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Purification of Transcript-Specific mRNP Complexes Formed In Vivo from *Saccharomyces cerevisiae*

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Abstract

RNA binding proteins play critical roles in shaping the complex life cycle of cellular transcripts. For most RNAs, the association with a distinct complement of proteins serves to orchestrate its unique pattern of maturation, localization, translation, and stability. A key aspect to understanding how transcripts are differentially regulated lies, therefore, in the ability to identify the particular repertoire of protein binding partners associated with an individual transcript. We describe here an optimized experimental procedure for purifying a single mRNA population from yeast cells for the characterization of transcript-specific mRNA-protein complexes (mRNPs) as they exist in vivo. Chemical cross-linking is used to trap native mRNPs and facilitate the co-purification of protein complexes associated with an individual transcript population that is captured under stringent conditions from cell lysates through hybridization to complementary DNA oligonucleotides. The resulting mRNP is highly enriched and largely devoid of non-target transcripts, and can be used for a number of downstream analyses including protein identification by mass spectrometry.

Keywords

RNA; RNA binding proteins; mRNA; mRNP; Affinity capture; Mass spectrometry; Yeast

1 Introduction

The posttranscriptional fate of an mRNA is strongly influenced by its protein binding partners. Indeed, RNA binding proteins (RBPs) play critical roles in mediating and regulating the processing, transport, localization, translation, and decay of all cellular protein-coding transcripts [1, 2]. Given their importance, significant efforts have been made over the years to experimentally identify RBPs and their targets, and to understand the physiological function of mRNA-protein complexes (mRNPs) in the life cycle of RNA.

In general, two approaches have been employed to isolate RNA-protein complexes (reviewed in [3, 4]). The first uses immunoprecipitation to collect a specific RBP of interest from cell lysates and the RNAs that co-purify with it. In this method, it is advantageous to covalently cross-link RNA-protein complexes (by treating intact cells with either formaldehyde or UV light) to increase the capture of proteins that associate with the target RNA only transiently and to minimize protein re-association during the purification that results in interactions that do not reflect bona fide in vivo mRNPs [5, 6]. When coupled with next-generation sequencing technologies, this approach can be used to identify targets of a

known RBP at the level of the entire transcriptome and map the binding position along the RNA at near-nucleotide resolution [7, 8]. In the second method, referred to generally as RNA capture, an RNA of interest is targeted and retrieved from cell lysates for the purpose of co-purifying and characterizing the novel RBPs that interact with it. For the isolation of RNA-protein complexes that exist in vivo, the target RNA is typically expressed as a transgene harboring sequence tags within its 3' UTR that bind with high affinity to either a protein (e.g., bacteriophage MS2 coat protein) or small molecule (e.g., tobramycin or streptavidin) that can be conjugated to a resin or magnetic bead [9–13]. The global and unbiased identification of proteins that co-purify with the target RNA can then be achieved using quantitative or nonquantitative mass spectrometry.

Recent advances in RNA capture methodologies have employed hybridization to complementary oligonucleotides as a means to affinity capture endogenous RNA targets and circumvent the need to ectopically express tagged transgenes. In addition, a cross-linking step is often added to allow purification under denaturing conditions so as to isolate native interactions and enhance specificity in the RNA capture. Using these additions, total cellular polyA⁺ RNA and its directly associated proteins have been purified through hybridization to oligo-d(T) for the systematic identification of mRNA-interacting proteins in a number of cell types [14–20]. In addition, mammalian long noncoding RNA *Xist* has been captured using antisense oligonucleotides to reveal novel protein interacting partners critical for its function in transcriptional silencing of the X chromosome [21, 22]. These studies clearly demonstrate the utility of purifying RNA-protein complexes that exist on distinct transcript populations as a means to unravel the function or events that contribute to the unique cellular lifetime of the RNA.

Here, we describe the application of RNA affinity capture in the purification of a single mRNA population from yeast cell lysates for the isolation of a transcript-specific mRNP complex as it exists in vivo. Importantly, growing cells are treated in situ with limiting formaldehyde to mediate formation of chemical cross-links between protein-RNA and protein-protein complexes as they are assembled in vivo [23]. This treatment traps highly dynamic and transient interactions that exist between proteins and the RNA target, enables the capture of proteins that associate both directly and indirectly (such as those from multi-subunit protein complexes) with the transcript, and allows for purification of native mRNP complexes under highly stringent and denaturing conditions. Target RNA is purified from whole cell lysates through hybridization to DNA oligonucleotides with complementarity to various regions along the transcript and which are biotinylated and immobilized to streptavidin-conjugated magnetic beads. Purified mRNPs are highly enriched for the RNA target (~10,000-fold, as demonstrated by northern blot analysis and quantitative RT-PCR) and protein components can be identified either individually by SDS-PAGE and western blot or globally by mass spectrometry (Fig. 1). We recently utilized this approach to identify proteins that associate with an mRNA targeted to nonsense-mediated mRNA decay (NMD) compared to a matched NMD-insensitive mRNA [24]. We found ~100 proteins associated with the control transcript—a majority of which are polyA⁺ RNA binding proteins defined in recent global studies [16, 18]. Critically, we found ~50 proteins enriched exclusively on the NMD target, including UPF1, a key component of the NMD machinery [24]. This method offers a facile approach to purify transcript-specific mRNP complexes from yeast

cells for the identification and characterization of RBPs that contribute to regulating the cellular fate of that RNA.

2 Materials

2.1 Cell Growth, Chemical Cross-Linking, and Harvesting

(see Note ¹).

1. Synthetic Dextrose (SD) media with appropriate amino acids supplemented: 0.17% Bacto-Yeast Nitrogen Base, 0.5% ammonium sulfate, 2% dextrose, 0.2% Amino Acid Dropout Mix (see item 2 below), pH to 6.5 with 10 N NaOH. Sterilized by autoclave.
2. Amino Acid Dropout Mix: Combine the following powder reagents: 0.25 g adenine: 1 g alanine: 1 g arginine: 1 g asparagine: 1 g aspartic acid: 1 g cysteine: 1 g glutamine: 1 g glutamic acid: 1 g glycine: 1 g inositol: 1 g isoleucine: 1 g lysine: 1 g methionine: 1 g para-aminobenzoic acid: 1 g phenylalanine: 1 g proline: 1 g serine: 1 g threonine: 1 g tyrosine: 1 g valine: 1 g uracil: 1 g histidine: 1 g tryptophan: 5 g leucine. Omit amino acid(s) as required for the maintenance of a plasmid-borne auxotrophic marker (see Note ²). Mix thoroughly using a mortar and pestle.
3. Appropriate yeast strain (see Note ³).
4. 30 °C shaker/incubator.
5. Visible light spectrophotometer capable of reading at 600 nm λ and cuvettes.
6. Formaldehyde (37% stock).
7. 2.5 M glycine. Sterilized by autoclave.
8. Clinical centrifuge (or similar) capable of processing sample volumes of 200 mL at speeds of $3200 \times g$.
9. 2.0 mL Eppendorf tubes.
10. Microcentrifuge capable of processing 2.0 and 1.5 mL Eppendorf tubes at speeds of up to $16,100 \times g$.
11. Dry ice.

¹Media, growth conditions, and lysis method are specific for the budding yeast, *S. cerevisiae*. Cell-type-specific methods could be substituted, in principle, but should be tested for compatibility.

²In the established system, cells expressed a heterologous *GFP* reporter from a high-copy plasmid also harboring *URA3* (complementing the uracil auxotrophy of the yeast strain). Thus, uracil was omitted from the Amino Acid Dropout Mix.

³The optimized protocol was performed using a yeast strain lacking three vacuolar proteases (*MATa*, *ade2-1*, *ura3*, *leu2*, *his3*, *pep4* ::*HIS3*, *prb1* ::*his3*, *prc1* ::*hisG*) to reduce protein degradation during the procedure (see Note 20). Target mRNA can be expressed from its endogenous chromosomal locus, or, alternatively, from a high-copy plasmid (2 μ origin of replication and harboring a gene marker for plasmid maintenance) to enhance mRNA expression and mRNP yield. In order to identify protein components specifically bound to the target mRNA, mRNP purification should also be performed on cells lacking the target transcript, such as those with the endogenous gene locus deleted or lacking the plasmid-encoded copy of the target gene. Alternatively, for essential genes, the use of a repressible promoter could be used to inhibit target expression. We have found that performing the mRNP purification protocol using cells that do not express target mRNA (i.e., no plasmid control) yielded negligible protein contamination as determined by mass spectrometry.

12. -80°C freezer (for storage of samples [cell pellets, lysates, RNA, or protein]).

2.2 Cell Lysis

1. RNase-free dH_2O (*see Note* ⁴).
2. $1\times$ Lysis Buffer: 10 mM Tris-HCl pH 7.4, 100 mM NaCl, 30 mM MgCl_2 , protease inhibitor. Prepared using RNase-free dH_2O .
3. 0.5 M dithiothreitol (DTT).
4. Sterile glass beads: 0.5 mm diameter.
5. Vortexer housed at 4°C .
6. 18 gauge needles.
7. Bunsen burner or similar heat source.
8. 15 mL conical bottom centrifuge tubes.
9. Clinical centrifuge (or similar) capable of processing 15 mL conical bottom centrifuge tubes at $1600\times g$, housed at 4°C .
10. 1.5 mL Eppendorf tubes.
11. 2.0 mL Eppendorf tubes.
12. Microcentrifuge capable of processing 2.0 and 1.5 mL Eppendorf tubes at speeds of up to $16,100\times g$.
13. UV spectrophotometer and quartz cuvettes, capable of reading at OD_{260} and OD_{280} (optional).

2.3 Magnetic Bead Preparation

1. MyOne Streptavidin C1 Dynabeads and magnet (*see Note* ⁵).
2. 1.5 mL Eppendorf tubes.
3. $1\times$ Binding and Washing Buffer ($1\times$ B&W Buffer): 5 mM Tris-HCl pH 7.5, 0.5 mM EDTA pH 8.0, 1 M NaCl. Prepared using RNase-free dH_2O .
4. Biotinylated oligonucleotides (*see Note* ⁶): custom sequence-specific DNA oligonucleotides, with a $3'$ biotinylation modification. Resuspended to 400 pmol/ μL using RNase-free dH_2O .

⁴It is critical to use RNase-free dH_2O for all reagents throughout the purification protocol beginning at cell lysis.

⁵Additional magnetic Dynabeads options were tested (Fig. 2a). The MyOne Streptavidin C1 Dynabeads demonstrated the highest binding capacity for this purification scheme (Fig. 2b).

⁶DNA oligonucleotides complementary to the target transcript should be individually tested for their ability to hybridize to and capture the RNA target. The recommended design for an oligonucleotide includes ~25 nucleotides of complementarity to the RNA of interest with a melting temperature of $\sim 55^{\circ}\text{C}$, a 15 nucleotide linker with no significant complementarity to genomic regions in the organism of interest, and a $3'$ biotinylation modification. Oligonucleotides should be evaluated in silico to ensure that no substantial intramolecular secondary structure can form. We have observed that, similar to PCR primers or probes, not all complementary oligonucleotides are equally effective, even when the above guidelines are followed. Thus, it is highly recommended that each oligonucleotide be tested individually for the ability to purify the RNA species of interest. The combination of several oligonucleotides with distinct regions of complementarity to the target is likely to be the most effective at depleting the RNA from the lysate (Fig. 3a, b).

5. 2× Binding and Washing Buffer (2× B&W Buffer): 10 mM Tris–HCl pH 7.5, 1 mM EDTA pH 8.0, 2 M NaCl. Prepared using RNase-free dH₂O.
6. Tube rotator capable of end-over-end rotation.

2.4 Hybridization/Affinity Capture

1. TE Buffer: 10 mM Tris–HCl, 1 mM EDTA, pH to 8.0. Sterilized by autoclave.
2. 15 mL conical bottom centrifuge tubes.
3. 5 M LiCl. Sterilized by autoclave.
4. 10% SDS. Filter sterilized.
5. 0.5 M EDTA pH 8.0. Sterilized by autoclave.
6. M Tris–HCl pH 7.5. Sterilized by autoclave.
7. 100% Formamide.
8. Fungal protease inhibitor.
9. Tube rotator capable of end-over-end rotation.
10. 1.5 mL Eppendorf tubes.
11. Wash Buffer 1: 10 mM Tris–HCl pH 7.5, 1 mM EDTA pH 8.0, 250 mM LiCl, 0.1% SDS. Prepared using RNase-free dH₂O.
12. Wash Buffer 2: 10 mM Tris–HCl pH 7.5, 1 mM EDTA pH 8.0, 100 mM LiCl. Prepared using RNase-free dH₂O.
13. Microcentrifuge capable of processing 2.0 and 1.5 mL Eppendorf tubes at speeds of up to $16,100 \times g$.

2.5 Elution of Purified mRNP Material

1. RNase-free dH₂O.
2. 70 °C heat block.
3. 1.5 mL Eppendorf tubes.

2.6 RNA Analysis (Optional, But Recommended)

1. 5 M NaCl. Sterilized by autoclave.
2. GlycoBlue nucleic acid coprecipitant.
3. 95% and 75% ethanol. Stored at –20 °C.
4. Microcentrifuge capable of processing 1.5 mL Eppendorf tubes at speeds of up to $16,100 \times g$.
5. LET Buffer: 25 mM Tris–HCl pH 8.0, 100 mM LiCl, 20 mM EDTA pH 8.0. Sterilized by autoclave.
6. 10% SDS. Filter sterilized.

7. 65 °C heat block.
8. P/C/L: 50% v/v phenol/50% v/v chloroform, equilibrated with LET Buffer.
9. Vortexer housed at room temperature.
10. 1.5 mL Eppendorf tubes.
11. Chloroform.
12. RNase-free dH₂O.

2.7 Protein Analysis by SDS-PAGE (Optional)

1. Vacuum concentrator.
2. 100% and 80% acetone. Stored at –20 °C.
3. Microcentrifuge capable of processing 2.0 and 1.5 mL Eppendorf tubes at speeds of up to $16,100 \times g$, housed at 4 °C.
4. 1× SDS Sample Buffer: 125 mM Tris–HCl pH 6.8, 2% SDS, 100 mM DTT, 10% glycerol, 0.05% bromophenol blue.
5. 70 °C heat block.
6. 95 °C heat block.

3 Methods

3.1 Cell Growth, Chemical Cross-Linking, and Harvesting

1. Grow a 200 mL culture of cells (*see* **Notes** ³ and ⁷) at 30 °C and 250 rpm in SD media supplemented with appropriate amino acids (*see* **Note** ²) to an optical density of 0.4 at 600 nm λ (OD₆₀₀).
2. Cross-link RNA-protein and protein-protein complexes by adding formaldehyde to cell culture at 0.25% (final concentration) and incubating for 15 min at 30 °C and 250 rpm (*see* **Note** ⁸).
3. Quench cross-linking reaction by adding glycine to the culture to 0.125 M (final concentration) and incubating for 5 min at 30 °C and 250 rpm.
4. Harvest cells by centrifugation in clinical centrifuge at $3200 \times g$ for 1 min at room temperature. Decant media from pelleted cells.
5. Resuspend cell pellet in residual media and transfer suspension to a 2 mL Eppendorf tube. Pellet cells at $16,100 \times g$ for 30 s to collect sample at bottom of

⁷Culture size can be adjusted; however, the downstream lysis procedure is optimized for a cell pellet resulting from a 200 mL culture grown to an OD₆₀₀ = 0.4. If increasing the scale of the experiment, it is recommended to harvest multiple cell pellets equivalent to a 200 mL culture and process in parallel. 200 mL of yeast culture is sufficient for downstream RNA analysis by northern blot or protein analysis by western blot; approximately 12 L of total cell volume was necessary and sufficient for three to four technical replicates of analysis by mass spectrometry.

⁸Depending on the particular mRNP complex of interest, formaldehyde cross-linking conditions may need to be optimized. This may include altering the concentration and/or duration of treatment. Possible ways to monitor the extent of cross-linking include measuring overall protein yield from cell extracts (yield decreases as cross-linking increases), or testing for the loss of protein epitopes by western blotting (increased cross-linking often results in the masking of exposed epitopes).

tube, and remove residual supernatant. Flash freeze pelleted cells on dry ice and store at -80°C .

3.2 Cell Lysis

1. Supplement ice-cold (*see Note*⁹) $1\times$ Lysis Buffer with fresh 0.5 M DTT to a final concentration of 1 mM.
2. Resuspend each cell pellet in 400 μL $1\times$ Lysis Buffer by vortexing (*see Note*¹⁰). Immediately place resuspended cells on ice.
3. Add $\frac{1}{2}$ volume (~ 200 μL volume) of sterile glass beads to resuspended cells.
4. Vortex for 3 min at 4°C and then return sample to ice for 2 min. Repeat two additional times.
5. Puncture the bottom of the 2 mL Eppendorf tube with a red-hot 18-gauge needle (heated using a Bunsen burner or other heat sources) and nest tube within the opening of a 15 mL conical bottom centrifuge tube that has been prechilled by placing in ice. Centrifuge at $1600\times g$ for 2 min at 4°C (*see Note*¹¹).
6. Transfer the supernatant to a cold 1.5 mL Eppendorf tube and adjust volume for any lost material by bringing up volume to 400 μL with $1\times$ Lysis Buffer. Place sample on ice.
7. Resuspend pelleted cell debris from the previous step in 0.5 mL $1\times$ Lysis Buffer and transfer to a 2 mL Eppendorf tube that has been prechilled by placing in ice. Pellet at $16,100\times g$ for 30 s, and remove the residual supernatant. Repeat lysis (Subheading 3.2, **steps 2–6**) and combine supernatant with lysate from first lysis (*see Note*¹²). The total lysate volume should now equal approximately 800 μL . Store yeast whole cell lysate on ice until steps in Subheading 3.4.
8. Optional: quantify lysate by measuring the OD_{260} and OD_{280} with a UV spectrophotometer using quartz cuvettes and diluting 5 μL in 995 μL dH_2O (*see Note*¹³). Use an equivalent dilution of $1\times$ Lysis Buffer in dH_2O as a blank.

3.3 Magnetic Bead Preparation

1. For each lysed 200 mL cell pellet, resuspend MyOne Streptavidin C1 Dynabeads completely by repeat pipetting, and transfer 250 μL beads to a 1.5 mL Eppendorf tube (*see Note*⁵).

⁹Ensure that all cell lysis steps are carried out on ice and with reagents prechilled to 4°C .

¹⁰Resuspension of cell pellets stored at -80°C may be difficult. It is recommended that samples be vortexed in short (~ 30 s) bursts and then returned to ice to ensure they remain at 4°C . Proceed to the next step once no solid pellet is visible and the cell suspension appears uniform.

¹¹Passage of lysates through the opening created by puncturing the tube with an 18-gauge needle allows physical separation of the lysate from the glass beads and increases yield of the supernatant. Low-speed centrifugation of lysates ensures minimal loss of cross-linked protein complexes while removing cellular debris.

¹²A second sequential lysis of the cellular material was found to significantly increase the yield and concentration of the cell extract. This is particularly important for formaldehyde cross-linked cells, which typically give rise to a lysate of lower concentration.

¹³Quantification of cell extracts at this step is useful for monitoring the reproducibility of lysis from one sample to another. However, normalization of recovered material between samples or replicates is not performed using values acquired at this step.

2. Place tube alongside magnet and allow beads to collect along side of tube. Remove the supernatant by pipetting.
3. Wash beads with an equal volume (250 μ L) of 1 $\times$ B&W Buffer (*see Note* ¹⁴) by pipetting up and down approximately 20 times.
4. Place tube alongside magnet and allow beads to collect along side of tube. Remove the supernatant by pipetting.
5. Repeat wash (**steps 3 and 4** above) two more times.
6. Immobilize biotinylated DNA oligonucleotides (*see Note* ⁶) to washed Dynabeads in an incubation volume 4 $\times$ the original bead volume used. For a 250 μ L starting volume of magnetic beads, add reagents as indicated below. Incubate with end-over-end rotation for 15 min at room temperature.

Reagent	Volume
2 $\times$ B&W Buffer	500 μ L
Biotinylated DNA oligonucleotides (400 pmol/ μ L; <i>see Note</i> ¹⁵)	25 μ L
RNase-free dH ₂ O	475 μ L

7. Place tube alongside magnet and allow beads to collect along side of tube. Remove supernatant by pipetting. Wash beads with 500 μ L 1 $\times$ B&W Buffer by pipetting up and down approximately 20 times. Repeat wash step with an additional 500 μ L 1 $\times$ B&W Buffer.
8. Resuspend beads in 250 μ L 1 $\times$ B&W Buffer. Set beads aside at room temperature until ready for use, or continue immediately to the next step.

3.4 Hybridization/Affinity Capture

1. Bring volume of yeast whole cell lysate (800 μ L total volume from the two extractions of a cell pellet from 200 mL of culture, from Subheading 3.2, **step 7**) up to 3.5 mL with 2.7 mL TE Buffer. Remove an aliquot as “input” and store at -80°C — ideal amounts are 1/10 volume for downstream RNA analysis or 1/20 for protein analysis (*see Note* ¹⁶).

¹⁴Wash buffers and procedures follow the manufacturer’s protocol for Dynabeads (ThermoFisher Scientific). While the manufacturer’s protocol also recommends a set of bead washes with Solution A (0.1 M NaOH, 0.05 M NaCl) and Solution B (0.1 M NaCl) for applications involving RNA purification, our experience demonstrated no RNase contamination when these steps were eliminated. In contrast, we observed an increase in sample degradation when these steps were included, perhaps due to unintentional carryover of NaOH from Solution A (data not shown). Therefore, we recommend bypassing these wash steps (which have been omitted here).

¹⁵If using multiple DNA oligonucleotides in combination, the total volume added at this step should remain at 25 μ L and the combined concentration of oligonucleotides should total 10 nmol. Exceeding this total concentration does not result in increased target mRNA capture.

¹⁶It is recommended that during both initial optimization of this protocol and for quality control during the experiment, aliquots of sample from each step of the procedure (input, supernatant, and eluate) be monitored for RNA or protein recovery, or both. Additionally, this step allows for the calculation of percent recovery and a determination of the integrity of the sample. Detailed procedures for sample processing to carry out RNA or protein analysis are described below.

2. Prepare 5 mL (*see Note* ¹⁷) Hybridization Reaction for each sample in a 15 mL conical tube as follows (*see Note* ¹⁸).

Reagent	Volume	Final concentration
Yeast lysate + TE buffer	3 mL	N/A
5 M LiCl	500 μ L	500 mM LiCl
10% SDS	250 μ L	0.5% SDS
0.5 M EDTA pH 8.0	500 μ L	50 mM EDTA pH 8.0
1 M Tris-HCl pH 7.5	50 μ L	10 mM Tris-HCl pH 7.5
100% formamide	700 μ L	14% formamide
Fungal protease inhibitor	5 μ L	N/A

3. Anneal RNA to bead-bound DNA oligonucleotides: place Dynabeads from Subheading 3.3, step 8 alongside magnet, allow beads to collect along side of tube, and remove supernatant. Use a 500 μ L aliquot of Hybridization Reaction to resuspend Dynabeads. Transfer aliquot and resuspended beads back to the 15 mL conical bottom centrifuge tube containing the remaining hybridization reaction. Close and seal lid with parafilm and incubate overnight at room temperature with end-over- end rotation (*see Notes* ¹⁹ and ²⁰).
4. Centrifuge 15 mL conical tube for 5 s at $1600 \times g$ to collect sample at bottom of tube.
5. Transfer 1 mL of the Hybridization Reaction/Dynabeads mixture to a 1.5 mL Eppendorf tube. Place tube alongside magnet and allow beads to collect along side of tube; remove the supernatant. Continue adding ~1 mL aliquots of Hybridization Reaction/Dynabeads mixture to the same 1.5 mL Eppendorf tube until the entire Hybridization Reaction sample is transferred and all the beads are collected into one Eppendorf tube. Save an aliquot of the supernatant (i.e., “supernatant”) for downstream analysis (e.g., to determine the efficiency of target RNA depletion)—ideally 1/10 volume for RNA analysis or 1/20 volume for protein analysis (*see Note* ¹⁶).

¹⁷The same reaction can be scaled to 10 mL in a single tube (i.e., extracts from two 200 mL cell pellets combined, also doubling the volume of Hybridization Reaction and beads) with no substantial loss of the capture of target transcript. Increasing the scale further was seen to result in a substantial decrease in the efficiency of RNA recovery. To increase the scale beyond 10 mL, it is recommended to carry out multiple purifications in parallel and combine samples prior to the final downstream analysis step selected.

¹⁸Hybridization conditions were optimized to maximize mRNP purification while minimizing nonspecific binding (Fig. 4a). This is accomplished through the use of a high salt concentration (500 mM LiCl) to decrease nonspecific protein binding but promote DNA:RNA hybridization, ionic detergent (0.5% SDS) to decrease nonspecific protein binding (with no expected effect on hybridization), and 14% formamide to increase the stringency of DNA:RNA hybridization. EDTA is included to chelate divalent cations, reducing the possibility of RNA degradation during the incubation.

¹⁹Overnight incubation at room temperature was found to result in the highest recovery of RNA from cell extracts (Fig. 4b). EDTA and SDS were sufficient to inhibit degradation of RNA in samples under these conditions. For mRNP purification from other cell types, the addition of a general RNase inhibitor may be warranted.

²⁰Protein degradation may occur in samples incubated overnight at room temperature. A combination of a protease-deficient yeast strain and a fungal-specific protease inhibitor was sufficient to inhibit protein degradation in our samples. It is recommended that an organism-specific protease inhibitor be used for samples not originating from yeast cells.

6. Wash beads twice with 500 μ L Wash Buffer 1 (*see Note* ²¹). For each wash, thoroughly resuspend beads using a micropipette, wash by pipetting up and down approximately 20 times, then place tube alongside magnet and allow beads to collect along side of tube. Remove the supernatant and discard.
7. Wash beads three times with 500 μ L Wash Buffer 2 (*see Note* ²²). Perform washes as in Subheading 3.4, **step 6**. Following removal of supernatant after each wash, pulse-spin sample for 10 s in a microcentrifuge, place tube alongside magnet and allow beads to collect along side of tube. Remove residual supernatant and discard.
8. For RNA analysis or protein analysis by western blot of the entire mRNP sample, proceed to steps in Subheading 3.5. For protein analysis by mass spectrometry, proceed to steps in Subheading 3.8.

3.5 Elution of Purified mRNP Material (See Note ²³)

1. Resuspend beads in 75 μ L dH₂O per 100 μ L beads (i.e., 187.5 μ L dH₂O for 250 μ L beads).
2. Heat beads at 70 °C for 2 min to disrupt DNA:RNA hybrid annealing interaction (*see Note* ²⁴).
3. Place beads immediately alongside magnet and allow beads to collect along side of tube. Transfer eluate to a new 1.5 mL Eppendorf tube and save for downstream processing. In the event that any beads are carried over into the eluate, place the new tube on a magnet and transfer eluate one additional time to a new 1.5 mL Eppendorf tube to ensure complete removal of beads.
4. Remove an aliquot of eluate for RNA recovery analysis (ideally 1/10 volume for RNA; *see Notes* ¹⁶ and ²⁵). This step may be omitted if RNA analysis is performed on the entire eluate (as in the case of optimization experiments).
5. Proceed to steps in Subheading 3.6 for processing samples for RNA analysis, and steps in Subheading 3.7 for protein analysis.

²¹Wash Buffer 1 removes excess salt and SDS from the sample, both of which can have adverse effects on downstream applications such as mass spectrometry. Additionally, decreasing the salt concentration during the wash steps increases the stringency of DNA:RNA hybridization, helping to eliminate mRNP complexes that may result from off-target hybridization.

²²Wash Buffer 2 removes additional excess salt and SDS, and further increases DNA:RNA hybrid stringency (*see Note* ²¹). It is particularly important to ensure that the supernatant from each of these washes is removed completely in order to minimize residual SDS in the sample.

²³Steps in Subheadings 3.5–3.7 are recommended to monitor the efficiency of target RNA capture and the integrity of the purified RNA samples. These steps are performed on the “input” and “supernatant” aliquots and the resulting purified mRNP from **step 8** in Subheading 3.4.

²⁴Elution temperature was optimized to maximize recovery (based on the melting temperature of the DNA:RNA hybrid) without degradation of the RNA. This temperature may need to be adjusted if oligonucleotides have a different melting temperature than what is recommended here.

²⁵Protein analysis of a fraction of the purified eluate is not recommended, as the quantity is typically low and difficult to detect by western blotting. To analyze individual purified protein components of the captured mRNP complex, it is necessary to use the entire sample (and proceed to steps in Subheading 3.7).

3.6 RNA Analysis (Optional But Recommended) (See Notes 16, 23, and 26)

1. Bring the eluate volume from steps in Subheading 3.5 up to 400 μL with RNase-free water. Add 5 M NaCl to 0.2 M final concentration and 1.5 μL GlycoBlue to this sample (eluate), the “input” sample (collected in **step 1** in Subheading 3.4) and “supernatant” sample (collected in **step 5** in Subheading 3.4) and mix well. Precipitate RNA by adding 2.5 volumes of ice-cold 95% ethanol and incubating overnight at $-20\text{ }^{\circ}\text{C}$.
2. Pellet RNA at $16,100 \times g$ for 10 min at room temperature. Wash pellet with 500 μL ice-cold 75% ethanol by vortexing for 10 s. Centrifuge at $16,100 \times g$ at room temperature for an additional 10 min. Remove ethanol by decanting, centrifuge again for 10 s at $16,100 \times g$ at room temperature to collect any residual ethanol, and remove with micropipette. Air dry pellet until residual ethanol has evaporated.
3. Resuspend RNA pellet in 450 μL LET Buffer + 50 μL 10% SDS (see **Note 27**) by vortexing for 5 min at room temperature.
4. Reverse chemical cross-links by heating the sample at $65\text{ }^{\circ}\text{C}$ for 1 h (see **Note 28**). 5. Extract RNA with phenol/chloroform by adding an equal volume (i.e., 500 μL) of P/C/L to each sample. Vortex 5 min at room temperature and separate phases by centrifugation at $16,100 \times g$ for 5 min at room temperature. Carefully draw off the aqueous (top) layer and transfer it to a new 1.5 mL Eppendorf tube. Repeat extraction, substituting the P/C/L with an equal volume of chloroform.
5. Precipitate RNA overnight as in **steps 1** and **2** in Subheading 3.6.
6. Resuspend RNA in 20 μL LET Buffer for northern blot analysis, or 20 μL RNase-free dH_2O for qRT-PCR analysis (see **Note 29**).

3.7 Protein Analysis by SDS-PAGE (Optional) (See Notes 16, 23, and 25)

1. Concentrate proteins in eluate from **step 3** in Subheading 3.5 by evaporation under vacuum (using a SpeedVac or similar) on high heat until volume is reduced to $\sim 20\text{ }\mu\text{L}$ (see **Note 30**). If multiple samples have been processed in parallel to increase scale, they may be combined prior to concentration at this step.

²⁶Alternative RNA isolation methods may be substituted. It is recommended, however, to begin any RNA isolation protocol with an ethanol precipitation step in order to remove residual Hybridization Reaction or Wash Buffer components that may interfere with downstream isolation or analysis.

²⁷The addition of SDS serves to improve resuspension of the RNA/protein pellet, which still contains cross-linked material.

²⁸A lower temperature is recommended for reversing chemical cross-links when RNA analysis is being performed. The high heat used to reverse cross-links in samples for downstream protein analysis (**step 7** in Subheading 3.7) is likely to result in RNA degradation.

²⁹Both northern and qRT-PCR analyses of input, supernatant, and eluate aliquots are appropriate for determining percent yield and enrichment during purification. Northern analysis is recommended to monitor the integrity of the RNA samples.

³⁰Concentration of the protein sample is critical at this step as this protocol results in an eluate with low protein levels that, without concentration, are typically immeasurable by standard biochemical techniques. Tests using acetone to precipitate the entire unconcentrated sample (with or without carriers) indicated that substantial protein loss occurs. Loss is minimized by acetone precipitation of concentrated samples. Depending on the volume of sample being concentrated, reducing the volume to $\sim 20\text{ }\mu\text{L}$ may take several hours.

2. Precipitate proteins from concentrated eluate (and “input” and/or “supernatant” samples from **steps 1** and **5**, Subheading 3.4, if collected), by adding 4 volumes of ice-cold 100% acetone and incubating for 1 h at -20°C .
3. Collect precipitated proteins by centrifugation for 10 min at $16,100 \times g$ at 4°C . Remove the supernatant, taking care to not disrupt the pellet.
4. Wash the protein pellet with ice-cold 80% acetone by vortexing for 60 s.
5. Repeat centrifugation as in **step 3** in Subheading 3.7. Remove the supernatant and air dry pellet to complete dryness (*see Note* ³¹).
6. Resuspend pellet in volume of $1 \times$ SDS Sample Buffer suitable for loading the full quantity into a single well of a SDS polyacrylamide gel (e.g., 20 μL) by vortexing for 5 min or until pellets are completely resuspended (*see Note* ³²).
7. Heat proteins at 70°C for 1 h to reverse chemical cross-links (*see Note* ²⁸) followed by 95°C for 5 min. Sample can now be analyzed by SDS-PAGE.

3.8 Protein Analysis by Mass Spectrometry

1. Resuspend beads in up to 100 μL Wash Buffer 2. Remove an aliquot of resuspended beads for RNA recovery analysis (ideally 1/10 volume) and process this sample (i.e., “eluate”) with the “input” and supernatant’ samples beginning at **step 2** in Subheading 3.5 (*see Note* ¹⁶). The remainder of the on-bead sample resuspended in Wash Buffer 2 can be stored at -80°C .
2. Purified mRNP sample can be further processed for downstream analysis by mass spectrometry (i.e., on-bead trypsin digestion and mass spectrophotometric analysis).

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³¹Take care to ensure that the protein pellet does not over-dry, as this makes the protein much more difficult to resuspend.

³²Resuspension of protein pellet by vortexing may require longer than the suggested 5 min, and can be aided by micropipette mixing and/or gentle heat (37°C). During resuspension, it is also recommended to run buffer along sides of tube to ensure entire sample is fully resuspended and material adhering to sides of tube is not lost.

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Fig. 1.

Schematic of transcript-specific mRNP affinity capture. Native protein-protein and protein-nucleic acid interactions are stabilized *in vivo* by covalent cross-linking with the addition of formaldehyde to growing yeast cells. Cells are harvested and lysed, and the extracts incubated under denaturing, stringent conditions with magnetic bead-bound DNA oligonucleotides complementary to a single RNA population. Affinity captured, transcript-specific mRNPs are washed with denaturing buffers and low salt and processed for downstream applications including targeted RNA or protein analysis to monitor mRNP integrity and specificity of capture, or global protein identification by mass spectrometry

Fig. 2.

Screening streptavidin-conjugated magnetic beads for RNA capture efficiency. **(a)** Table highlighting differences between magnetic bead systems tested. All beads are streptavidin-conjugated Dynabeads (ThermoFisher Scientific; product numbers indicated). Bead diameter: large = 2.8 μm ; small = 1.05 μm . **(b)** Northern blot analysis of *GFP* mRNA (target RNA) captured from total purified yeast RNA using a single complementary DNA oligonucleotide conjugated to each magnetic bead system. Percent recovery of RNA from input is indicated below

Fig. 3. Screening of complementary DNA oligonucleotides for efficiency of affinity capture of *GFP* mRNA. **(a)** Schematic of the 5' region of the *GFP* open reading frame (ORF; from the start codon through codon 69). Approximate site of complementarity for each tested DNA oligonucleotide is indicated. **(b)** Northern blot analysis of *GFP* mRNA (target RNA) captured from total purified yeast RNA using different complementary DNA oligonucleotides (oligos) either alone or in combination. Oligonucleotide numbers correspond to the labels in **(a)**. Percent target RNA remaining in the supernatant or captured in the eluate are quantified relative to the input and are indicated below

Fig. 4.

Optimization of conditions for transcript-specific affinity capture. **(a)** Northern blot analysis of *GFP* mRNA (target RNA) captured from total purified RNA under varying hybridization conditions. Variables shown include bead volume, hybridization volume, salt concentration, and the addition of formamide to hybridization reactions. Percent recovered in eluate relative to input indicated below. *ND* not detected. Note, a number of additional optimization conditions were tested (not shown) and all conditions leading to increased capture of the target RNA were incorporated into the final protocol. **(b)** Northern blot analysis of *GFP* mRNA (target RNA) captured from total purified RNA under varying hybridization times and temperatures. *RT* room temperature, *O/N* overnight, *NC* not calculated