Chapter 30

RNA Tagging: Preparation of High-Throughput Sequencing Libraries

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Abstract

Protein–RNA networks, in which a single protein binds and controls multiple mRNAs, are central in biological control. As a result, methods to identify protein–RNA interactions that occur in vivo are valuable. The "RNA Tagging" approach enables the investigator to unambiguously identify global protein–RNA interactions in vivo and is independent of protein purification, cross-linking, and radioactive labeling steps. Here, we provide a protocol to prepare high-throughput sequencing libraries for RNA Tagging experiments.

Key words RNA Tagging, High-throughput sequencing, Protein–RNA interactions, Protein–RNA networks, RNA regulatory networks, Poly(U) polymerases, RNA, RNA-binding proteins

1 Introduction

Protein–RNA interactions underlie fundamental cellular functions. Single proteins often bind to hundreds of RNAs inside the cell to control when the RNAs are made, where they are located, when they are degraded, and what they do [1–3]. These "protein–RNA networks" have important roles in the activity of protein-coding genes, and thus underlie a diverse range of biological processes. As a result, methods to identify transcriptome-wide protein–RNA interactions in vivo are valuable and multiple strategies have been described, including several forms of CLIP (cross-linking and immunopurification) [4–9].

We developed a facile and unambiguous approach, called RNA Tagging, which enables an investigator to identify and analyze global protein–RNA interactions in vivo [10]. In the version of RNA Tagging described here, we express an RNA-binding protein (RBP) of interest fused to a poly(U) polymerase (PUP) . The RBP–PUP chimera covalently marks RNAs it binds in vivo with 3′ terminal uridines, which we refer to as a "U-tag." The U-tagged RNAs are then identified from a pool of total RNA extracted from

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the cell using high-throughput sequencing. For applications involving mRNA networks, we commonly purify poly(A)-containing RNAs to obtain an enriched pool of mRNAs, but this can be dispensed with to allow nonadenylated RNAs to be captured. The RNA Tagging strategy does not require cross-linking, protein purification, or radioactive-labeling steps. The data are unambiguous because the U-tags are directly detected via sequencing and cells are lysed under denaturing conditions, which ensures RNAs are U-tagged in vivo and not in the cell lysate. Furthermore, the approach highlights mRNAs that likely are regulated from those that likely are not. The TRIBE approach developed by McMahon et al. is conceptually similar and uses ADAR as the tagging agent [11].

We describe here a detailed protocol to prepare RNA Tagging high-throughput sequencing libraries. We first outline our protocol to isolate total RNA from *Saccharomyces cerevisiae* in denaturing conditions (Subheading 3.1) (Fig. 1). We next describe our protocols to enrich for polyadenylated RNAs using poly(A) selection (Subheading 3.2) and rRNA depletion (Subheading 3.3) steps. To enable transcriptome-wide analyses, we then G–I tail all RNAs (Subheading 3.4), which both captures U-tags and ensures all RNAs have a common sequence at their 3′ termini. Next, we selectively reverse transcribe U-tagged RNAs using a U-select oligo that preferentially hybridizes to U-tags and the G–I tail (Subheading 3.5), synthesize the second strand of DNA (Subheading 3.6), and PCR amplify and purify the DNA libraries (Subheading 3.7). At the conclusion of this protocol, users will have RNA Tagging libraries that are ready for analysis by high-throughput sequencing.

2 Materials

Use nuclease-free water to make all buffers. Test buffers to ensure they are RNase-free before use.

2.1 Total RNA Isolation from Yeast

1. Vortex.
2. Nuclease-free 1.75 mL microcentrifuge tubes.
3. Refrigerated general purpose centrifuge.
4. Refrigerated microcentrifuge.
5. UV-Vis spectrophotometer (e.g., NanoDrop).
6. Agarose gel electrophoresis setup.
7. −20 to −50 °C freezer, and −80 °C freezer.
8. Optional: bioanalyzer.
9. Ice cold water.

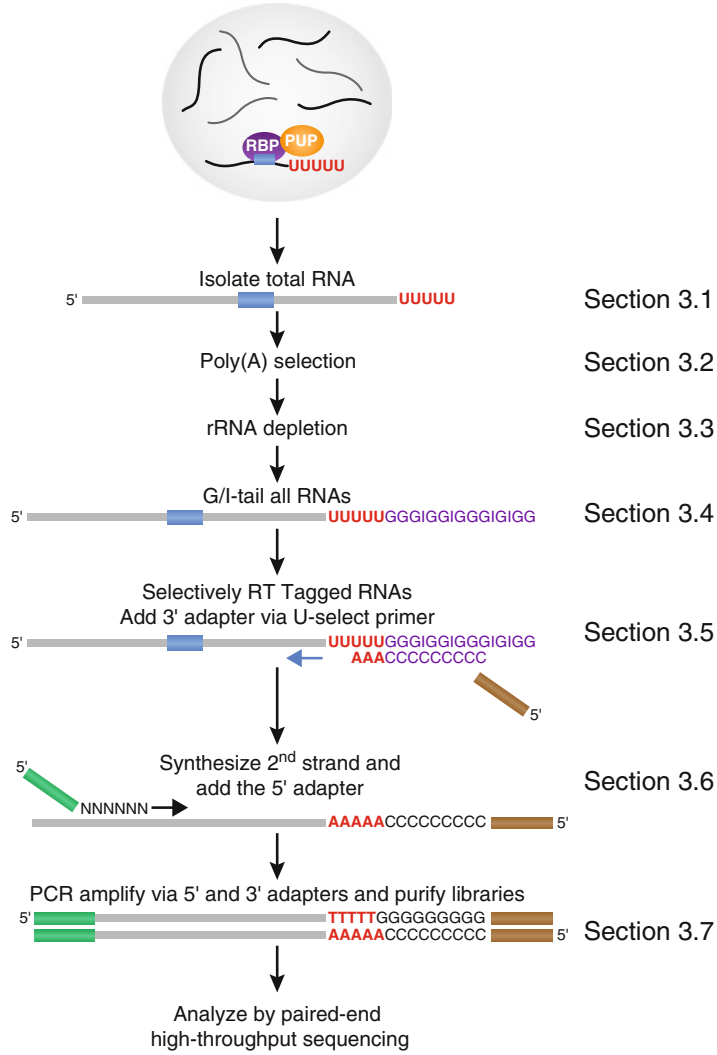

Fig. 1 Schematic of the RNA Tagging library preparation workflow. Adapted from Fig. 1 in Lapointe et al. [10]

10. 425–600 μm acid-washed glass beads.
11. Phenol–chloroform–isoamyl alcohol (25:24:1) pH 6.7.
12. Chloroform.
13. 100% ethanol.
14. 80% ethanol.
15. TURBO DNaseI.
16. Nuclease-free water.
17. RNA Purification kit (e.g., GeneJET, ThermoFisher Scientific).

18. RNA ISO Buffer: 0.2 M Tris–HCl pH 7.5, 0.5 M NaCl, 0.01 M EDTA, 1 v/v % SDS. Filter-sterilize before first use (0.2 μm filter), and store at room temperature (*see* **Note 1**).

2.2 Poly(A) Selection

1. Adjustable temperature thermomixer.
2. Magnetic stand for 1.75 mL microcentrifuge tubes.
3. Dynabeads mRNA purification kit (ThermoFisher Scientific).

2.3 rRNA Depletion

1. Adjustable temperature thermomixer.
2. Nuclease-free 1.75 mL microcentrifuge tubes.
3. Thermocycler (0.2 mL tube volume).
4. Ribo-Zero Magnetic Gold kit for yeast (Epicentre/Illumina) (*see* **Note 2**).
5. Agencourt RNAclean XP beads (Beckman Coulter).
6. 80% ethanol (*see* **Note 3**).

2.4 G–I Tailing of RNA

1. Nuclease-free 0.2 mL PCR strip tubes.
2. Refrigerated microcentrifuge.
3. Yeast poly(A) polymerase (PAP) (Affymetrix, 74225Y).
4. 3 M sodium acetate (NaOAc) pH 5.5.
5. 15 mg/mL GlycoBlue.
6. G-I tailing master mix (per sample): 4 μL 5× PAP Reaction buffer, 2 μL nuclease-free water, 1 μL 10 mM GTP, and 1 μL 3.3 mM ITP.

2.5 U-Select Reverse Transcription

1. Reverse transcriptase (e.g., SuperScript III ThermoFisher Scientific).
2. Ribonuclease H (e.g., ThermoFisher Scientific).
3. PCR Purification kit (e.g., GeneJET, ThermoFisher Scientific).
4. 1 μM U-select oligo 5′- *GCCTTGGCACCCGAGAATTCCA*CCCCCCCCCCAAA-3′.

   The three adenosines on the 3′ end of the U-select oligo preferentially anneal to RNAs that end in uridines (prior to the G/I-tailing), thus selectively enriching for U-Tagged RNAs. The nine cytosines anneal to the G–I tail. The underlined portion corresponds to Illumina 3′ adapter sequence vital for PCR amplification.
5. RT master mix (per sample): 4 μL 5× SuperScript III Reaction Buffer, 1 μL 100 mM DTT, and 1 μL RNaseOUT (e.g., ThermoFisher Scientific).

2.6 Second Strand Synthesis of DNA

1. 5 U/μL Exo-Klenow Fragment DNA Polymerase I.
2. 80% ethanol (*see* **Note 3**).

3. Second strand synthesis oligo 5′-GTTCAGAGTTCTACAGTCCGACGATCNNNNNN-3′.

   The underlined portion corresponds to Illumina 5′ adapter sequence vital for PCR amplification.
4. 10× Klenow Buffer: 500 mM Tris–HCl pH 7.5, 100 mM $MgCl_2$, 10 mM DTT, and 0.5 mg/mL BSA. Store buffer at −20 °C.
5. S3 master mix (per sample):12 μL nuclease-free water, 10 μL 10× Klenow Buffer 5 μL 10 mM dNTPs, and 10 μL 10 μM second strand synthesis oligo.

2.7 PCR Amplification and Size-Selection of Libraries

1. GoTaq Green 2× PCR Master Mix (Promega).
2. 10 μM 5′ PCR primer

   5′-*AATGATACGGCGACCACCGAGATCTACACGTTCAGAGTTCTACAGTCCGA-3′*
3. 10 μM 3′ Barcoded PCR Primer

   5-*CAAGCAGAAGACGGCATACGAGAT**NNNNNN**GTGACTGGAGTTCCTTGGCACCCGAGAATTCCA-3′*. The ***NNNNNN*** represents the unique barcode. Use a unique barcode for each sample.
4. 80% ethanol (*see* **Note 3**).

3 Methods

This protocol requires that the relevant RBP–PUP fusion proteins already be engineered and introduced into cells. Upon request, we provide a kit that includes plasmids that encode the PUP-2 open-reading frame, free of charge to any academic lab.

3.1 Isolate Total RNA from S. cerevisiae

Isolate total RNA from yeast in completely denaturing conditions (*see* **Note 4**).

Timing: **steps 3–21**: 1.5–2 h; **steps 22–31**: 3–4 h.

1. Place 25 mL of A_{660} ~ 0.5–0.8 cultures on ice for 5 min.
2. Harvest cultures by centrifugation at 1900 rcf for 5 min at 4 °C.
3. Wash yeast pellets once with 40 mL of ice-cold water.
4. Resuspend yeast in 500 μL RNA ISO buffer.
5. Add ~200 μL of acid-washed beads.
6. Add 500 μL of phenol–chloroform–isoamyl alcohol.
7. Vortex for 20 s at room temp., then incubate for 20 s on ice. Repeat for a total of ten cycles.
8. Split into two tubes. Each sample is now in two tubes.

9. Add 375 µL RNA ISO buffer and 375 µL PCA.
10. Mix gently by several inversions.
11. Spin at max speed (16,100 × *g*) at 4 °C for 10 min.
12. Transfer aqueous phase to new tube.
13. Add equal volume PCA and mix gently.
14. Spin at max speed at 4 °C for 10 min.
15. Remove aqueous phase to new tube.
16. Add equal volume chloroform and mix gently.
17. Spin at max speed at 4 °C for 10 min.
18. Remove aqueous phase to new tube.
19. Add 1 mL of 100% ethanol, mix gently. Incubate for at least 1 h at −50 °C (*see* **Note 5**).
20. Spin at max speed at 4 °C for 20 min. Remove supernatant.
21. Wash pellet at least once in 80% ethanol (*see* **Note 6**).
22. Resuspend RNA pellets in 43 µL of nuclease-free water.
23. Combine the two tubes for each sample (back to one tube per sample), add 10 µL of 10× TURBO DNase Buffer and 4 µL (8 U) of TURBO DNase.
24. Incubate for 1 h at 37 °C (*see* **Note 7**).
25. Clean reactions with an RNA Purification kit (e.g., using the "RNA cleanup protocol" of the GeneJET kit).
26. Elute in 30 µL of nuclease-free water.
27. Determine the concentration of total RNA (*see* **Note 8**).
28. Analyze isolated RNA by agarose gel electrophoresis to assess RNA quality. Load 500 ng of total RNA (*see* **Note 9**).
29. Store RNA at −80 °C until next step.

3.2 Poly(A) Selection

Many small, noncoding RNAs end in several uridine residues. To decrease the number of these "background" RNAs in our libraries, we enrich for polyadenylated RNAs (*see* **Note 10**). This step can be omitted, but the resulting libraries will be primarily composed of small noncoding RNAs, such as SCR1 (≈90% of the sample). Thus deeper sequencing (larger numbers of reads) will be required to identify the complete collection of U-tagged RNAs.

Timing: ~1 h.

1. Prior to starting, let the aliquots of beads and buffers warm to room temp (~15 min), slow thaw RNAs on ice (15–20 min), and set a thermomixer to 65 °C.
2. Use 75 µg of total RNA per sample and adjust volume to 100 µL using nuclease-free water.

3. Heat RNA solution to 65 °C in the thermomixer for 2 min to disrupt secondary structures.
4. Place samples on ice until use in **step 9**.
5. Set the thermomixer to 70 °C.
6. Transfer 200 μL of well-resuspended Dynabeads to a microcentrifuge tube.
7. Place tube on magnet. Discard supernatant.
8. Add 100 μL of Binding Buffer to calibrate the beads. Mix well.
9. Place tube back on magnet and discard the supernatant.
10. Add 100 μL of Binding Buffer to the beads. Mix well.
11. Add the RNA (from **step 3**) to the bead solution. It's important to have a 1:1 ratio of RNA volume to Binding Buffer volume.
12. Mix beads thoroughly.
13. Gently vortex samples briefly every 30 s for 5 min at room temp.
14. Place tube on magnet to separate beads. Remove the supernatant.
15. Wash with 200 μL Washing Buffer B. Mix thoroughly.
16. Place tube on magnet. Remove all of the supernatant.
17. Repeat **steps 15–16**.
18. After removing all of the supernatant from the second wash, add 28 μL of water.
19. Resuspend the beads well.
20. Heat the samples to 70 °C in the thermomixer for 2 min.
21. Place the samples on the magnet *immediately*.
22. Collect the supernatant. This is your poly(A)+ mRNA.

3.3 rRNA Depletion

The poly(A) selection efficiently removes small, noncoding RNAs (such as snRNAs and SCR1) but there is bleed through of rRNAs. Thus, we use this step to remove the remaining rRNAs (*see* **Note 10**). This step can be omitted, but the resulting libraries will be primarily composed of rRNAs (90–95% of the sample).

Timing: ~2 h.

1. *Prior to starting:* let the magnetic beads and associated buffers warm to room temperature (~15 min), slow thaw reagents stored at −80 °C on ice (15–20 min), set a thermocycler to 68 °C, set a thermomixer to 50 °C, and aliquot the needed volume of RNA Clean XP beads (*see* Subheading 3.3.4) and store them at room temp until use in Subheading 3.3.4 (*see* **Note 11**).

3.3.1 Bead Washing

1. For each reaction, pipet 225 μL of Ribo-Zero magnetic beads into a 1.75 mL centrifuge tube. Pipet slowly to avoid air bubbles. Store the unused beads at 4 °C.
2. Place tubes on magnetic stand for >1 min.
3. Remove and discard the supernatant.
4. Remove the tube from the stand and add 225 μL of water to each tube. Mix well by repeated pipetting or vortexing at medium speed.
5. Repeat **steps 2–4**. Remove the tube from the stand. Add 65 μL of Magnetic Bead Resuspension Solution to each tube. Mix well by pipetting or vortexing on medium speed.
6. Add 1 μL of RiboGuard RNase Inhibitor and mix briefly by vortexing.
7. Keep the tubes at room temp until needed in Subheading 3.3.3.

3.3.2 Treatment of Total RNA with Ribo-Zero rRNA Removal Solution

1. For each sample, combine the following in an RNase-free 0.2 mL PCR strip-tube (volumes reflect 1 reaction):

4 μL	Ribo-Zero Reaction Buffer
26 μL	poly(A)+ RNA
10 μL	Ribo-Zero rRNA Removal Solution

2. Gently mix the reaction by pipetting and incubate at 68 °C for 10 min in a thermocycler.
3. Store the rest of the unused kit at −80 °C.
4. Remove the reaction tube and incubate at room temperature for 5 min.

3.3.3 Magnetic Bead Reaction and rRNA Removal (See Note 12)

1. Using a pipette, add the treated RNA sample to the washed magnetic beads and *immediately* mix by pipetting at least ten times to thoroughly mix the sample. Then, vortex the tube at medium setting for 10 s and place at room temperature.
2. Incubate the samples at room temperature for 5 min.
3. Then, mix the reactions by vortexing at medium speed for 10 s and place at 50 °C for 5 min. Avoid any significant condensation during this step (e.g., make sure the cover for the thermomixer is on the instrument during the incubation to keep the lids of the tubes exposed to warm air).
4. Remove the tubes from the 50 °C heat block and place on a magnetic stand for >1 min.
5. While on the stand, carefully remove the supernatant (*this is your rRNA-free RNA!)* and place in a labeled, RNase-free tube.

6. If there are residual beads in the supernatant, repeat the magnetic separation.
7. Place the RNA on ice and immediately proceed to RNA cleanup.

3.3.4 Agencourt RNAClean XP Bead Mediated RNA Cleanup

1. Mix the Agencourt RNAClean XP beads well by vortexing.
2. Add 160 μL of the mixed beads to each reaction containing 85–90 μL of rRNA-depleted sample. Mix thoroughly by pipetting >10 times. Vortex gently.
3. Incubate at room temperature for 15 min. During the incubation prepare a fresh 80% ethanol solution.
4. Place the tube on a magnetic stand for >5 min.
5. Remove the supernatant without disturbing the beads.
6. With the tube still on the stand, add 400 μL of fresh 80% ethanol without disturbing the beads.
7. Incubate at room temperature for 1 min.
8. Remove the ethanol supernatant.
9. Repeat the 80% ethanol wash for a total of two wash steps.
10. Allow the tube to air dry on the magnetic stand (*see* **Note 13**).
11. Add 12 μL of RNase-free water to the tube and immediately and thoroughly mix.
12. Incubate the tubes at room temperature for 2 min.
13. Place the tubes on the magnetic stand for at least 5 min. Transfer the clear supernatant to a new tube, always leaving 1–2 μL behind to prevent carryover of the beads to the next steps.

3.4 G-I Tailing of RNA

This protocol adds a known sequence to the 3′ end of all RNAs that can be exploited to reverse transcribe the RNA (*see* **Note 14**).

Timing: **steps 1–22**: ~2.5 h; **steps 23–28**: ~1 h.

1. For each sample, aliquot 8 μL of the G-I tailing master mix into nuclease-free 0.2 mL PCR strip-tubes.
2. For each sample, add 10 μL of the appropriate poly(A)+/rRNA-depleted RNA and mix.
3. Add 2 μL of 600 U/μL Yeast PAP to each reaction.
4. Incubate at 37 °C for 90 min.
5. Add an additional 2 μL of 600 U/μL Yeast PAP to each reaction.
6. Incubate at 37 °C for 30 min.
7. Add 80 μL of nuclease-free water to each reaction (volume should now be ~100 μL).

8. Transfer reactions to 1.75 mL microcentrifuge tubes.
9. Add 100 µL of phenol–chloroform–isoamyl alcohol (25:24:1) pH 6.7 to each reaction.
10. Gently vortex or mix thoroughly.
11. Spin at max speed for 5 min at 4 °C.
12. Collect the aqueous phase (top layer, aim for 95–100 µL) and transfer to a new tube (*see* **Note 15**).
13. Add an equal volume of chloroform.
14. Gently vortex or mix thoroughly.
15. Spin at max speed for 5 min at 4 °C.
16. Collect the aqueous phase (top layer, aim for 90 µL) and transfer to a new tube.
17. Add 10 µL of 3 M NaOAc, 1 µL of 15 mg/mL GlycoBlue, and 500 µL of 100% ethanol.
18. Mix thoroughly.
19. Incubate for at least 1 h at −50 °C (*see* **Note 5**).
20. Spin at max speed for 25 min at 4 °C.
21. Remove supernatant. Wash pellet with 70–80% ethanol.
22. Spin at max speed for 25 min at 4 °C.
23. Remove supernatant.
24. Pulse-spin and remove residual ethanol.
25. Resuspend pelleted RNA in 10 µL of nuclease-free water.

3.5 U-select Reverse Transcription

Use the G-I nucleotides on the 3′ end of the RNA to prime reverse transcription via the U-select oligo. The three adenosines on the 3′ end of the U-select oligo preferentially anneal to RNAs that end in uridines (prior to the G/I-tailing), thus selectively enriching the U-Tagged RNAs. The U-select oligo also contains Illumina 3′ adapter sequence (underlined).

Timing: ~2.5 h

1. *Recommended*: Also prepare –RT reactions for each sample as a comparison.
2. Assemble the following reaction in strip tubes (volumes reflect one reaction):

1 µL	1 µM U-select oligo
5 µL	G-I-tailed RNA
1 µL	10 mM dNTP mix
6 µL	nuclease-free water

3. Heat the reactions and the RT master mix to 65 °C for 5 min.

4. Cool the reactions and the RT master mix to 50 °C for 5 min.

   Important: Perform steps 5 and 6 while the RNA/primer mix and the RT master mix are in the thermomixer. It's important to keep the reactions at 50 °C to maintain the U-selection.

5. Add 6 μL of the preheated (50 °C) RT master mix in the thermocycler to each reaction.
6. Add 1 μL of 200 U/μL SuperScript III reverse transcriptase to each reaction.
7. Incubate at 50 °C for 60 min.
8. Incubate at 85 °C for 5 min.
9. Cool reactions to 4 °C.
10. Add 1 μL RNase H to each reaction.
11. Incubate at 37 °C for 20 min.
12. Add 80 μL of water to increase reaction volume to ~100 μL.
13. Clean cDNA using the GeneJET PCR Purification kit. (We do not add isopropanol.)
14. Add 32 μL nuclease-free water to the dry column.
15. Incubate the water on the column for a least 2 min at room temperature.
16. Centrifuge at max speed for 2 min.
17. Repeat **steps 14–16**.
18. Combine elution fraction to get ~60 μL of cDNA for each reaction.

3.6 Second Strand Synthesis

Randomly synthesize the second strand of DNA that is complementary to the cDNA sequence, while at the same time adding the Illumina 5′ adapter sequence.

Timing: ~2 h

1. *Prior to starting:* aliquot the required volume of Agencourt RNAClean XP beads and keep them at room temperature until use.
2. Aliquot 37 μL of S3 master mix for each reaction into 0.2 mL nuclease-free PCR strip tubes.
3. Add 60 μL purified cDNA to the S3 master mix for each sample.
4. Add 3 μL of 5 U/μL Exo-Klenow fragment DNA polymerase I to each reaction.
5. Incubate at 37 °C for 30 min.
6. Cool to 4 °C.
7. Warm reactions to room temperature (*see* **Note 16**).
8. Mix the Agencourt RNAClean XP beads well by vortexing.

9. Add 100 μL of the mixed beads to each 100 μL of sample (*see* **Note 17**).
10. Mix thoroughly by pipetting >10 times. Vortex gently.
11. Incubate at room temperature for 15 min. During the incubation prepare a fresh 80% ethanol solution.
12. Place the tube on a magnetic stand for >5 min.
13. Remove the supernatant without disturbing the beads.
14. With the tube still on the stand, add 400 μL of fresh 80% ethanol without disturbing the beads.
15. Incubate at room temperature for 1 min while still on the magnetic stand.
16. Remove the ethanol supernatant.
17. Repeat the 80% ethanol wash for a total of two wash steps.
18. Allow the tube to air dry on the magnetic stand (*see* **Note 13**).
19. Add 100 μL of nuclease-free water to the tube and immediately and thoroughly mix.
20. Incubate the tubes at room temperature for 2 min.
21. Place the tubes on the magnetic stand for 5 min. Transfer the clear supernatant to a new tube, always leaving 1–2 μL behind to prevent carryover of the beads to the next steps.
22. Repeat **steps 8–18**.
23. Add 50 μL of nuclease-free water to the tube and immediately and thoroughly mix.
24. Incubate the tubes at room temperature for 2 min.
25. Place the tubes on the magnetic stand for 5 min. Transfer the clear supernatant to a new tube, always leaving 1–2 μL behind to prevent carryover of beads to the next steps.

3.7 PCR Amplification and Purification

Amplify the dsDNA and add the remaining 5′ and 3′ Illumina adapter sequences for subsequent high-throughput sequencing. The PCR purification step efficiently removes adapter–adapter products (5′ Illumina adapter–3′ Illumina adapter with no RNA insert) that will preferentially sequence.

Timing: ~2.5 h.

1. Assemble the following reaction for each sample:

83.3 μL	2× GoTaq Master Mix
6.7 μL	10 μM 5′ PCR primer
6.7 μL	10 μM 3′ barcoded PCR primer
20 μL	Nuclease-free water
50 μL	Purified cDNA

2. Aliquot 20 μL of the PCR reaction mix into eight separate 0.2 mL nuclease-free PCR tubes.
3. Amplify via the following protocol (*see* **Note 18**):
   (a) 94 °C: 2 min.
   (b) 94 °C: 10 s.
   (c) 40 °C: 2 min.
   (d) 72 °C: 1 min.
   (e) Go to **step b** once.
   (f) 94 °C: 10 s.
   (g) 55 °C: 30 s.
   (h) 72 °C: 1 min.
   (i) Go to **step f** seven times.
   (j) 94 °C: 15 s.
   (k) 55 °C: 30 s.
   (l) 72 °C: 1 min.
   (m) Go to **step j** fourteen times.
   (n) 72 °C: 5 min.
   (o) 4 °C: pause.
4. Warm Agencourt RNAClean XP beads and PCR samples to room temperature before proceeding (≈15 min).
5. Combine individual PCRs for each sample into a single 1.75 mL microcentrifuge tube (*see* **Note 19**).
6. Measure the volume of each sample using a pipette.
7. Mix the Agencourt RNAClean XP beads well by vortexing.
8. Add 0.8 volumes (relative to sample volume) of the pre-warmed, mixed beads to each sample (*see* **Note 20**).
9. Mix thoroughly by pipetting >10 times. Vortex gently.
10. Incubate at room temperature for 15 min. During the incubation prepare a fresh 80% ethanol solution.
11. Place the tube on a magnetic stand for >5 min.
12. Remove the supernatant without disturbing the beads.
13. With the tube still on the stand, add 400 μL of fresh 80% ethanol without disturbing the beads.
14. Incubate at room temperature for 1 min while still on the magnetic stand.
15. Remove the ethanol supernatant.
16. Repeat the 80% ethanol wash for a total of two wash steps.
17. Allow the tube to air dry on the magnetic stand (*see* **Note 13**).
18. Add 100 μL of nuclease-free water to the tube and immediately and thoroughly mix.

19. Incubate the tubes at room temperature for 2 min.
20. Place the tubes on the magnetic stand for 5 min. Transfer the clear supernatant to a new tube, always leaving 1–2 μL behind to prevent carryover of the beads to the next steps.
21. Repeat **steps 6–17**.
22. Add 15 μL of nuclease-free water to the dried beads and immediately and thoroughly mix.
23. Incubate the tubes at room temperature for 2 min.
24. Place the tubes on the magnetic stand for 5 min.
25. Transfer the clear supernatant to a new tube, always leaving 1–2 μL behind to prevent carryover of the beads to the next steps.
26. Store reactions at −80 °C until submission for high-throughput sequencing.
27. Analyze 2–4 μL of the libraries on a 1% agarose gel to check library quality. *See* Fig. 2 for an image of our analysis with an example library.
28. Optional: Topo–Cloning analysis of initial library preps is recommended to ensure libraries are constructed correctly.
29. Analyze libraries by paired-end high-throughput sequencing.

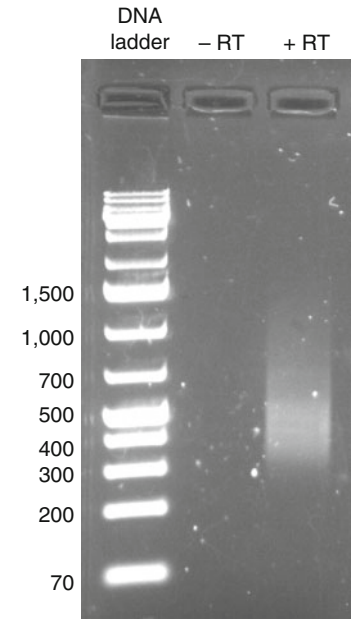

Fig. 2 Image of ethidium bromide-stained agarose gel that shows an RNA Tagging library ready for analysis via high-throughput sequencing. The "+RT" lane is the sample, and the "−RT" lane is the negative control that lacked reverse transcriptase. The numbers to the left of the DNA ladder lane indicate the size of the band (nucleotides)

4 Notes

1. The SDS in the buffer may precipitate at room temperature. If it does, heat the buffer very briefly in a 37 °C water buffer until the solution is clear.
2. For non-yeast experiments, substitute the yeast rRNA depletion kit with an rRNA depletion kit appropriate for the desired organism.
3. Prepare fresh 80% ethanol solution prior to each use.
4. If not using *S. cerevisiae*, proceedYeast directly to the poly(A) selection Subheading 3.2. Ensure total RNA was isolated in denaturing conditions.
5. If desired, an overnight incubation at −50 °C works well as a stopping point.
6. Two washes with 80% ethanol work well.
7. We do this in 1.75 mL microcentrifuge tubes in a 37 °C incubator.
8. We analyze 1:10 dilutions of total RNA using a NanoDrop spectrophotometer.
9. Optionally, total RNA can be analyzed using a BioAnalyzer.
10. The poly(A) selection protocol is essentially done as recommended by the manufacturer.
11. Mix the Agencourt RNAClean XP beads very well prior to aliquoting. Also, pipette slowly to help avoid air bubbles.
12. Always add the treated RNA sample to the washed magnetic beads and immediately mix by pipetting. Immediate and thorough mixing prevents the beads from forming clumps that can significantly impact rRNA removal efficiency.
13. The beads shouldn't over dry, but make sure there is no ethanol remaining in the tube. The elution in the next step is only 12 μL, so even very small volumes of ethanol could negatively impact the downstream enzymatic reactions. We typically add the water once the beads go from glossy to more matte-like appearance, just before they start cracking (thin white lines). It typically takes about 5 min. According to the manufacturer, overdrying reduces elution efficiency.
14. The G/I-tailing step can be replaced by a 3′ ligation step.
15. The aqueous phase will likely have a white, cloudy precipitate. Do not worry about it. Just collect as much of the aqueous phase as possible, even if it includes the precipitate. The precipitate will disappear once the second extraction is complete.
16. We let the cooled reactions sit on a lab bench for 5 min.

17. Use a 1:1 bead-to-reaction ratio (by volume) to efficiently remove the second strand synthesis oligo.
18. We most often run this PCR protocol overnight. A standard cycling protocol (94 °C for 10 s; 55 °C for 30 s; 72 °C 1 min; repeated 25 times) would likely work fine, too.
19. Add the eight PCR reactions per sample into a single 1.75 mL tube, but keep each of the samples in separate 1.75 mL tubes. There should be 140–150 μL per sample.
20. For example, add 120 μL of beads to 150 μL of sample.

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