 4–7 h.
6. Dry the plate in chemical hood overnight. Wrap the plate in saran wrap and expose the plate using phosphorimager (*see* Note 21). A sample result is shown in Fig. 2.

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¹⁸The uridine to be pseudouridylated can be site-specifically ³²P-labeled. This can be achieved by cleaving the full-length RNA at the site 5' of the target uridine, followed by phosphorylation of the 3' fragment with [gamma-³²P]ATP and ligation of the labeled 3' fragment with the 5' fragment [23, 25].

¹⁹Yeast tRNA is needed to protect substrate RNA from degradation (Fig. 2, compare lane 1 with lane 3).

²⁰Use the Geiger counter to detect radiolabeled RNA and make sure RNA is precipitated.

²¹Make sure the TLC plate is dried completely before being wrapped and exposed to a phosphorimaging screen. The residual developing buffer could otherwise damage and reduce the sensitivity of the phosphorimaging screen.

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Fig. 1.

(a) Typical structure of a box H/ACA RNA (bound with its substrate RNA). Box H/ACA RNA is typically ~ 200-nts long and folds into a hairpin-hinge-hairpin-tail structure (*black and blue lines*). Within each hairpin, there is an internal loop (*blue*) that serves as a guide to base-pair with its substrate RNA (*red*). The arrows indicate the target uridines (U) to be pseudouridylated. N stands for any nucleotide. Here, four short DNA oligos are used to construct a DNA template (by PCR) for the synthesis of an artificial box H/ACA RNA (with desired guide sequences). For illustrative purposes, the box H/ACA RNA is marked at several positions by black letters (A–H), and oligos are designed according to the markers (*see b*). (b) Design of oligos (one forward sense oligo, F1, and three reverse antisense oligos, R1, R2, and R2) for template DNA amplification. The overlapping complementary region (between B and D) is indicated. The overlapping regions between C and F and between E and H are also shown. The blue segments represent guide sequences that can be altered

according to the substrate sequences. The T7 promoter (for in vitro transcription) is also indicated

Fig. 2.

In vitro pseudouridylation assay. Pseudouridylation is carried out at 37 °C for 1 h. The RNA substrate contains a single ^{32}P at the site 5' of target uridine. After the reaction, the RNA is recovered, cleaved by RNase P1, and analyzed by TLC. Lane 1, in vitro assay performed as described in the text (Subheading 3.5) where the pseudouridylation reaction contains 50 ng of in vitro transcribed box H/ACA RNA. Lane 2, same as lane 1, except that the reaction contains 10 ng of box H/ACA RNA. Lane 3, same as lane 1, except that tRNA is not

included. Lane 4, substrate RNA used directly for RNase P1 digestion (no pseudouridylation)

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