Chapter 31

RAP-MS: A Method to Identify Proteins that Interact Directly with a Specific RNA Molecule in Cells

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Abstract

RNA molecules interact with proteins to perform a variety of functions in living cells. The binding partners of many RNAs, in particular the newly discovered class of long noncoding RNAs (lncRNAs), remain largely unknown. RNA antisense purification coupled with mass spectrometry (RAP-MS) is a method that enables the identification of direct and specific protein interaction partners of a specific RNA molecule. Because RAP-MS uses direct RNA–protein cross-linking methods coupled along with highly denaturing purification conditions, RAP-MS provides a short list of high confidence protein interactors.

Key words RNA–protein interactions, UV cross-linking, Mass spectrometry, RNA purification, Protein purification, Antisense nucleic acid capture, RNA-binding proteins

1 Introduction

Long noncoding RNAs (lncRNAs) are emerging as a new class of cellular regulators that play important roles in gene regulation, chromatin structure, and cell fate during development [1], yet the mechanisms by which most lncRNAs work remain unknown. Addressing this question requires knowledge of the protein interaction partners that these RNA molecules use to achieve their functions.

There have been technical challenges in addressing this goal because of a lack of available methods that can successfully isolate direct RNA interacting proteins that occur in vivo (for a review of methods *see* McHugh et al. [1]). Briefly, methods that measure in vitro association of an RNA with cellular proteins fail to separate interactions that occur in vivo from those that occur in solution. Purifications of RNA-protein complexes from formaldehyde cross-linked samples identify both direct and indirect protein interactors, leading to a potentially long list of proteins making functional characterization of these interactions challenging [1].

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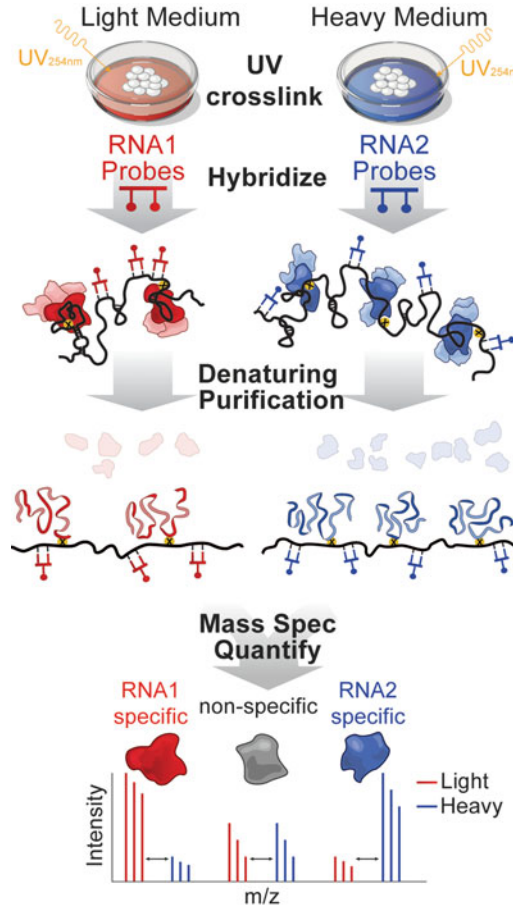

Fig. 1 Schematic of RAP-MS purification procedure from SILAC labeled mouse ES cells. Target RNA and control RNA are captured from cross-linked SILAC labeled cell lysates and purified under denaturing conditions. The resulting protein preparations are mixed and analyzed by mass spectrometry to identify proteins that bind specifically and directly to the target RNA versus the control RNA. Reproduced from [4]

In recent years, methods such as cross-linking and immunoprecipitation (CLIP) have been developed that have become the gold standard for studying direct RNA–protein interactions in vivo [2]. These methods utilize UV cross-linking to create a covalent link between RNA and protein interactions and are coupled with stringent purification conditions to enable the precise genome-wide mapping of the RNA binding sites of a specific protein [2]. Despite their success, CLIP methods are of more limited utility for identifying new protein interaction partners for a specific lncRNA.

To address this goal, we developed the RNA antisense purification coupled with mass spectrometry (RAP-MS) method to identify proteins that directly and specifically interact with a target RNA molecule (Fig. 1). The RAP-MS protocol uses ultraviolet light to

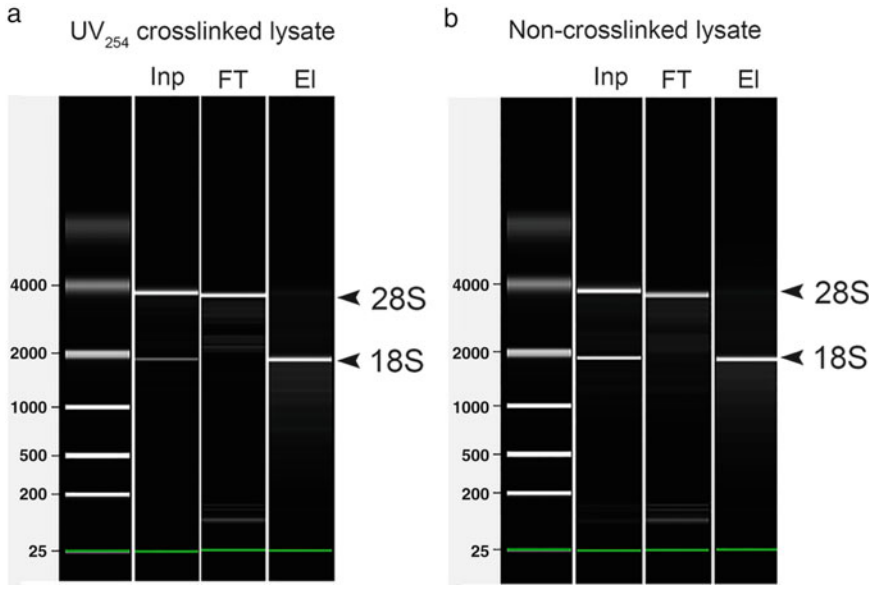

Fig. 2 Example of purified RNA captured by RAP-MS for 18S rRNA. Agilent Bioanalyzer gel-like images from RNA 6000 Pico chip for RNA Input (Inp), Flow-Through (FT), and Elution (El) samples from RAP-MS captures of 18S performed in (**a**) UV_{254} cross-linked or (**b**) non-cross-linked control (20 million cells each). The target RNA is efficiently captured and recovered from both UV cross-linked and non-cross-linked lysates

cross-link zero-distance interacting RNA and protein partners, followed by capture of the RNA of interest through hybridization with biotin-labeled DNA probes on streptavidin beads (Fig. 2). RAP-MS incorporates stringent washing with buffers containing high concentrations of denaturing and reducing agents to isolate only direct and specific proteins that are covalently cross-linked in vivo to the target RNA (Fig. 3). Stable isotope labeling of amino acids in cell culture (SILAC) tagging [3] is used to compare multiple protein capture samples in a single mass spectrometry experiment (Fig. 4), reducing instrument time and resulting in highly accurate quantitation of relative protein levels in each sample. At the end of the experiment, a short list of high-confidence direct protein interactors can be identified for a target RNA, simplifying follow-up analysis and subsequent functional assays.

The RAP-MS method has been validated on several well-characterized cellular RNAs (18S rRNA, U1 snRNA, and 45S preribosomal RNA) and was successfully used to identify the key functional proteins that interact with the Xist lncRNA during the initiation of X chromosome inactivation during development [4].

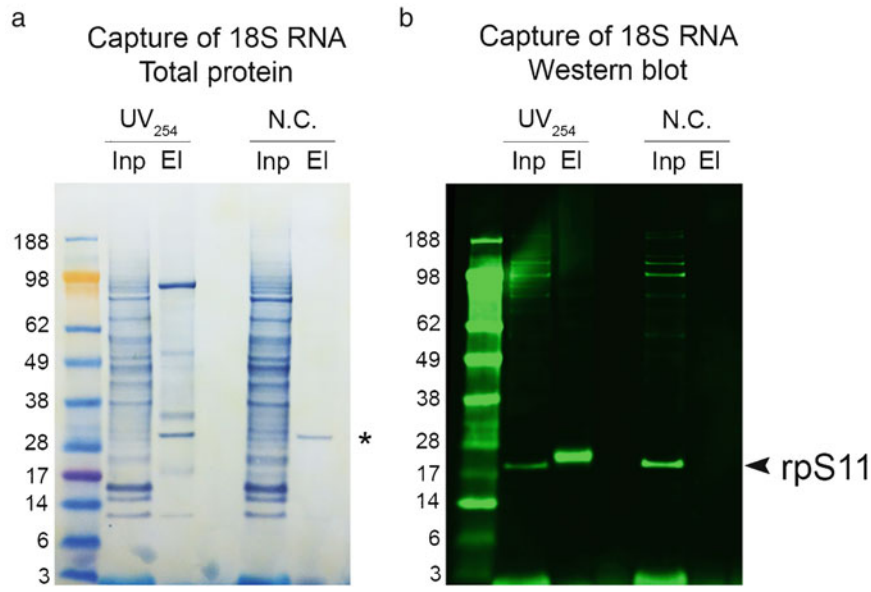

Fig. 3 Example of interacting proteins captured by RAP-MS for 18S rRNA. Proteins from RAP-MS captures of 18S in UV_{254} cross-linked and non-cross-linked N.C. control were separated by SDS-PAGE and transferred to nitrocellulose membrane. Each capture was performed from 20 million cells lysate. (**a**) Total protein staining of input (Inp) and elution (El) samples was performed with Blot FastStain (G Biosciences). *Asterisk* (*) indicates benzonase enzyme that was added to the elution sample. (**b**) For Western blotting, the same membrane was probed with an antibody (Abcam ab175213) against the rpS11 protein that is known to interact with 18s rRNA. Direct and specific 18S interacting proteins are recovered from the UV cross-linked sample, but not from the non-cross-linked lysate

2 Materials

Prepare all solutions using ultrapure water and the highest possible purity reagents. Whenever possible, use certified RNase- and DNase-free tubes and water for preparing samples. Please follow all safety and waste disposal guidelines when disposing of waste materials.

2.1 Specialized Equipment and Reagents

1. Custom-designed 90-mer DNA oligonucleotides with 5′-biotin modification (*see* **Note 1**).
2. Sonicator with microtip (e.g., Branson).
3. Thermomixer with heating and shaking functions.
4. Magnetic separation rack for Eppendorf tubes, 15 mL tubes, and 96-well plates (e.g., Life Technologies DynaMag).
5. Peptide HPLC column (e.g., Michrom Bioresources peptide MicroTrap column).
6. HPLC system (e.g., Agilent 1200 HPLC system), with Buffer A (0.2% formic acid) and Buffer B (100% acetonitrile).

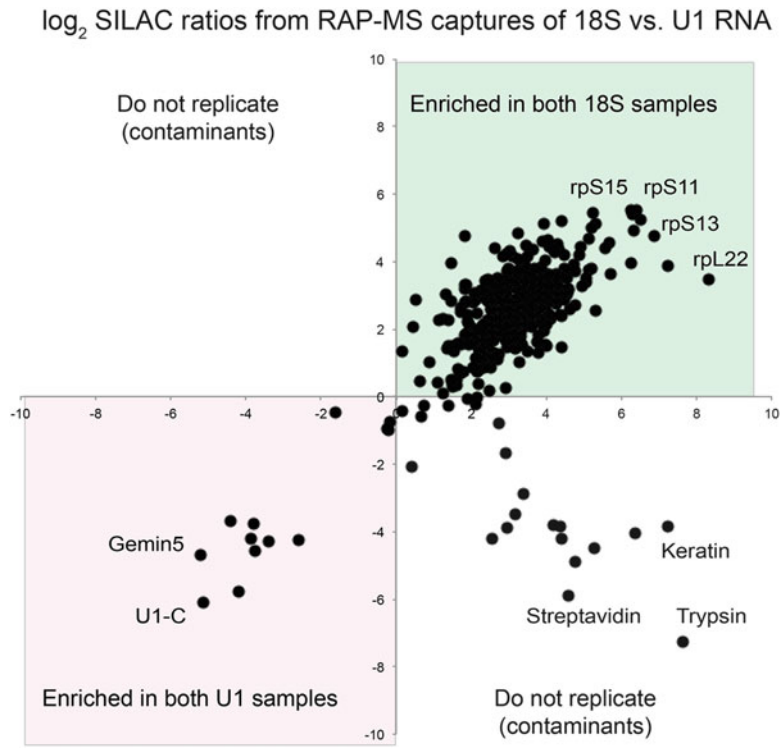

Fig. 4 Example of SILAC ratio plot for proteins from RAP-MS captures. Captures from more than one RNA target can be mixed and quantitated by mass spectrometry. RAP-MS captures for U1 and 18S were performed in both heavy and light lysates and resulting proteins were mixed together. Proteins identified in two replicates with label swap (18S light label vs. U1 heavy label, 18S heavy label vs. U1 light label) are plotted by their $\log_2$ SILAC ratio. Proteins that replicate in label-swap captures are high-confidence interactors for the target RNA molecule. Known contaminants (keratins, trypsin, benzonase, and streptavidin) are always purified in the light labeled sample and can be excluded from the final list of interactors. Adapted from [4]

7. q-PCR and/or Agilent Bioanalyzer.
8. TurboDNase with high salt tolerance.
9. Glass dounce homogenizer, 2 mL size.
10. UV cross-linker with 254 nm wavelength bulbs (e.g., Spectrolinker).
11. Vacuum lyophilizer.
12. Streptavidin-coated magnetic beads (e.g., Life Technologies Dynabeads).
13. Protease inhibitor cocktail set III, EDTA free.
14. Benzonase nuclease.
15. Detergent removal columns (e.g., Life Technologies HiPPR).

16. Sequencing grade modified trypsin (e.g., Promega).
17. Lysyl endopeptidase, mass spectrometry grade (e.g., Wako).
18. Liquid nitrogen.

2.2 SILAC Medium Recipes for Cell Culture

1. Heavy mouse embryonic stem cell SILAC medium: custom DMEM/F-12 without lysine and arginine (Dundee Cell Products), 0.398 mM heavy arginine (Sigma #608033), 0.798 mM heavy lysine (Cambridge Isotope Laboratories #CNLM-291-H), 0.2 mg/mL proline, 0.5× B-27 supplement (Life Technologies #17504-044), 1× N2 supplement (Life Technologies #17502-048), 2 mg/mL bovine insulin, 1.37 μg/mL progesterone, 5 mg/mL BSA Fraction V, 0.1 mM 2-mercaptoethanol, 5 ng/mL murine LIF (GlobalStem #GSR-7001), and 0.1 μM PD0325901 inhibitor (SelleckChem #S1036), 0.3 μM CHIR99021 inhibitor (SelleckChem #S2924).
2. Light mouse embryonic stem cell SILAC medium: Custom DMEM/F12 without lysine and arginine, 0.398 mM light arginine, 0.798 light lysine, 0.2 mg/mL proline, 0.5× B-27 supplement, 1× N2 supplement, 2 mg/mL bovine insulin, 1.37 μg/mL progesterone, 5 mg/mL BSA Fraction V, 0.1 mM 2-mercaptoethanol, 5 ng/mL murine LIF, 0.1 μM PD0325901 inhibitor, and 0.3 μM CHIR99021 inhibitor.

2.3 Buffer Recipes for RAP-MS Captures

1. Phosphate-buffered saline (1× PBS): 1 mM monobasic potassium phosphate, 155 mM sodium chloride, and 3 mM dibasic sodium phosphate.
2. 10 mM Tris–HCl pH 7.5.
3. 100 mM Tris–HCl pH 8.5.
4. Tris(2-carboxyethyl)phosphine (TCEP).
5. 8 M urea dissolved in 100 mM Tris–HCl pH 8.5.
6. 100 mM $CaCl_2$.
7. 500 mM iodoacetamide.
8. 80 w/v % aqueous solution tricholoroacetic acid (TCA).
9. 98% formic acid.
10. 200× DNase salt solution: 500 mM $MgCl_2$, 100 mM $CaCl_2$.
11. Cell lysis buffer I: 10 mM HEPES pH 7.4, 20 mM KCl, 1.5 mM $MgCl_2$, 0.5 mM EDTA, 1 mM TCEP, and 0.5 mM phenylmethylsulfonyl fluoride (PMSF).
12. Cell lysis buffer I with dodecyl maltoside (DDM): 10 mM HEPES pH 7.4, 20 mM KCl, 1.5 mM $MgCl_2$, 0.5 mM EDTA, 1 mM TCEP, 0.5 mM PMSF, and 0.1% DDM.
13. Cell lysis buffer II: 20 mM Tris–HCl pH 7.5, 50 mM KCl, 1.5 mM $MgCl_2$, 2 mM TCEP, 0.5 mM PMSF, 0.4% sodium deoxycholate, 1% DDM, and 0.1% *N*-lauroylsarcosine (NLS).

14. 1× Hybridization buffer: 10 mM Tris–HCl pH 7.5, 5 mM EDTA, 500 mM LiCl, 0.5% DDM, 0.2% SDS, 0.1% sodium deoxycholate, 4 M urea, and 2.5 mM TCEP.
15. Total cell lysis buffer: 10 mM Tris–HCl pH 7.5, 500 mM LiCl, 0.5% DDM, 0.2% SDS, 0.1% sodium deoxycholate, 1× protease inhibitor cocktail, and 1000 U/mL murine RNase inhibitor.
16. Benzonase elution buffer: 20 mM Tris–HCl pH 8.0, 0.05% NLS, 2 mM $MgCl_2$, and 0.5 mM TCEP.
17. NLS elution buffer: 20 mM Tris–HCl pH 8.0, 10 mM EDTA, 2% NLS, and 2.5 mM TCEP.
18. Bead based nucleic acid purification kit (We use Life Technologies Dynabeads® MyOne™ SILANE).

3 Methods

Maintain samples on ice and perform all centrifugation steps at 4 °C unless otherwise noted. All buffers should be prepared before starting the protocol. Buffers containing urea should be freshly prepared, or alternatively, can be prepared then immediately aliquoted and frozen at −20 °C. Do not store urea buffers at room temperature for extended periods of time. Before beginning experiments, select the target RNA of interest and controls as needed (*see* **Note 2**) and determine number of input cells to be used for each capture (*see* **Note 3**). If the cellular localization of the RNA is already known, select the appropriate lysis method for the target RNA; otherwise, use the whole cell lysis method (*see* **Note 4**).

3.1 Preparation of SILAC Labeled Cell Pellets

1. Initiate culture of cell line of interest. Once cells are growing well, split into two parallel cultures in SILAC Heavy and SILAC Light medium and grow for at least three passages to incorporate SILAC labels (*see* **Note 5**).
2. Seed adherent cells on 15 cm tissue culture plate. Grow to 70–90% confluence then remove medium from plate and replace with 10 mL ice-cold 1× PBS. Rock gently for 10 s then remove PBS wash. Add another 10 mL ice-cold PBS to plate to prevent cells from drying during cross-linking.
3. Place plates on a shallow tray of ice and UV cross-link at 254 nm for a total energy of 0.8 J/cm^2. Remove plates from cross-linker and keep on ice for the remainder of the procedure.
4. Scrape cells from the plate using a cell lifter and transfer to a sterile 15 mL tube. Cell suspensions from multiple plates can be pooled at this point.
5. Pellet cells by centrifugation at 1000 × *g* for 5 min.

6. Remove supernatant and resuspend cells in cold PBS to a final concentration of 50 million cells per 1 mL of buffer, pipetting gently to break up pellet.
7. Aliquot 1 mL of PBS/cell mixture into microcentrifuge tubes and centrifuge at 1000 × *g* for 5 min. Remove supernatant by aspirating or pipetting gently. At this point, pellets may be flash frozen in liquid nitrogen and stored at −80 °C.

3.2 Preparation of Nuclear Lysates

1. Resuspend each cell pellet in 1 mL of Cell Lysis Buffer I.
2. Centrifuge at 3300 × *g* for 10 min. Discard supernatant and resuspend cell pellet in 1 mL of Cell Lysis Buffer I with 0.01% DDM.
3. Incubate for 10 min on ice, then transfer sample to a tissue homogenizer and dounce with B (small clearance) pestle 20 times to break cells (*see* **Note 6**).
4. Transfer sample to microcentrifuge tube, and pellet nuclei by centrifugation at 3300 × *g* for 10 min.
5. Discard supernatant and resuspend pellet in 580 μL of Cell Lysis Buffer II.
6. Incubate for 10 min on ice, then sonicate on ice with microtip using 5 W of power (25% duty) for 60 s total in pulses of 0.7 s on, followed by 3.3 s off.
7. Add 1× DNase salt solution (3.75 μL) and 330 U TurboDNase (165 μL).
8. Incubate for 12 min at 37 °C.
9. Mix lysate with equal volume of 2× Hybridization Buffer (750 μL).
10. Centrifuge at 16,000 × *g* for 10 min at 4 °C.
11. Transfer supernatant to fresh tube and flash freeze in liquid nitrogen.
12. Store lysate at −80 °C until ready to perform RAP captures.

3.3 Preparation of Whole Cell Lysates

1. Resuspend each cell pellet in 900 μL Total Cell Lysis Buffer and incubate on ice for 10 min.
2. Pass cell suspension 3–5 times through a 26-G needle, then sonicate with a microtip at 5 W power for 30 s in pulses of 0.7 s on followed by 1.3 s off.
3. Perform DNase treatment as described for nuclear lysate (**steps 7** and **8** of Subheading 3.2), then add salt and detergents to adjust sample buffer to match 1× Hybridization Buffer.
4. Centrifuge at 16,000 × *g* for 10 min to pellet insoluble material.
5. Transfer the supernatant to a fresh tube and flash freeze in liquid nitrogen.
6. Store lysate at −80 °C until ready to perform RAP captures.

3.4 Preclearing of Lysate

1. On the day of the experiment, warm frozen aliquots of either whole cell lysate or nuclear lysate to 37 °C using a thermomixer. Pool samples in a single tube.
2. Transfer 1.2 mL of streptavidin-coated magnetic beads per 200 million cell sample into a fresh microfuge tube (*see* **Note 7**).
3. Separate on magnetic rack and remove storage buffer from beads.
4. Resuspend beads in 1 mL of 10 mM Tris–HCl pH 7.5 with gentle pipetting, then separate on magnetic rack and remove supernatant.
5. Repeat bead washes (**step 4**) for a total of four washes in 10 mM Tris pH 7.5, and two washes in 1× Hybridization Buffer (*see* **Note 8**).
6. Magnetically separate and remove last wash from beads, then transfer lysate to beads and resuspend by pipetting gently.
7. Incubate for 30 min at 37 °C with intermittent mixing at 1100 rpm on thermomixer (30 s shaking, 30 s off).
8. Magnetically separate beads and transfer supernatant to fresh tubes. Repeat this step to transfer lysate to fresh tubes a second time, to remove all traces of beads from sample.
9. Remove sample of 100,000 cells worth of lysate and transfer to PCR strip tube. This is the "RNA Input" sample.

3.5 RAP Captures of Target RNA–Protein Complexes

1. Denature appropriate quantity of probe by heating at 85 °C for 3 min, then place on ice (*see* **Note 7**).
2. Mix lysate and probe, then incubate for 2 h at 67 °C with intermittent mixing at 1100 rpm on thermomixer (30 s shaking, 30 s off).
3. During the 2 h incubation, prepare streptavidin beads as previously described (**steps 4** and **5** of Subheading 3.4).
4. Magnetically separate beads and remove final wash from beads.
5. At the end of the 2 h incubation, remove sample of 100,000 cells worth of lysate and transfer to PCR strip tube. This is the "RNA Input + Probe" sample.
6. Resuspend beads in lysate (*see* **Note 8**).
7. Incubate for 30 min at 67 °C with intermittent mixing at 1100 rpm on thermomixer (30 s shaking, 30 s off).
8. Magnetically separate beads and remove supernatant. Take sample of 100,000 cells worth of supernatant and transfer to PCR strip tube. This is the "RNA Flow-Through" sample.

9. Wash beads 3–6 times, with at least one bead volume of 1× Hybridization Buffer per wash. Incubate each wash for 5 min at 67 °C.
10. Remove a sample of beads between 0.5% and 1% of the total volume and transfer to a PCR strip tube. This is the "RNA elution" sample.

3.6 Elution of Captured Protein

1. Magnetically separate remaining beads and remove supernatant.
2. Resuspend beads in 1 mL of Benzonase Elution Buffer.
3. Add 125 U of benzonase nuclease to sample.
4. Incubate for 2 h at 37 °C with intermittent mixing at 1100 rpm on thermomixer (30 s shaking, 30 s off) to digest nucleic acids and release proteins from beads.
5. Magnetically separate beads and transfer supernatant to a fresh microcentrifuge tube. Repeat this step for a total of six transfers to fresh tubes to remove all traces of streptavidin beads. The last supernatant is the "protein elution" sample.
6. If desired, a second nonspecific elution can be performed by boiling the bead sample in NLS elution buffer for 2 min at 95 °C. This elution will remove all remaining RNA as well as the streptavidin and bound proteins from the beads and can be used to test for remaining RNA or protein after the benzonase elution.

3.7 Elution of Captured RNA

1. Take the "RNA elution" sample of beads from the final step of Subheading 3.5 and separate on magnetic rack.
2. Remove and discard supernatant. Resuspend beads in 20 μL of NLS Elution Buffer.
3. Heat samples for 2 min at 95 °C.
4. Magnetically separate and transfer supernatant containing eluted RNA to a fresh PCR strip tube.
5. Take the previously collected samples (RNA Input, Input + Probe, and Flow-Through) and dilute each sample to 20 μL total volume with NLS Elution Buffer.
6. Add 1 mg/mL Proteinase K to each sample.
7. Incubate for 1 h at 52–55 °C to digest proteins.
8. Store samples at −20 °C for short term or −80 °C for long term.

3.8 Quantitation of Captured RNA

1. Perform SILANE bead based nucleic acid cleanup using the following steps (for a 20 μL sample):
2. Aliquot 20 μL of beads per RNA sample into clean PCR strip tubes. Magnetically separate beads and remove storage buffer.

3. Resuspend beads in 60 μL RLT Buffer (3× original sample volume).
4. Transfer beads in RLT to 20 μL RNA sample and mix well.
5. Add 120 μL of 100% ethanol (6× original sample volume). Wait for 2 min for sample to bind beads.
6. Wash beads two times with 150 μL of 70% ethanol.
7. Remove supernatant and allow to air-dry for approximately 5 min.
8. Elute RNA in water or desired buffer. In this case, elute RNA samples by adding 26 μL of 1× TURBO DNase Buffer (dilute from 10× stock supplied by manufacturer).
9. Leave beads in tube. Perform DNase treatment to remove background DNA by adding 1 μL of murine RNase inhibitor and 3 μL of TURBO DNase to each sample (30 μL total reaction volume). Incubate for 20 min at 37 °C.
10. Perform a second SILANE cleanup using beads already in the tube:
11. Add 90 μL RLT Buffer to each 30 μL sample.
12. Add 180 μL of 100% ethanol and mix well.
13. Wait 2 min for sample to bind beads.
14. Wash beads two times with 70% ethanol.
15. Remove supernatant and allow beads to air dry approximately 5 min.
16. Elute in 10 μL of UltraPure water.
17. Analyze RNA samples using Agilent Bioanalyzer or by RT-qPCR (*see* **Note 9**).

3.9 Mass Spectrometry of Captured Proteins

3.9.1 Protein Precipitation

1. Add 10% final concentration of TCA to protein elution sample. Incubate at 4 °C overnight.
2. Centrifuge at 16,000 × *g* for 30 min to pellet protein.
3. Remove supernatant and replace with 1 mL of cold acetone.
4. Centrifuge at 16,000 × *g* for 15 min.
5. Remove supernatant and allow pellet to dry in open tube in fume hood or on bench. Store protein elution samples at −20 °C.

3.9.2 In-Solution Digest of Protein Samples for Mass Spectrometry

1. Resuspend protein elution sample in 40 μL of freshly prepared 8 M urea dissolved in 100 mM Tris–HCl pH 8.5.
2. Add 3 mM TCEP and incubate 20 min at room temperature.
3. Add 11 mM freshly prepared iodoacetamide and incubate for 15 min at room temperature in the dark.

4. Digest samples with 0.1 μg Lysyl endopeptidase for 4 h at room temperature.
5. Dilute samples to final concentration of 2 M urea by adding appropriate volume of 100 mM Tris–HCl pH 8.5.
6. Add 1 mM $CaCl_2$ to sample.
7. Digest with 0.1–0.5 g of trypsin overnight at room temperature.

3.9.3 Purify Peptides to Remove Detergent

1. Use HiPPR resin spin columns to remove detergent according to manufacturer's instructions.
2. Add 5% formic acid and centrifuge for 1 min at 16,000 × *g*.
3. Desalt by HPLC (*see* **Note 10**).
4. Collect fractions containing peptides.
5. Lyophilize peptides in SpeedVac.
6. Store samples at −20 °C until ready for mass spectrometry.
7. Resuspend samples in 0.2% formic acid and 5% acetonitrile.
8. Mix SILAC heavy labeled target RNA capture sample with SILAC light labeled control RNA capture sample, or vice versa (*see* **Note 11**).
9. Analyze mixed samples by mass spectrometry and quantify peptide ratios using MaxQuant or similar analysis software (*see* **Note 12**).

4 Notes

1. *Oligonucleotide probe design for RAP-MS captures.* Design 90-nucleotide oligos that tile across the target RNA sequence of interest without overlapping. Probe design software is available at www.lncRNA.caltech.edu/software.php. To avoid off-target hybridization, use BLAST, or similar alignment programs, to remove sequences that contain a perfect 30 base pair match or an imperfect (90%) identity 60 base pair match with another transcript or genomic region. Compare the oligos to RepeatMasker annotations and remove probes that contain more than 30 bases that overlap with a repeat annotation. Order oligos with 5′ biotin standard modification from an oligonucleotide synthesis company such as Integrated DNA Technologies. Individual probes should be resuspended at 500 μM or 1 mM concentration depending on the synthesis scale. Dilute probe stocks 1:100 from 96-well plates or individual tubes into UltraPure water. Mix all individual probes together to create a probe stock to cover the length of the target RNA. We usually make several aliquots of probe stock mixtures and store at −20 °C,

avoiding multiple freeze–thaw cycles. Sequences for control capture oligonucleotide probes for mouse RNA targets (U1, 18S, and 45S) are available upon request.

2. *Selection of appropriate controls.* The U1 snRNA control and non-cross-linked capture control are generally used as our standard negative controls. Antisense probes that do not bind the target RNA, probes targeting a known mRNA, or other controls could also be used to evaluate the level and identify of background or nonspecific proteins in the cell type of interest. Negative controls like antisense probes and non-cross-linked samples are important to perform to ascertain the level of background but generally do not yield enough protein for useful quantitation in mixed samples. We use U1 as a standard nuclear control for calculating SILAC ratios in mixing experiments, but 18S or other RNA capture controls could also be used.
3. *Number of cells per capture.* Volumes indicated are for 200 million cells per capture. Increase or decrease the cell number used for each capture depending on the abundance of the target RNA. For high abundance RNAs (U1, 18S, and 45S), between 20 and 200 million mouse embryonic stem cells are usually sufficient per capture. For lower abundance RNAs (Xist or other lncRNAs), we used between 200 and 800 million mouse embryonic stem cells per capture. Other RNA targets may require different cell input numbers to reliably obtain sufficient quantities of captured protein for mass spectrometry analysis.
4. *Cell lysis method*. Select either the nuclear lysis or whole cell lysis method as needed for the target RNA. For nuclear RNAs that are chromatin associated, like Xist, the nuclear lysis method is optimal. If the lncRNA is not chromatin associated or a whole cell extraction method is preferred, the whole cell lysis procedure can be followed. For 18S rRNA captures shown here, the whole cell lysis method was used. For Xist lncRNA captures shown in McHugh et al. 2015 [4], the nuclear lysis method was used. Alternative methods for nuclear extraction, particularly the method described in [5], are also compatible with RAP-MS as long as the guidelines for removing detergents and salts from proteins before mass spectrometry analysis are followed.
5. *Initiating SILAC cultures.* Sample SILAC medium recipes for mouse ES cell culture are provided above. SILAC medium should be adapted to fit the requirements of the desired cell line. The most important factor is to use base medium without lysine and arginine amino acids. Serum that has been dialyzed to remove unlabeled amino acids may be required to support the growth of some cell types. Cells should be grown in SILAC

medium for at least three generations and the incorporation should be tested by mass spectrometry to ensure >95% labeling of peptides. It is also possible to perform experiments in label-free samples and identify and quantitate captured proteins by intensity of their peptides only. However, this approach requires more controls and experimental replicates to obtain a list of high-confidence interactors with a target RNA, since nonspecific contaminant proteins will likely dominate the total mass spectrum by intensity.

6. *Douncing for cell lysis.* Number of strokes for douncing to lyse cells but retain nuclear integrity should be optimized for the cell type so that the cell membrane is broken but the nuclei remain intact. When establishing any cell lysis procedure, check the cells under a microscope to confirm appropriate lysis.
7. *Selection of appropriate probe to lysate and bead to lysate ratios.* The exact concentration of probe and streptavidin beads needed for a particular experiment will vary depending on the target concentration. Many targets will require a lower probe and bead quantity than is suggested here. Optimization of the RNA capture in small-scale lysates is advisable before scaling up for protein identification experiments. The quantity of streptavidin beads and probe can be adjusted up or down relative to the amounts given here, and capture temperatures ranging from 45 to 67 °C can be evaluated to determine the best combination for efficient capture of a particular target RNA molecule.
8. *Sample handling during magnetic bead captures.* The buffers used for hybridization and washes contain detergents that may create bubbles and make magnetic separation of beads challenging. Pipette gently to avoid creating bubbles when washing beads, and ensure magnetic beads have separated sufficiently from the liquid phase before removing and discarding supernatants.
9. *Samples for analysis of RNA yield.* In the first experiment for a new target RNA, do a small-scale capture in 1–10 million cells lysate to test that probes and washes are performing as expected. Collect and evaluate the RNA Input lysate, Input plus Probe, Flowthrough, and Elution samples to evaluate where the target RNA is located at each step of the procedure. Captured RNA is usually of sufficient quantity to detect by Agilent Bioanalyzer or RT-qPCR analysis but may not be enough to detect on a standard agarose gel. If performing qPCR analysis, ensure that primers are designed such that they do not amplify a region contained within a single probe sequence. Highly stable DNA:RNA hybrids formed during RAP capture are not always completely removed during SILANE cleanups, even after DNase treatment. Inclusion of

the Input plus Probe sample during RT-qPCR controls for any DNA probe that was not removed during RNA cleanup.

10. *Cleaning of peptides for mass spectrometry.* It is imperative that peptide samples be fully cleaned to remove all detergents and salts that may interfere with analysis. The method described here uses a combination of detergent-binding resin and a desalting column, but Stop-and-Go extraction tips [6] or other methods may also be used as an alternative.
11. *Mixing samples for mass spectrometry.* Accurate mixing is important to achieve correct SILAC peptide ratios. Take a small amount of unmixed peptide sample (about one tenth of the total sample) and perform a short quantitation run on the mass spectrometer to get an accurate measurement of the amount of protein present in each sample. Perform peptide searches for each sample. Filter to remove common contaminants using the database provided by MaxQuant or other analysis software. Finally, calculate the median peptide intensity for each sample and mix heavy and light SILAC samples based on the median intensity of peptides in the sample. Protein concentration measurement methods like Bradford or Coomassie assays could be used to estimate sample concentration instead of a quantitation MS run, but these methods may not be accurate enough to precisely measure the concentration of small amounts of captured protein in the final RAP-MS sample.
12. *Mass spectrometry data analysis and identification of final RNA–protein interactors list.* At the end of this protocol, samples are ready for LC-MS measurements using the desired instrument. For example, a nanoflow LC system (Proxeon EASy-nLC1000) coupled to a hybrid linear ion trap Orbitrap Elite mass spectrometer (Thermo Fisher Scientific) was used for the experiments described in McHugh et al. [4]. MaxQuant analysis software was used for peptide searches and to calculate SILAC median peptide ratios for proteins with at least two matched SILAC pairs. Other software may be used to identify and quantitate peptides after SILAC labeling. Judicious use of background controls including non-cross-linked samples and a variety of nontarget RNAs (U1, 18S, or others) can help distinguish real and specific interactors from proteins that interact with many RNA molecules in the cell. We exclude known contaminants from the final protein list, including keratins and proteins introduced during the sample purification and preparation process (such as streptavidin, benzonase, and trypsin, Fig. 4), as well as naturally biotinylated proteins like histones that can contaminate the preparation by binding to streptavidin beads. The threshold SILAC ratio was set at $\geq$3.0 for Xist vs. U1 experiments but high or lower cutoff may be appropriate for other combinations of target RNA and control samples.

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