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Chapter 12

Non-radioactive In Vivo Labeling of RNA with 4-Thiouracil

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Abstract

RNA molecules and their expression dynamics play essential roles in the establishment of complex cellular phenotypes and/or in the rapid cellular adaption to environmental changes. Accordingly, analyzing RNA expression remains an important step to understand the molecular basis controlling the formation of cellular phenotypes, cellular homeostasis or disease progression. Steady-state RNA levels in the cells are controlled by the sum of highly dynamic molecular processes contributing to RNA expression and can be classified in transcription, maturation and degradation. The main goal of analyzing RNA dynamics is to disentangle the individual contribution of these molecular processes to the life cycle of a given RNA under different physiological conditions. In the recent years, the use of nonradioactive nucleotide/nucleoside analogs and improved chemistry, in combination with time-dependent and high-throughput analysis, have greatly expanded our understanding of RNA metabolism across various cell types, organisms, and growth conditions.

In this chapter, we describe a step-by-step protocol allowing pulse labeling of RNA with the nonradioactive nucleotide analog, 4-thiouracil, in the eukaryotic model organism *Saccharomyces cerevisiae* and the model archaeon *Haloferax volcanii*.

Key words 4-thiouracil, RNA, Pulse labeling, RNA tagging, *Haloferax volcanii*, *Saccharomyces cerevisiae*

1 Introduction

RNA homeostasis is a fundamental process for the regulation of gene expression and therefore for the physiology of living organisms [1, 2]. Several methods enable the characterization of the cellular RNA composition in a quantitative (relative amount) and qualitative manner (specific molecule identity) [1–3]. Classically, the presence and the relative amount of an RNA of interest can be easily assessed by northern blot or quantitative RT-PCR analyses

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[1–3]. More recently, the emergence of high-throughput technologies, like DNA microarrays [4–6] or next generation sequencing analysis [7, 8], have leveraged our capacity to obtain a faithful qualitative and quantitative overview of the ensemble of RNA molecules present at equilibrium in varying conditions. However, all these methods are inherently lacking temporal resolution and hardly permit in depth systematic analysis of the relative contribution of the individual molecular processes underlying the life cycle of an RNA [9, 10].

This problem was recognized very early in the history of molecular biology. Pioneering studies have ingeniously taken advantage of metabolic labeling to reveal the importance of RNA dynamic for gene expression. For example, the seminal work of Brenner, Jacob, and Meselson in 1961 enabled the discovery of mRNA as a dynamic intermediate molecule required for protein synthesis [11]. Metabolic labeling of RNA using radioactively labeled molecules, like nucleotides/nucleosides, has been the method of choice to explore RNA dynamics. However, country-dependent administrative regulations of radioactive isotopes constrain the use of metabolic labeling using radioactive-labeled nucleotides. In addition, the inherent difficulty of this technique to analyze dynamics of individual RNAs in a global and systematic fashion, has often confined this methodology to assess global transcription output or production and maturation of abundant cellular RNA (like ribosomal RNA maturation [12–19]). Alternative methods using nonradioactive nucleotide analogs for the metabolic labeling of RNA were explored early on [20]. However, it is only recently, thanks to improvement of chemistry, labeled-RNA enrichment and detection methods, that the full potential of this approach is unfolding [9, 21–24].

Several nonradioactive nucleotide analogs providing different advantages/disadvantages have been described in the literature [9, 21]. Among them, uracil derivatives have been widely used [9]. Due to their versatile chemical properties, thiouracil/uridine and uracil/uridine derivatives compatible with click-chemistry have emerged as molecules of choice to perform RNA dynamic analysis [9]. These uracil/uridine derivatives allow for orthogonal chemistry with a large battery of reagents to facilitate downstream visualization, isolation and system-wide characterization of labeled RNA, thereby providing an unprecedented time and resolution depth of RNA metabolism [9, 10, 21–28].

Furthermore, the chemical properties of the uracil/uridine derivatives can be further harnessed in combination with other methodologies (e.g., UV cross-linking, structure probing, mass spectrometry, next-generation sequencing) to investigate the dynamics of structure and composition of ribonucleoprotein particle (RNP) [23, 24, 28–34]. Overall, the potential of using chemical

biology strategies and their combinations is still emerging and will undoubtedly provide new molecular insights into the life cycle of RNA molecules.

In this chapter, we describe step-by-step conditions to successfully perform 4-thiouracil labeling in two model organisms, *Saccharomyces cerevisiae* and *Haloferax volcanii*, and provide general technical advice, which should facilitate application of this methodology (Fig. 1).

2 Materials

There are no specific preferences of sources of chemical reagents or materials, unless stated otherwise. Use ultrapure water with 18 MΩ·cm resistivity at 25 °C, unless stated otherwise.

2.1 Microbiological Cultures

2.1.1 Strains

1. *Haloferax volcanii* H26 [35].
2. *Saccharomyces cerevisiae* (any genetic background prototroph for uracil).

2.1.2 Haloferax Volcanii Enhanced Casamino Acids (Hv-Ca$^+$)

1. 1 M Tris–HCl pH 7.0.
2. 1 M Tris–HCl pH 7.5.
3. 1 M KOH.
4. 30% (w/v) salt water: 4.1 M NaCl; 147.5 mM $MgCl_2$; 142 mM $MgSO_4$; 94 mM KCl; 20 M Tris–HCl pH 7.5.
5. 10× casamino acids solution: 10 g/L casamino acids.
6. *Haloferax volcanii* minimal carbon source: 10% sodium-DL-lactate; 9% succinic acid; 1% glycerol; pH 7.5. Sterilize by filtration.
7. *Haloferax volcanii* minimal salts: 417 mM NH_4Cl; 250 mM $CaCl_2$; 152 μM $MnCl_2$; 127 μM $ZnSO_4$; 689 μM $FeSO_4$; 16.7 μM $CuSO_4$. Sterilize by filtration.
8. Hv-Trace elements solution: 1.8 mM $MnCl_2$; 1.53 mM $ZnSO_4$; 8.27 mM $FeSO_4$; 0.2 mM $CuSO_4$.
9. 0.5 M KPO_4 buffer, pH 7.0.
10. 1 mg/L thiamine solution. Sterilize by filtration.
11. 1 mg/L biotin solution. Sterilize by filtration.
12. Uracil stock solution (500 mM in DMSO).
13. 4-thiouracil stock solution (500 mM in DMSO).
14. Sterile filters (0.22 μm) and syringes.

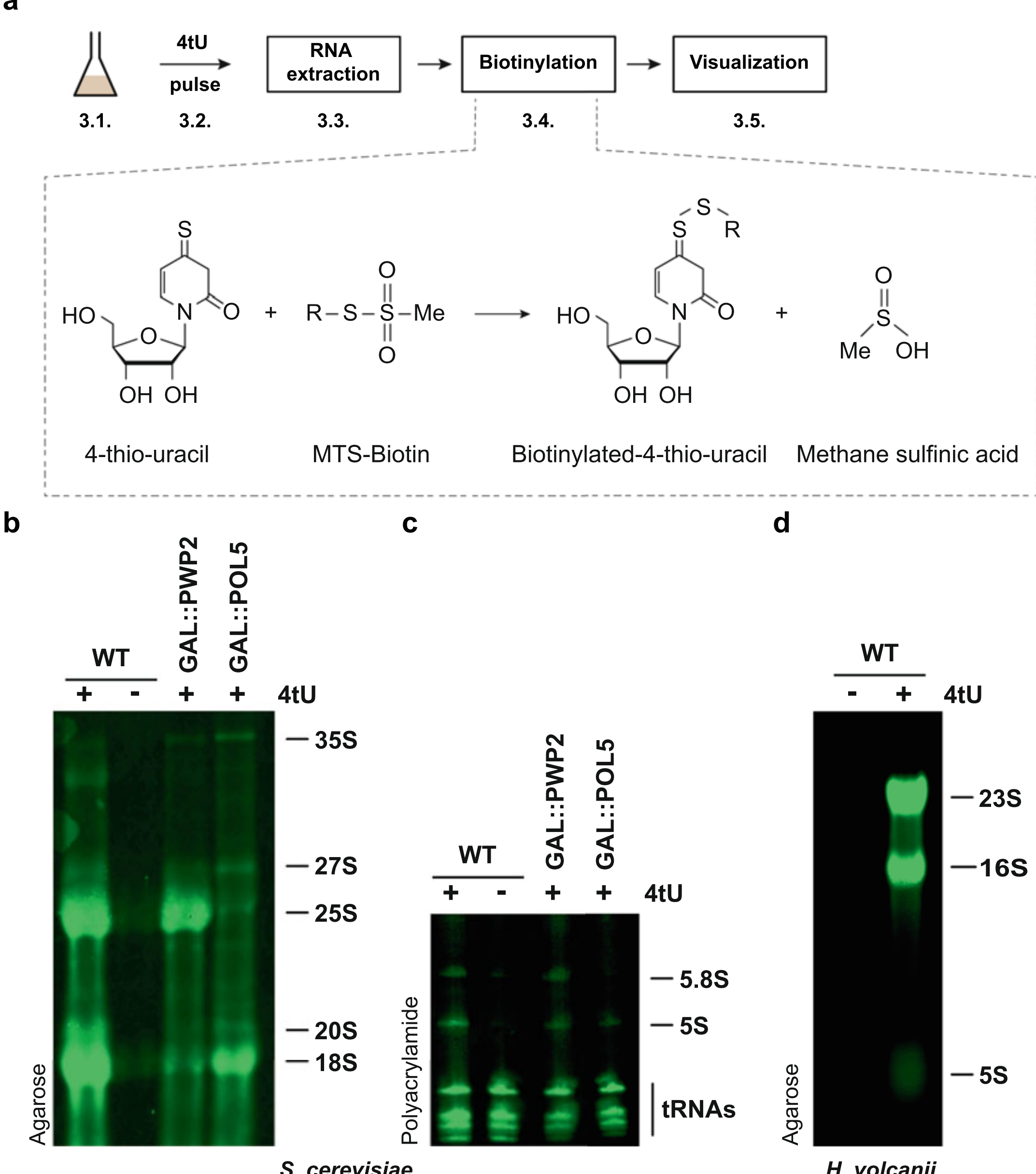

Fig. 1 Exemplary 4-thiouracil (4-TU) labeling of total RNA in *S. cerevisiae* and *H. volcanii*: (**a**) Workflow summary of 4-TU labeling. 4-thiouracil labeling and biotinylation workflow is briefly depicted. Growing cells (*see* Subheading 3.1.) are pulse labeled with 4-thiouracil as described in Subheading 3.2. After RNA extraction (*see* Subheading 3.3), sulfhydryl groups are specifically biotinylated in the presence of methylthiosulfonate activated Biotin derivate (MTS-Biotin) (*see* Subheading 3.4). After biotinylation RNA are separated by gel electrophoresis and visualized as described in Subheading 3.5 and below. (**b** and **c**) Exemplary 4-TU labeling applied on the eukaryotic model organism *S. cerevisiae*. The indicated yeast cells, wild-type (WT), cells depleted for the small and large ribosomal subunit biogenesis factors Pwp2 (*GAL::PWP2*) and Pol5 (*GAL::POL5*), respectively [39, 40] were labeled for 20 min with 4-thiouracil as indicated in Subheading 3.2. Labeled total RNA were separated by denaturing agarose (**b**) or polyacrylamide (**c**) gel electrophoresis, respectively. Biotinylated RNA was detected as described in Subheading 3.5. Ribosomal RNA precursors (35S, 27S, and

2.1.3 *Saccharomyces cerevisiae* Minimal Medium (See **Note 1**)

1. 0.65 g/L Complete Supplement Mixture w/o histidine, leucine, uracil (CSM −His -Leu -Ura).
2. 6.7 g/L yeast nitrogen base containing ammonium sulfate as a nitrogen source (5 g/L) (YNB).
   (a) 20 g/L glucose (D) or galactose (G).
   (b) 0.1 g/L histidine.
   (c) 0.1 g/L leucine.
   (d) Uracil stock solution (500 mM in DMSO).
   (e) 4-thiouracil stock solution (500 mM in DMSO).

2.2 Chemical Reagents and Materials for RNA Extraction

1. AE buffer (50 mM Na-acetate pH 5.3; 10 mM EDTA pH 8).
2. AE buffer-saturated phenol (*see* **Note 2**).
3. 10% sodium dodecyl sulfate (SDS) solution.
4. 3 M Na-acetate pH 5.3 solution.
5. RNA Precipitation mix (96% ethanol; 120 mM Na-acetate pH 5.3).
6. RNA sample solubilization buffer (95% deionized formamide, 20 mM EDTA, 0.05% bromophenol blue) (*see* **Note 3**).
7. Chloroform p.a.
8. Thermomixer.
9. 1.5 mL Safe-Lock tubes.

2.3 Chemical Reagents for Biotinylation of 4TU-Labeled RNA

1. MTSEA-Biotin-XX (Biotium—Cat Number:90066) (1 mg/mL solved in *N*,*N*-dimethylformamide).
2. 10× biotinylation buffer (100 mM Tris–HCl 7.4; 10 mM EDTA pH 8).
3. RNase-free H_2O.
4. Dark box.

Fig. 1 (continued) 20S rRNA) and mature ribosomal RNA (25S, 18S, 5.8 and 5S rRNA) are indicated. Please note that depletion of the small ribosomal subunit biogenesis factor, Pwp2, for 14 h leads to the reduction of small ribosomal subunit pre- and mature rRNAs (20S and 18S rRNA, respectively) [39]. In contrast depletion of the large ribosomal subunit biogenesis factor, Pol5, for 9 h leads to the reduction of the common large ribosomal subunit precursor and corresponding mature rRNA (27S, 25S, and 5.8S, respectively) [39–41]. Additional application examples using this protocol and *S. cerevisiae* is described in [41]. (**d**) Exemplary 4-TU labeling applied on the archaeal model organism *H. volcanii*. *Haloferax volcanii* wildtype cells were labeled for 3 h with 4-thiouracil as indicated in Subheading 3.2. Labeled total RNA was separated by denaturing agarose electrophoresis. Biotinylated RNA was detected as described in Subheading 3.5. Mature ribosomal RNA (23S, 16S, and 5S rRNA) observed in these conditions are indicated. Additional application examples using this protocol and *H. volcanii* has been described previously [36]

2.4 Separation and Immobilization of RNA

2.4.1 Agarose Gel Electrophoresis and Capillary Transfer

1. RNase-free agarose.
2. 10× MOPS buffer (0.2 M MOPS, 20 mM Na-acetate, 10 mM EDTA, pH 7).
3. 37% formaldehyde p.a.
4. Running buffer (20 mM MOPS, 2 mM Na-acetate, 1 mM EDTA, 2% formaldehyde, pH 7).
5. Power pack.
6. Gel Chamber (20 × 25 cm gel tray).
7. 0.05 M NaOH.
8. 10× SSC (1.5 M NaCl, 0.15 M sodium citrate, pH 7).
9. Whatman paper.
10. Positively charged Nylon membrane.
11. UV cross-linking device.

2.4.2 Polyacrylamide Gel Electrophoresis and Electro Transfer

1. Urea.
2. 5× TBE buffer (445 mM Tris, 445 mM boric acid, 10 mM EDTA pH 8).
3. 30% acrylamide mix (acrylamide–bisacrylamide 37.5:1).
4. *N*,*N*,*N*′,*N*′-tetramethyl ethylenediamine (TEMED).
5. 10% ammonium persulfate (APS) solved in H_2O.
6. Gel system apparatus including glass plates (20 × 10 cm), comb, spacers.
7. Power pack.
8. Whatman paper.
9. Positively charged nylon membrane.
10. Wet blot transfer cell with cassette clamping system.
11. Magnetic stirrer.
12. Magnetic stir bar.
13. UV cross-linking device.

2.5 Detection of 4-TU Labeled RNA

1. Blocking solution: 1× PBS pH 7.5, 1 mM EDTA pH 8; 10% SDS.
2. IR-dye conjugated Streptavidin (1 mg/mL).
3. Wash solution I: 1× PBS pH 7.5, 1 mM EDTA pH 8; 1% SDS.
4. Wash solution II: 1× PBS pH 7.5, 1 mM EDTA pH 8; 0.1% SDS.
5. Infrared detection system.

3 Methods (*See* Notes 4 and 5)

3.1 Microbiological Methods (See Note 6)

3.1.1 *Haloferax volcanii* Medium and Cultivation

1. Prepare 30% (w/v) Salt water by dissolving in 4 L of Millipore water 1200 g NaCl; 150 g $MgCl_2 \cdot 6H_2O$; 175 g $MgSO_4 \cdot 7H_2O$; 35 g KCl; 100 mL 1 M Tris–HCl, pH 7.5. Fill up with H_2O to 5 L. Autoclave and store at room temperature.
2. Prepare Hv-Min carbon source by dissolving in 150 mL H_2O: 41.7 mL 60% sodium-DL-lactate, 37.5 g succinic acid Na_2 salt-$\cdot 6H_2O$; 3.15 mL 80% glycerol. Carefully adjust to pH 7.5 with first 5 M NaOH and then 1 M NaOH. Fill up with H_2O to a final volume of 250 mL and sterilized by filtration. Aliquot in 50 mL and store at 4 °C.
3. Prepare Hv-Trace elements solution by adding to 100 mL water a few drops of 37% concentrated HCl. Dissolve the following salts one by one in the following order: 36 mg $MnCl_2 \cdot 4H_2O$; 44 mg $ZnSO_4 \cdot 7H_2O$; 230 mg $FeSO_4 \cdot 7H_2O$; 5 mg $CuSO_4 \cdot 5H_2O$. Sterilize by filtration and store at 4 °C (*see* **Note 7**).
4. Prepare Hv-minimal salts by mixing 30 mL 1 M NH_4Cl; 36 mL 0.5 M $CaCl_2$ and 6 mL Hv-Trace elements solution (*see* **step 3**). Sterilize by filtration and store at 4 °C.
5. Prepare 0.5 M KPO_4 buffer (pH 7.0). Mix 61.5 mL 1 M K_2HPO_4 and 38.5 mL 1 M KH_2PO_4. Check pH and adjust to pH 7.0, if required. Add an equal volume of H_2O (100 mL). Autoclave and store at room temperature.
6. Prepare thiamine–biotin mix by combining 9.5 mL thiamine (1 mg/mL) and 1.2 mL biotin (1 mg/mL).
7. Prepare 10× casamino acids solution. For 100 mL dissolve 5.1 g casamino acids in ~75 mL H_2O. Add 2.4 mL 1 M KOH. Fill up to 100 mL with H_2O. Sterilize by filtration.
8. Prepare Hv-Ca^+ medium. For 1 L medium mix 600 mL 30% salt water, 225 mL H_2O and 30 mL Tris–HCl pH 7.0. Autoclave. When cooled down add 100 mL 10× casamino acids solution (*see* **step 7**), 25.5 mL Hv-Min Carbon Source (*see* **step 2**), 12 mL Hv-Min Salts (*see* **step 4**), 1.95 mL 0.5 M KPO_4 buffer pH 7.0 (*see* **step 5**), 900 μL thiamine–biotin mix (*see* step **6**). Store at room temperature in a dark cupboard.
9. Scratch the surface of an *Haloferax volcanii* glycerol cryo-stock with a sterile inoculating loop and inoculate 5 to 10 mL Hv-Ca^+ medium supplemented with Uracil (end concentration = 400 μM) as a start culture. Incubate at 42 °C under agitation. Dilute with prewarmed medium or let grow until the culture has reached the desired cell density (OD_{600nm} = 0.5–0.8).

3.1.2 *Saccharomyces cerevisiae* Medium and Cultivation

1. Prepare SCG-Ura medium for precultures (*see* **Note 8**). For 300 mL medium dissolve 0.195 g CSM-His-Leu-Ura, 2.01 g YNB, 6 g galactose, 0.03 g histidine, and 0.03 g leucine in 250 mL H_2O. Fill up to 300 mL with H_2O. Aliquot in 50 mL, autoclave, and store at room temperature.
2. Prepare SCD-Ura medium for main cultures. For 800 mL medium dissolve 0.52 g CSM-His-Leu-Ura, 5.36 g YNB, 16 g glucose, 0.08 g histidine, and 0.08 g Leucine in 700 mL H_2O. Fill up to 800 mL with H_2O. Aliquot in 200 mL, autoclave, and store at room temperature.
3. Inoculate uracil prototroph yeast strains in 50 mL SCG-Ura medium with a sterile inoculating loop by picking one single colony from agar plate and grow yeast cells overnight at 30 °C under shaking.
4. Measure absorbance at 600 nm (OD_{600nm}) of overnight cultures and dilute them to $OD_{600nm} = 0.2$ in 50 mL SCG-Ura medium. Let cells grow to exponential phase to $OD_{600nm} = 0.5–0.8$.
5. Inoculate main cultures in 200 mL SCD-Ura medium to the appropriate cell density using exponentially growing precultures (*see* above **step 4**). Incubate yeast cells until the culture has reached the desired cell density.

3.2 In Vivo Pulse Labeling with 4-TU (See Note 9)

3.2.1 Uracil and 4-Thiouracil Stocks

1. Prepare 500 mM uracil stock solution. For 5 mL, dissolve 280.2 mg uracil powder in 3 mL DMSO. Fill up to 5 mL with DMSO. Store aliquots at −20 °C.
2. Prepare 500 mM 4-thiouracil stock solution. For 5 mL, dissolve 320.4 mg 4-thiouracil powder in 3 mL DMSO. Fill up to 5 mL with DMSO. Store aliquots at −20 °C.

3.2.2 *Haloferax volcanii* Labeling

1. Grow *H. volcanii* cells in $Hv\text{-}Ca^{+}$ medium containing 400 μM uracil.
2. Centrifuge cells for 8 min at 4,500 × *g*.
3. Resuspend cell pellet in prewarmed $Hv\text{-}Ca^{+}$ supplemented with 100 μM uracil.
4. Split the cells in an appropriate number of cultures.
5. Add 300 μM uracil (mock control) or add 300 μM 4-thiouracil (pulse).
6. Incubate at 42 °C under agitation.
7. Centrifuge cells (~2 OD_{600nm} equivalent) 5 min at 10,000 × *g*.
8. Discard supernatant.
9. Freeze the cells in liquid nitrogen.
10. Proceeds with RNA extraction (*see* Subheading 3.3).

3.2.3 *Saccharomyces cerevisiae* Labeling

1. Take a volume corresponding to 30 OD_{600nm} of exponentially growing yeast culture and centrifuge for 3 min at 1,700 × *g*.
2. Discard supernatant and resuspend cell pellets in 40 mL SCD-Ura medium and transfer to 100 mL Erlenmeyer flasks.
3. Add either 100 μM uracil (mock control) or 100 μM 4-thiouracil (pulse).
4. Incubate for 20 min at 30 °C under agitation.
5. Centrifuge cells for 5 min at 3,000 × *g*.
6. Discard supernatant.
7. Freeze the cells in liquid nitrogen.
8. Proceed with RNA extraction (*see* Subheading 3.3).

3.3 RNA Extraction [36]

Perform all RNA extractions as fast as possible in a controlled RNase-free environment (*see* **Note 10**).

1. Label a total of four 1.5 mL safe-seal tubes (1–4) for each sample condition.
2. Prewarm Thermomixer to 65 °C.
3. Add reagents as described in Table 1.
4. Dissolve cell pellets in 500 μL AE buffer and keep on ice.
5. Add the dissolved pellet to the corresponding prepared Tube 1 (*see* Table 1).

   Close the tube properly and vortex briefly. Keep at room temperature until all the Tube 1 series is completed.
6. Incubate at 65 °C (Thermomixer) for 5 min with full speed agitation.
7. Vortex all the samples and keep on ice for 1 min.
8. Centrifuge 2 min at 18,000 × *g*, room temperature (*see* **Note 11**).
9. Transfer ~450 μL aqueous phase (upper phase) (*see* **Note 12**) to the corresponding prepared Tube. Vortex briefly. Keep at room temperature until all the Tube 2 series is completed.

Table 1
Tube series required for RNA extraction (*see* Subheading 2.2)

	Sample X
Tube 1	500 μL AE buffer-saturated phenol 50 μL 10% SDS
Tube 2	500 μL AE buffer-saturated phenol
Tube 3	500 μL chloroform
Tube 4	780 μL RNA precipitation mix

10. Centrifuge for 1 min at 18,000 × *g*, room temperature.
11. Transfer ~360 μL aqueous phase (upper phase) (*see* **Note 12**) to the corresponding prepared Tube 3. Vortex briefly. Keep at room temperature until all the Tube 3 series is completed.
12. Centrifuge 1 min at 18,000 × *g*, room temperature.
13. Transfer ~300 μL aqueous phase (upper phase) (*see* **Note 12**) to the corresponding prepared Tube 4. Invert to mix and precipitate the RNA at least 60 min at −20 °C.
14. Centrifuge for 20 min at 18,000 × *g* at 4 °C.
15. Discard supernatants.
16. Optional recommended step: wash pellets with cold (−20 °C) 75% Ethanol. Centrifuge if necessary and discard supernatants.
17. Let the pellet dry at room temperature.
18. Dissolve RNA pellet in 20–40 μL RNase-free water. Store at −20 °C or −80 °C.
19. Measure RNA concentration.

3.4 4-TU–Labeled RNA Biotinylation

1. Mix 25–50 μg extracted RNA, 5 μL MTSEA-Biotin-XX (5 μg), 25 μL 10× biotinylation buffer in a total volume of 250 μL.
2. Incubate for 30 min in the dark at room temperature.
3. Add 250 μL AE buffer (*see* **item 1** Subheading 2.2).
4. Proceed with RNA extraction by adding the sample to a prepared tube containing 500 μL AE buffer-saturated phenol and 50 μL 10% SDS (*see* Subheading 3.3).
5. Dissolved RNA pellet in RNA loading buffer (*see* **item 6** Subheading 2.2).

3.5 Separation and Immobilization of RNA (See Note 11)

3.5.1 Preparation of Denaturing Agarose Gel

1. Mix 1.3% agarose in 0.85 final volume of H_2O.
2. Dissolve the agarose by heating the mixture in the microwave.
3. Let cool down under agitation (~50 °C) and add 0.1 volume of 10× MOPS running buffer and 0.054 volume of 37% Formaldehyde (final concentration 2%).
4. Cast the gel.
5. Prepare running buffer (1× MOPS, 2% formaldehyde).
6. Load the RNA samples (obtained in Subheading 3.4).
7. Run the gel overnight at 40 V and room temperature.

3.5.2 Preparation of Denaturing Polyacrylamide Gel

1. Prepare urea–polyacrylamide gel solution (6% acrylamide, 7 M urea solved in 0.5× TBE). Mix by stirring at room temperature until urea is properly dissolved.
2. Add 0.001% (v/v) TEMED and 0.1% (v/v) APS.

3. Cast gel immediately and store it after polymerization at 4 °C overnight.
4. Prepare running buffer (0.5× TBE).
5. Load the RNA samples (obtained in Subheading 3.4). Prior to sample loading, flush pockets very well with 0.5× TBE using an hollow needle to remove urea.
6. Run the gel for 1 h and 15 min at 150 V.

3.5.3 Northern Blotting (Agarose Gel)

1. Rinse gel in 5 gel volumes of H_2O for 5 min.
2. Hydrolyzed RNA in 5 gel volumes of 0.05 M NaOH for 20 min under mild agitation.
3. Rinse twice with 5 gel volumes of 10× SSC for 20 min under mild agitation.
4. Prepare nylon membrane, Whatman paper, and soaking paper.
5. Transfer RNA overnight onto the Nylon membrane by capillary transfer.
6. Cross-link RNA to the membrane by exposing the membrane twice with 0.5 J/cm^2.

3.5.4 Northern Blotting (Polyacrylamide Gel)

1. Rinse gel in 5 gel volumes of 0.5× TBE for 5 min.
2. Equilibrate Nylon membrane, Whatman paper, and clamping nets in 0.5× TBE.
3. Set up blotting sandwich and fill transfer tank with blotting buffer (0.5× TBE).
4. Transfer RNA at room temperature for 1 h and 20 min at 50 V. Blotting buffer should be stirring constantly with the help of a magnetic stir device.
5. Cross-link RNA to the membrane by exposing the membrane twice with 0.5 J/cm^2.

3.5.5 Detection of Biotinylated RNA

1. Incubate membrane for 20 min in blocking buffer under mild agitation.
2. Add IR-dye conjugated Streptavidin (0.1 μg/mL) and incubate for 20 min under mild agitation in the dark.
3. Wash the membrane twice 10 min with blocking buffer under mild agitation in the dark.
4. Wash the membrane twice 10 min with washing buffer I under mild agitation in the dark.
5. Wash the membrane twice 10 min with washing buffer II under mild agitation in the dark.
6. Visualized biotinylated RNA with an infrared detection system (Fig. 1b–d).

4 Notes

1. Increased labeling is obtained when using a prototroph yeast strain. When using an uracil prototroph yeast strains, all used media lack uracil.
2. AE buffer-saturated phenol is obtained by equilibrating phenol water 3 times with 1/10th volume of AE buffer (50 mM Na-acetate pH 5.3; 10 mM EDTA pH 8). The equilibrated phenol solution should be stored at 4 °C in the dark in a safety cabinet. Phenol can cause severe burns. Always work with phenol in a fume hood and wear safety glasses, gloves, and a lab coat. Avoid direct contact.
3. Deionized formamide is essential to resolve RNA correctly on polyacrylamide gel and can be purchased from most providers. For agarose gel deionized formamide is not required. Formamide is a moderate irritant and is hazardous for health. Handle using the necessary safety precautions.
4. Use ultrapure water with 18 MΩ cm resistivity at 25 °C unless stated otherwise.
5. All solutions/media should be autoclaved, unless stated otherwise.
6. Use standard microbiological practices required for the cultivation of noninfectious or low-risk microorganisms (Risk Group 1 applies for *H. volcanii* and *S. cerevisiae*). In other cases, apply specific additional safety measures related to the organism used and according to biological safety law. In case of doubt, contact your local biology safety officer. Ensure that glassware used for the cultivation of *H. volcanii* are well cleaned and do not contain residual traces of detergents/chemicals.
7. Order is critical to ensure proper solubility of the chemicals.
8. In this example, precultures must necessarily be cultivated in galactose containing medium to avoid premature depletion of proteins of interest in glucose containing medium. Otherwise, SCD medium containing 2% dextrose instead of galactose can be used.
9. 4-thiouracil is toxic at different concentrations depending on the organism used [36–38]. Make sure to determine the optimal amounts of uracil analog for the organism used. *H. volcanii* cells can tolerate up to 75% of thiouracil as uracil source (total concentration of uracil and analog 400 μM) [36]. In absence of uracil, uracil prototroph *S. cerevisiae* cells can tolerate up to 200 μM of thiouracil in 24 h cultures with only a 15% decrease on cell growth.

10. For best results keep all reagents free of RNases. If possible, keep glassware and materials separated and, set up a dedicated area for RNA work only. Keep the working place tidy.
11. Temperature is critical to allow correct phase separation. Do not use a cooled centrifuge since SDS will tend to precipitate.
12. For best results avoid phase contamination by collecting the upper phase using 3 fractions of equal volumes (3× 150 μL, 3× 120 μL, and 3× 100 μL, respectively).
13. To avoid high level of autofluorescence background, avoid staining the gel with staining reagents like ethidium bromide and SYBR Safe.

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