

Journal of Visualized Experiments

Identification of footprints of RNA:protein complexes via RNA Immunoprecipitation in Tandem followed by sequencing (RIPiT-Seq) --Manuscript Draft--

Article Type:	Invited Methods Article - JoVE Produced Video
Manuscript Number:	JoVE59913R2
Full Title:	Identification of footprints of RNA:protein complexes via RNA Immunoprecipitation in Tandem followed by sequencing (RIPiT-Seq)
Keywords:	RNA footprint, RIPiT, RNA-binding protein, RBP, RNA:protein complexes, RNP
Corresponding Author:	Amrit Singh UNITED STATES
Corresponding Author's Institution:	
Corresponding Author E-Mail:	singh.734@osu.edu
Order of Authors:	Lauren Woodward Pooja Gangras Guramrit Singh
Additional Information:	
Question	Response
Please indicate whether this article will be Standard Access or Open Access.	Standard Access (US\$2,400)
Please indicate the city, state/province, and country where this article will be filmed . Please do not use abbreviations.	Columbus, OH, USA



Department of Molecular Genetics

Guramrit Singh, Ph.D.
484 West Twelfth Avenue
Columbus, OH 43210-1292

Phone (614) 292-2640
Fax (614) 292-4466
Email: singh.734@osu.edu

Dr. Stephanie R. Weldon
Science Editor
JoVE

February 26, 2019

Dear Dr. Weldon,

This letter accompanies the manuscript titled “*Identification of footprints of RNA:protein complexes via RNA Immunoprecipitation in Tandem followed by sequencing (RIPiT-Seq)*” by Lauren Woodward, Pooja Gangras, and Guramrit Singh for publication alongside an instructional video in *JoVE*.

Within eukaryote cells, RNA is bound directly or indirectly by a myriad of RNA-binding proteins (RBPs) to form a dynamic ribonucleoprotein (RNP) network. Post-transcriptional gene regulation through RBPs is an ever-expanding frontier across many biological disciplines. Investigators often seek to identify endogenous targets of an RBP in mammalian cell culture, for which various options exist—the most famous being CLIP-Seq (UV-Crosslinking followed by Immunoprecipitation). However, the identification of RNA targets of an RBP that does not bind the RNA directly or does not UV-crosslink well is a major limitation in the field. Further, RBPs generally have multiple roles and interaction partners. As such, it is challenging to deduce which RNA binding events represent a biologically active RBP binding network.

Our method, RIPiT, bypasses the need for UV-crosslinking and is designed to enrich compositionally distinct RBP complexes from a pool of RNPs with overlapping protein composition. This tool has been used successfully to characterize the biological role of compositionally distinct varieties of the exon junction complex (EJC). The EJC is a protein complex that interacts with many RBPs and participates in multiple compositionally distinct RNP structures.

In this manuscript, we describe in detail the steps, reagents, and controls an investigator will need to successfully perform RIPiT-Seq on their protein complex of interest. Schematic figures have been included to illustrate the major steps in the RIPiT workflow. RIPiT yields a low amount of RNA, so in addition to the RIPiT protocol, this manuscript includes our method for library preparation for deep sequencing. While our sample data focuses on EJC RIPiTs, the procedure is adaptable to virtually any other protein complex, and the *JoVE* video will provide a useful tool for someone new to the procedure.

We are excited about the opportunity to make this approach more widely accessible through *JoVE*, and look forward to hearing from you.

A handwritten signature in black ink, appearing to read 'Guramrit Singh'.

Guramrit Singh
Assistant Professor
Department of Molecular Genetics
Center for RNA Biology
The Ohio State University

TITLE:

Identification of Footprints of RNA:Protein Complexes via RNA Immunoprecipitation in Tandem Followed by Sequencing (RIPiT-Seq)

AUTHORS AND AFFILIATIONS:

Lauren Woodward¹, Pooja Gangras¹, Guramrit Singh¹

¹Department of Molecular Genetics, Center for RNA Biology, The Ohio State University, Columbus, OH, USA

Corresponding author:

Guramrit Singh (singh.734@osu.edu)

Email addresses of co-authors:

Lauren Woodward (woodward.178@osu.edu)

Pooja Gangras (gangras.1@osu.edu)

KEYWORDS:

RNA footprint, RIPiT, RNA-binding protein, RBP, RNA:protein complexes, RNP

SUMMARY:

Here, we present a protocol to enrich endogenous RNA binding sites or “footprints” of RNA:protein (RNP) complexes from mammalian cells. This approach involves two immunoprecipitations of RNP subunits and is therefore dubbed RNA immunoprecipitation in tandem (RIPiT).

ABSTRACT:

RNA immunoprecipitation in tandem (RIPiT) is a method for enriching RNA footprints of a pair of proteins within an RNA:protein (RNP) complex. RIPiT employs two purification steps. First, immunoprecipitation of a tagged RNP subunit is followed by mild RNase digestion and subsequent non-denaturing affinity elution. A second immunoprecipitation of another RNP subunit allows for enrichment of a defined complex. Following a denaturing elution of RNAs and proteins, the RNA footprints are converted into high-throughput DNA sequencing libraries. Unlike the more popular ultraviolet (UV) crosslinking followed by immunoprecipitation (CLIP) approach to enrich RBP binding sites, RIPiT is UV-crosslinking independent. Hence RIPiT can be applied to numerous proteins present in the RNA interactome and beyond that are essential to RNA regulation but do not directly contact the RNA or UV-crosslink poorly to RNA. The two purification steps in RIPiT provide an additional advantage of identifying binding sites where a protein of interest acts in partnership with another cofactor. The double purification strategy also serves to enhance signal by limiting background. Here, we provide a step-wise procedure to perform RIPiT and to generate high-throughput sequencing libraries from isolated RNA footprints. We also outline RIPiT’s advantages and applications and discuss some of its limitations.

INTRODUCTION:

Within cells, RNA exists in complex with proteins to form RNA:protein complexes (RNPs). RNPs are assembled around RNA binding proteins (RBPs, those that directly bind RNA) but also comprise of non-RBPs (those that bind RBPs but not RNA), and are often dynamic in nature. RBPs and their cofactors function collectively within RNPs to execute regulatory functions. For example, in the nonsense-mediated mRNA decay (NMD) pathway, the UPF proteins (UPF1, UPF2, and UPF3b) recognize the prematurely terminated ribosome. Each of the UPF proteins can bind to RNA, but it is only when they assemble together that an active NMD complex begins to form. Within this complex, UPF1 is further activated by phosphorylation by a non-RBP SMG1, and such UPF1 activation eventually leads to recruitment of mRNA decay inducing factors^{1,2}. In this example, RBPs require non-RBP cofactors for recruitment and activation of the RNP complex that triggers NMD. Yet another property of RNPs is their compositional heterogeneity. Consider the spliceosome, which exists in distinct E, A, B or C complexes. Different spliceosome complexes have overlapping and distinct proteins³. To study RNP functions, it is important to elucidate which RNAs are bound by an RBP and its associated proteins. Many methods exist to accomplish this, with each approach having its distinct advantages and disadvantages⁴⁻⁷.

The widely popular methods to identify RBP binding sites — crosslinking followed by immunoprecipitation (CLIP) and its various variations — rely on ultraviolet (UV) light to crosslink an RBP to RNA⁸. However, this is not an effective approach for non-RBPs within RNPs, which do not contact the RNA directly. Here, we describe an alternative approach that is applicable to RBPs and non-RBPs alike, to isolate and identify their RNA binding sites. This approach termed RNA immunoprecipitation in tandem (RIPiT) consists of two immunoprecipitation steps, which help achieve higher specificity as compared to a single purification (**Figure 1**). As the individual immunoprecipitation (IP) steps can be carried out at a lower stringency as compared to CLIP, RIPiT does not depend on availability of antibodies that can withstand presence of strong detergents during immunoprecipitation. The most unique advantage of RIPiT is the ability to target two different proteins in two purification steps; this provides a powerful way to enrich a compositionally distinct RNP complex from other similar complexes⁹.

Small variations to the RIPiT procedure can further enhance RNP enrichment. For instance, some RNA-protein or protein-protein interactions within RNPs are transient and it may be difficult to efficiently purify footprints of such complexes. To stabilize such interactions, RNPs can be crosslinked within cells with formaldehyde prior to cell lysis and RIPiT. For example, we have observed that a weak interaction between the exon junction complex (EJC) core factor, EIF4AIII and the EJC disassembly factor, PYM¹⁰ can be stabilized with formaldehyde treatment such that more RNA footprints are enriched (data not shown). Prior to cell harvesting and RIPiT, cells can also be treated with drugs to stabilize or enrich RNPs in a particular state. For example, when studying proteins that are removed from mRNA during translation (e.g., the EJC¹¹, UPF1¹²), treatment with translation inhibitors such as puromycin, cycloheximide or harringtonine can lead to

increased occupancy of proteins on RNAs.

The amount of RNA recovered from RIPit is usually low (0.5–10 pmoles, i.e., 10–250 ng RNA considering an average RNA length of 75 nt). The primary reason for this is that only a small fraction of a given protein is present in complex with other proteins within RNPs (any “free” protein IP’ed in the first step is lost during the second IP). To generate RNA-Seq libraries from this RNA, we also outline here an adaptation of previously published protocol suitable for such low RNA inputs^{13,14} (**Figure 2**), which yields high-throughput sequencing ready samples in 3 days.

PROTOCOL:

1. Establishment of stable HEK293 cell lines expressing tetracycline-inducible FLAG-tagged protein of interest (POI)

1.1. Seed HEK293 cells with a stably integrated Flp recombination target (FRT) site at a density of 10×10^4 cells/mL in growth medium (Dulbecco’s modified Eagle’s medium [DMEM] + 10% fetal bovine serum [FBS] + 1% penicillin-streptomycin [penn/strep]) in 6-well plates. Allow cells to grow overnight in a humidified incubator at 37 °C and 5% CO₂ (standard growth conditions for all subsequent steps).

1.2. The next day, cells should be ~70% confluent. According to transfection reagent protocol, transfect the FRT site-containing HEK293 cells with a 9:1 ratio of pcDNA5-TETO-FLAG-POI:pOG44.

NOTE: The FLAG tag has the sequence motif DYKDDDDK (D = aspartic acid, Y = tyrosine, and K = lysine).

1.3. After 24 h, begin antibiotic selection. Remove media and add fresh growth medium supplemented with 100 µg/mL hygromycin. Within 72 h, untransfected cells should begin to die.

1.4. Every 48–72 h, change growth media and supplement with fresh hygromycin.

1.5. After ~2 weeks of selection, discrete colonies of stably transfected cells will begin to appear. Once colonies are visible to naked eye, add 1 mL of trypsin to the plate and incubate for 5 min at 37 °C. Resuspend cells in DMEM, transfer cells into a new 10 cm plate, and adjust volume to 10 mL fresh growth medium supplemented with 100 µg/mL hygromycin. Allow the plate to reach ~80% confluency to further expand cells to prepare permanent stocks.

1.6. Determine the amount of tetracycline (Tet) required to obtain the optimal level of FLAG-POI expression for the experiment. Grow cells in 12-well format and conduct a titration of Tet between 0–1000 ng/mL range for 16–24 h. Perform western blots on the

titration samples with antibody against the POI.

NOTE: For proteins up to ~60 kDa, the FLAG-tagged and the endogenous copy of the protein can be resolved on the sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE) to compare expression levels of FLAG-POI to its endogenous counterpart. For larger proteins, signal intensity in Tet-induced samples can be compared to uninduced sample. The tetracycline stock solution can be prepared at 1 mg/mL in 100% ethanol. Dilutions of the stock for cell culture work should be in sterile water or phosphate-buffered saline (PBS). Tetracycline stock should be prepared fresh once every month.

2. Culturing cells for tetracycline induction and RIPit procedure

2.1. Seed stably integrated FLAG-POI HEK293 at a density of 3×10^5 cells/mL in growth medium in 15 cm plates. Allow cells to grow at 37 °C and 5% CO₂.

NOTE: In general, three to five 15 cm plates will yield ~2–20 pmol of RNA footprints depending on the abundance of the RNP of interest. If RNPs will be formaldehyde crosslinked and purified under stringent conditions, then twice as much input may be required.

2.2. Add tetracycline to pre-determined optimal concentration to the media to induce expression of the FLAG-POI (see step 1.6) 16–24 h before cells will be harvested.

NOTE: It is not necessary to change growth medium. Cells should be ready to harvest about 72 h after seeding or when plates are ~80% confluent. It is important to avoid cells from becoming confluent.

3. Cell harvesting, formaldehyde treatment, and cell lysis

3.1. Wash the monolayer cells gently with 15 mL of chilled PBS for each 15 cm plate. Then scrape off cells in 30 mL of PBS. If cells were treated with drugs prior to harvesting, supplement PBS with the drug. Collect all cells into 50 mL conical tube.

NOTE: For preserving weak interactions, cells can be crosslinked with formaldehyde: add formaldehyde to the cell suspension from step 3.1 to a final concentration of 0.1% and incubate on a rocker at room temperature for 10 min. Add 3 mL of the quenching buffer (Table 1) and rock for 5 additional minutes.

3.2. Pellet cells at 400 x g for 10 min at 4 °C and discard supernatant.

3.3. Lyse cells in 4 mL of ice-cold hypotonic lysis buffer (HLB; Table 1). Use a P1000 pipette to resuspend cells. Transfer to a 5 mL tube and incubate lysate on ice for 10 min.

3.4. Place the lysate in an ice bath and sonicate at 10% amplitude for 30 s with 1-s pulses with 2-s pauses. Then, adjust salt concentration to 150 mM by adding 108 μ L of 5 M NaCl.

NOTE: Formaldehyde crosslinked samples can be subject to more stringent lysis and purification by including 0.1% each of SDS and sodium deoxycholate in the lysis buffer.

3.5. Clear the lysate by centrifugation at 21,000 $\times g$ for 10 min at 4 $^{\circ}$ C. Collect 20 μ L supernatant (cell extract) in a labeled tube for western blot analysis of protein levels in input (see **Figure 3A**).

NOTE: While lysate is in centrifuge, FLAG agarose beads can be washed (see step 4.1). FLAG agarose beads should be pre-washed 3x in 4 mL of ice-cold isotonic wash buffer (IsoWB, **Table 1**).

4. FLAG immunoprecipitation

4.1. Apply remaining supernatant from step 3.5 to 750 μ L pre-washed FLAG agarose beads in a 5 mL tube (bed volume of washed FLAG agarose beads will be 375 μ L). Incubate FLAG agarose beads and cell extract for 1–3 h at 4 $^{\circ}$ C with gentle mixing.

NOTE: This volume of FLAG agarose beads should be sufficient for a wide-range of proteins, but may be optimized, if needed.

4.2. Pellet FLAG agarose beads by centrifugation at 400 $\times g$ for 1 min at 4 $^{\circ}$ C. Collect 20 μ L of the supernatant in a labeled tube for Western blot analysis of protein levels in depleted cell extract (see **Figure 3A**). Discard the remaining supernatant.

4.3. To wash FLAG agarose beads, add 4 mL of IsoWB and resuspend. Pellet beads at 400 $\times g$ for 1 min at 4 $^{\circ}$ C. Carefully remove supernatant. Repeat 4x.

NOTE: For stringent washes of the formaldehyde crosslinked IPs, 0.1% each of SDS and sodium deoxycholate can be included in the wash buffer for the first two wash steps.

5. RNase I digestion

5.1. Dilute RNase I to 0.002–0.01 units/mL in 750 μ L of IsoWB (the appropriate concentration for desired footprint sizes needs to be empirically determined). Add IsoWB-RNase I to washed FLAG agarose beads and incubate with gentle mixing at 4 $^{\circ}$ C for 10 min.

5.2. Pellet beads at 400 $\times g$ for 1 min at 4 $^{\circ}$ C. Collect 20 μ L supernatant (RNase I elution) in a labeled tube for Western blot analysis. Discard IsoWB-RNase I and wash FLAG agarose beads 4x with IsoWB as described in step 4.3.

6. Affinity elution

6.1. Prepare a stock of elution buffer (FLAG peptide at 250 ng/mL in IsoWB). Apply 375 μ L of elution buffer to FLAG agarose beads and shake gently at 4 °C for 1–2 h. Pellet FLAG agarose beads and collect a 15 μ L aliquot of the elution for Western blot of proteins in FLAG IP (see **Figure 3A**).

NOTE: When the affinity elution is underway, section 7 can be performed.

7. Magnetic bead-antibody conjugation

7.1. Wash 50 μ L magnetic beads (i.e., Dynabeads; **Table of Materials**) 3x in 1 mL IsoWB in 1.5 mL tube. Resuspend magnetic beads in 100 μ L of conjugation buffer. Add appropriate amount of antibody (exact amount of antibody to use for IP will need to be empirically determined for each antibody).

NOTE: Protein A magnetic beads are optimal for antibodies produced in rabbit whereas Protein G magnetic beads are more appropriate for mouse antibodies. Magnetic beads compatibility chart is available at supplier website to choose beads appropriate for each antibody.

7.2. Wash magnetic beads 2x in conjugation buffer (**Table 1**). Resuspend magnetic beads in 375 μ L of RIPiT dilution buffer. Store on ice until next step.

8. Second immunoprecipitation

8.1. Apply remaining FLAG affinity elution from step 6.1 to magnetic beads coupled to antibodies against the protein of interest. Incubate with gentle mixing at 4 °C for 1–2 h. Capture magnetic beads on a magnet and collect 15 μ L of supernatant for analysis of unbound proteins via Western blot. Wash magnetic beads 7x with 1 mL of IsoWB.

9. Denaturing elution

9.1. Add 100 μ L of clear sample buffer (**Table 1**) to magnetic beads and resuspend with a P200 pipette. Incubate on ice for 10 min. Flick gently to resuspend beads periodically.

9.2. Capture magnetic beads on a magnet and collect 15 μ L of elution for analysis of proteins in RIPiT elution via western blot (see **Figure 3A**). Transfer remaining elution into a new labeled 1.5 mL tube.

NOTE: If samples were formaldehyde crosslinked, then samples must be incubated at 65 °C for 1 h to reverse crosslinking.

9.3. Perform western blots on samples collected at various steps (input, FLAG IP

depletion, FLAG IP, second IP depletion, second IP elution). Blot with antibodies against the two bait proteins, their other interactors if known, and at least one non-interacting RBP as a negative control (**Figure 3A**).

10. RNA extraction and end curing

10.1. To the RIPiT elution, add 320 μL of RNase-free ddH₂O, 400 μL of phenol-chloroform isoamyl alcohol (PCIAA, pH 4.5), and vortex for 30 s and spin at room temperature at 12,000 $\times g$ for 5 min. Collect 350 μL of aqueous phase into a separate tube. Add 35 μL of 3 M sodium acetate, 1 μL of 1 M MgCl₂, 10 μg of glycogen, and 1 mL of 100% ethanol. Incubate overnight at -20 °C.

10.2. To pellet RNA, centrifuge at 12,000 $\times g$ for 30 min at 4 °C. Wash RNA in 70% ethanol.

10.3. To remove 3' phosphate left on RNA after RNase I cleavage, resuspend RNA pellet in 17 μL of RNase-free ddH₂O, and add 2 μL of 10x T4 polynucleotide kinase (PNK) buffer (**Table of Materials**) and 1 μL of T4 PNK. Incubate at 37 °C for 30 min.

NOTE: The 3'phosphatase activity of T4 PNK has optimal activity at pH 6¹⁵. The PNK reaction buffer is optimized for the 5' kinase activity of T4 PNK and has a pH of 7.6. While adjusting the pH of the end-curing reaction has been attempted, this step can be further optimized.

10.4. Add 380 μL of RNase-free ddH₂O and 400 μL of PCIAA pH 4.5 to the tube. Vortex for 30 s, centrifuge at 12,000 $\times g$ for 5 min. Collect aqueous phase and add 35 μL of 3 M sodium acetate, 1 μL of 1 M MgCl₂, 10 μg of glycogen, and 1 mL of 100% ethanol.

10.5. Incubate overnight at -20 °C. Pellet and wash RNA with 70% ethanol as above. Resuspend RNA in 4.5 μL of RNase-free water.

11. Estimation of RNA footprint size and abundance

11.1. A successful RIPiT is expected to yield 1 pmol or more of RNA fragments. To quantify actual yield, transfer 0.7 μL of RIPiT RNA (~1/6 of total yield) into a new tube. Add 2 μL of 10x T4 PNK buffer, 1 μL of 1 mM ATP, 40 μCi $\gamma^{32}\text{P}$ -ATP (0.5–1.0 μL of the stock), and 1 μL of T4 PNK. Adjust volume to 10 μL and incubate at 37 °C for 30 min.

11.1.1. In parallel PNK reactions, label a low range DNA Ladder and 0.1 pmol of a synthetic RNA or DNA oligo (20–40 nt) to use as size and quantity standards.

11.2. Resolve labeled RNA/DNA on 26% urea-PAGE gel (20 x 27 x 0.45 mm). Gel must be pre-run for 30 min at 35 W. Flush wells before pre-run and before loading samples and run at 35 W until bromophenol blue dye front has almost reached the end of the gel.

11.3. Carefully remove gel from glass plates on to a piece of 8 x 11 inch filter paper. With gel on top of paper, place in gel drying apparatus and cover with a piece of plastic wrap. Dry gel at 80 °C for 1 h with vacuum.

11.4. Expose dried gel to a phosphoscreen overnight or until adequate signal is detected. Image phosphoscreen. Good quality RNA from a RIPiT should appear as a smear in the lane, with minimal prominent bands (**Figure 3B**). To quantify RNA, compare signal intensity of the desired size RNA fragments in RIPiT lane to the signal from 0.1 pmol of labeled synthetic oligo.

NOTE: Alternatively, RNA footprint size and amounts can be verified using a high-sensitivity bioanalyzer (**Figure 3C**).

12. Adapter ligation

12.1. Prepare RIPiT RNA such that at least 3 pmol of RNA is dissolved in 3.8 µL of water.

12.2. In a 0.2 mL polymerase chain reaction (PCR) tube, combine 3.8 µL of RNA, 1 µL of miR-CAT-33 pre-adenylated adapter (7 µM) (**Table of Materials**). Incubate the mix on a thermal cycler at 65 °C for 10 min, 16 °C for 5 min, then hold at 4 °C.

NOTE: The pre-adenylated linker can be either ordered from oligo synthesis service, or a custom unadenylated DNA oligo from any oligo synthesis service can be adenylated using Mth RNA ligase (**Table of Materials**) and gel purified.

12.3. To the same tube, add 1.5 µL of 10x T4 RNA ligase buffer, 7.5 µL of 50% polyethylene glycol 8000 (PEG-8000), 0.75 µL of 20 mM dithiothreitol (DTT), and 0.45 µL of T4 RNL2 Tr. K227Q (**Table of Materials**).

NOTE: 50% PEG-8000 comes with RNA ligase and buffer purchased. PEG-8000 solutions are viscous and should be pipetted slowly.

12.4. Incubate reaction in the thermal cycler at 30 °C for 6 h, heat inactivate the ligase at 65 °C for 10 min, then hold at 4 °C.

13. Reverse transcription

13.1. To the tube with the ligation mix from step 12.4, add 11.25 µL of 4x deoxynucleotide triphosphate (dNTP) mix, which contains a mix of regular and biotinylated dNTPs (see **Table 1**), 1.0 µL of 10 µM RT primers (**Table of Materials**), and 6.8 µL of RNase-free water. Incubate at 65 °C for 5 min, then hold at 4 °C.

13.2. Transfer tubes to ice and add 9.0 µL of 5x first-strand (FS) buffer without MgCl₂ (**Table 1**), 2.25 µL of 100 mM DTT, 1.2 µL of reverse transcriptase enzyme to a final volume

of 45 μ L (**Table of Materials**).

13.3. Incubate in a thermal cycler at 55 °C for 30–60 min. Heat inactivate reverse transcriptase at 70 °C for 15 min and hold the sample at 4 °C.

14. Purification of RT product

14.1. Add 45 μ L of 2x urea load buffer (**Table 1**) to RT reaction. Dilute 1 μ g of a low range DNA Ladder in 45 μ L and add 45 μ L of 2x urea load buffer.

14.2. Prepare 10% urea-PAGE gel (20 x 28 x 0.15 cm; **Table 1**) with 8-well comb. Using syringe or pipet, flush wells with 0.5x Tris/borate/EDTA (TBE) buffer.

NOTE: The homemade gels above offer better resolution in separating the extended RT product from the unextended RT primer. As an alternative, pre-cast urea-PAGE gels can also be used (**Table of Materials**). As pre-cast gels allow smaller maximum volumes per well, so samples will need to be divided into multiple wells. Pre-cast gels should be run at 150–200 V.

14.3. Pre-run the gel at 35 W for 30 min. Flush wells again, load samples and run gel at 35 W until bromophenol blue dye front has migrated to about 1 inch from the end of the gel.

NOTE: Use metal heat sink during the pre-run and the final run to prevent gel overheating.

14.4. Stain the gel for 5 min in 1x gold nucleic acid gel stain solution prepared in 0.5x TBE. This dye is light sensitive, so avoid light exposure.

14.5. Image gel on fluorescent scanner for documentation purposes using 520 nm excitation and 580 nm emission filters. If a fluorescent scanner is unavailable, use a blue light transilluminator. The RT product should appear as a smear starting above the unextended RT primer (**Figure 4**).

NOTE: Although the gold nucleic acid gel staining dye is easily visualized on a gel doc with UV-light source, it is vital to not expose the valuable RT product to UV to prevent damage.

14.6. Visualize the gel on a blue light transilluminator and excise the RT product from the gel. It is recommended to cut DNA with extensions ranging from 30–200 nt (**Figure 4**). Place the excised gel pieces on a clean surface and mince the slice into small pieces to increase surface area. Carefully transfer to a 1.5 mL tube and add 800 μ L of DNA elution buffer (**Table 1**).

14.7. Incubate the gel pieces with gentle mixing with DNA elution buffer over night at room temperature.

397 14.8. Separate the elution buffer from the gel by passing the slurry through a cellulose
398 acetate filter column (**Table of Materials**) placed in a 2 mL collection tube.

399
400 14.9. In 1.5 mL tube, wash 10 μ L of streptavidin magnetic beads with 500 μ L of
401 streptavidin bead wash buffer (**Table 1**). Repeat for three total washes. Do not let beads
402 dry out. Resuspend beads in 10 μ L of DNA elution buffer (**Table 1**).

403
404 14.10. Transfer the elution buffer separated from the gel pieces in step 14.8 to the tube
405 containing the washed streptavidin magnetic beads.

406
407 14.11. Incubate with gentle mixing for at least 8 h at room temperature. Capture beads
408 on a magnet, remove supernatant and resuspend magnetic beads in 10 μ L of RNase-free
409 water and transfer to a 0.2 mL PCR tube.

410 411 15. Circularization of RT product

412
413 15.1. RT products captured on streptavidin beads are circularized while bound on beads.
414 To the magnetic bead slurry, add 2.0 μ L of 10x circularization reaction buffer, 1.0 μ L of 1
415 mM ATP, 1.0 μ L of 50 mM $MnCl_2$, 4.0 μ L of 5 M betaine, 1.0 μ L of ssDNA ligase I (**Table of**
416 **Materials**), and 1.0 μ L of RNase-free water.

417
418 15.2. Incubate the circularization reaction on a thermal cycler at 60 $^{\circ}C$ for 4 h. Heat
419 inactivate the ssDNA ligase I by heating at 80 $^{\circ}C$ for 10 min then hold at 4 $^{\circ}C$.

420 421 16. Test PCR

422
423 16.1. Before proceeding to a large-scale PCR, use a portion of circularized product to
424 determine the ideal number of amplification cycles for each sample. This step helps
425 prevent over-amplification and limit PCR artifacts as PCR reaction components become
426 limiting at higher PCR cycles.

427
428 16.2. Prepare a 45 μ L PCR reaction with 4.0–6.0 μ L of the circularized product from step
429 15.2, 9.0 μ L of 5x reaction buffer, 0.9 μ L 10 M dNTPs, 2.25 μ L of 10 μ M PE1.0 primer (**Table**
430 **of Materials**), 2.25 μ L of 10 μ M PE2.0 primer (**Table of Materials**), and 0.045 μ L of high-
431 fidelity DNA polymerase (**Table of Materials**), and water.

432
433 16.3. Mix reaction well and split into three 15 μ L reactions. Each of these three reactions
434 will be subject to a variable number of PCR cycles. The ideal number of cycles is expected
435 to be between 7 and 14. So perform test PCRs for 8, 11, and 14 cycles.

436
437 16.4. Use the following PCR conditions: 98 $^{\circ}C$ —30 s; 98 $^{\circ}C$ —5 s; 65 $^{\circ}C$ —10 s; 72 $^{\circ}C$ —15 s;
438 72 $^{\circ}C$ —2 min; 12 $^{\circ}C$ —hold.

439
440 16.5. Add 3 μ L of 6x gel loading dye and resolve on 10% native PAGE gel until blue dye

front has migrated 3/4 of the gel. Stain the gel using gold nucleic acid gel stain and image as in steps 14.4 and 14.5 (**Figure 5**).

16.6. To choose the ideal number of PCR cycles, compare PCR products from increasing number of cycles. Choose the cycle number that yields the greatest amount of product of the expected size without overamplification artifacts (e.g., DNA smear much larger than expected product, and where no appreciable depletion of the PE1.0 and PE2.0 primers is seen (see red arrow in **Figure 5**).

17. Large-scale PCR

17.1. Prepare a 45 μ L PCR reaction, as in step 16.2 and repeat the PCR. Resolve PCR on 10% native PAGE at 150 V, stain with 1x gold nucleic acid gel stain and image on a blue light transilluminator.

17.2. Excise the PCR product from the gel and transfer to a 3 mL syringe. Use the syringe to crush the gel and extrude into a 1.5 mL tube.

NOTE: The unextended RT product upon circularization yields PCR product of 151 bp. Thus, at this step one should size select products that are larger than 151 bp (**Figure 6**).

17.3. Add 900 μ L of DNA elution buffer and incubate at room temperature overnight with gentle mixing.

17.4. Transfer the gel slurry to a cellulose acetate filter column placed in a 2 mL collection tube. Spin at 12,000 x *g* for 3 min, collecting supernatant into a fresh tube.

17.5. Add another 400 μ L of DNA elution buffer to the crushed gel and transfer to a 1.5 mL tube. Incubate with gentle mixing for an additional 4 hours for a second elution.

17.6. Pool all elutions and split into 3 tubes with 400 μ L each. Precipitate DNA by adding 1 mL of 100% ethanol and 10 μ g of glycogen. Vortex and incubate at least 2 h at -20 °C.

17.7. Pellet DNA at 12,000 x *g* for 30 min at 4 °C. Wash DNA pellet with 70% ethanol.

17.8. Carefully remove all ethanol by pipetting and quickly resuspend DNA pellet in 20 μ L of water.

NOTE: At this stage, it is important to not let the DNA pellet become dry, as drying DNA out can denature it.

17.9. Use a small portion of DNA sample to determine size and concentration of the PCR product via fluorometer and high sensitivity DNA bioanalyzer. Samples can now be submitted for sequencing on one of the platforms.

17.10. The sequenced reads can be processed (e.g., adapter removal, trimming to keep sequences >30 Phred score), aligned to the reference genome, and visualized on a browser such as UCSC genome browser (**Figure 7**).

REPRESENTATIVE RESULTS:

A successful RIPiT will result in the immunoprecipitation of both proteins of interest and other known interacting proteins, and the absence of non-interacting proteins. As seen in **Figure 3A**, both Magoh and EIF4AIII were detected in the RIPiT elution, but HNRNPA1 was not (lane 6). In parallel, RNA footprints that have co-purified with the RNP complexes was detected via autoradiography (**Figure 3B**) or bioanalyzer (**Figure 3C**). Puromycin treatment is expected to increase EJC occupancy on RNA, and a stronger RNA footprint signal was observed in the puromycin treated RIPiT in **Figure 3B** (compare lanes 2 and 3). Generating samples for deep sequencing requires ligating an adapter to the RNA, and then reverse transcribing the RNA into DNA using a primer that anneals to the adapter sequence. The reverse transcription step incorporates biotinylated nucleotides, for purification of reverse transcription product. Following the reverse transcription, the product must be separated from the unextended adapter by urea-PAGE (**Figure 4**). The reverse transcription product is then circularized and PCR amplified. The appropriate number of PCR cycles must not overamplify the circularized product. Overamplification will result in primer depletion and aberrant PCR product (see **Figure 5**, lane 4). The number of cycles with the greatest amplification without evidence of overamplification is most appropriate to use for a large-scale PCR (**Figure 5** lane 3 and **Figure 6**).

FIGURE AND TABLE LEGENDS:

Figure 1: Schematic outlining the main steps in RIPiT.

Figure 2: Schematic depicting the workflow for conversion of RIPiT RNA into libraries for high-throughput sequencing.

Figure 3: Estimation of RNA and protein yields from RIPiT. (A) Western blot of the proteins purified from each major step in the RIPiT procedure. **(B)** Autoradiograph image of RNA footprints from a FLAG-MAGOH:EIF4AIII RIPiT comparing puromycin treated and untreated cells. Red box indicates the RNA footprint size that will ultimately be converted into sequencing libraries. **(C)** Profile of RNA eluted from RIPiT as in lane 2 in panel B when visualized using a bioanalyzer.

Figure 4: Reverse transcription (RT) product resolved on a 10% urea-PAGE gel and stained with gold nucleic acid gel stain. Red box indicates gel region excised for gel purification of extended RT products.

Figure 5: Test PCR resolved on 8% non-denaturing PAGE. Note the aberrantly large PCR products which appear at 14 cycles (red box) and the parallel depletion of primers (red arrow) indicative of overamplification. For this sample, 11 cycles was chosen for the large-

scale PCR (blue box).

Figure 6: Large-scale PCR resolved on 8% non-denaturing PAGE. Red box indicates the gel piece excised for gel purification.

Figure 7: Genome browser screenshot of the MAPK1 gene showing distribution of FLAG-MAGOH:EIF4AIII footprints as representative results from a RIPiT. Red arrows denote the expected canonical EJC binding sites.

Table 1: Buffers.

DISCUSSION:

We discuss here some key considerations to successfully perform RIPiT. Foremost, individual IPs must be optimized to achieve highest possible efficiency at each step. The amount of FLAG agarose beads for the input number of cells described here has proven to be robust for a wide range of proteins we have tested. As only a small fraction of partner proteins is co-immunoprecipitated with the FLAG protein, the amount of antibody needed for efficient second IP is usually low (less than 10 micrograms). Small-scale RIPiT (from one 10 cm plate) followed by western blot verification of proteins in each fraction during the two immunoprecipitation steps prove extremely useful to assess efficiency and specificity of the procedure before scaling up. Both targeted proteins as well as any other expected interacting proteins in the complex should be detected in the elution. It is also beneficial to assay for proteins in the depleted lysates (unbound to the FLAG-agarose or magnetic beads) to have a good estimate of the immunoprecipitation efficiency and the percentage of proteins assembling into a complex. Further, this analysis also informs if the RNase digestion conditions are sufficient to separate RNA-dependent interactions from RNA-independent interactions within an RNP. Therefore, it is just as important to include a negative control, ideally an RBP unrelated to the RNP of interest. For example, in **Figure 3A**, HNRNPA1 is present in the input but is not detected in the RIPiT elution. HNRNPA1 is an RBP that does not directly interact with the EJC but interacts indirectly with the EJC when the EJC and HNRNPA1 are bound to the same RNA molecule. Detection of the negative control protein in the elution indicates either poor RIPiT specificity or insufficient RNA footprinting. In such a case, the RNA footprints obtained will not completely reflect the footprints of the protein of interest. Footprints of size 50–200 nt are recommended for subsequent RNA-Seq. Duration of RNase I treatment or the amount of enzyme used can be optimized to obtain desired size footprints. Note that the best-case scenario will be to obtain good signal in the desired size range, and it is unavoidable to have longer and shorter RNAs even in the most optimal conditions. RIPiT can also be used to obtain binding sites of a single RBP. In such a case, the same protein can be immunoprecipitated with two different antibodies, first using an antibody against an affinity tag and then with antibodies against the protein itself¹⁶. Finally, a negative control RIPiT can be performed in parallel from cells expressing a FLAG-tagged control protein (e.g., green fluorescent protein) in combination with antibody against a protein against an unrelated protein in the second IP.

Despite its many advantages, it is important to consider some limitations of the RIPiT approach, and possible remedies. The requirement of affinity elution after the first purification necessitates the biological source to express a tagged protein. If a site-specific recombination system is not available in the cell line or organism of interest, a short affinity tag such as a FLAG tag (8 amino acids) can be introduced at the endogenous gene locus using CRISPR/Cas-based genome editing approach¹⁷. The FLAG tag is an ideal epitope for this approach, because the FLAG antibody is well-suited for affinity elution and can withstand high ionic strengths and mild denaturing conditions that can be used in combination with formaldehyde crosslinking. Another limitation of the RIPiT approach is the requirement for a large input of cellular material. This may remain unavoidable to some extent as only a small percentage of an RBP likely interacts with other proteins in the RNP. Still improved library preparation approaches can help to bring down the large input requirement. Possible ideas to further streamline these steps include carrying out the RNA 3'-end dephosphorylation and adapter ligation on the magnetic beads immediately after second IP washes and prior to the final elution of RNP. Such an approach is successfully implemented in current CLIP-Seq procedures and in a recently described variation of RIPiT¹⁸. Such changes will also remove several time-consuming RNA purification steps from the early phases of library preparation procedure. Further, unlike CLIP, which provides a nucleotide level resolution of crosslinking site of an RBP on the RNA, resolution of RIPiT footprints will remain at the level of tens of nucleotides. Finally, as RNPs may include multiple RBPs, the RIPiT enriched RNA sites include a mixture of binding sites of many RBPs. As consensus sequences bound by individual RBPs are being uncovered at a rapidly increasing pace and are now readily available^{19–21}, this information can be leveraged to deconvolve the assortment of RBP sites enriched in RIPiT outputs. Notwithstanding these challenges, RIPiT-Seq is an effective procedure for capturing RNA footprints of dynamic, heterogeneous, and even transient RNP complexes, which can provide unique insights into the inner workings of RNA machineries that control cellular function.

ACKNOWLEDGMENTS:

This work was supported by the NIH grant GM120209 (GS). The authors thank the OSUCCC Genomics Shared Resources Core for their services (CCC Support Grant NCI P30 CA16058).

DISCLOSURES:

The authors have nothing to disclose.

REFERENCES:

1. Karousis, E. D., Nasif, S., Mühlemann, O. Nonsense-mediated mRNA decay: novel mechanistic insights and biological impact. *Wiley Interdisciplinary Reviews: RNA*. **7** (5), 661–682 (2016).
2. Ivanov, P.V., Gehring, N.H., Kunz, J.B., Hentze, M.W., Kulozik, A.E. Interactions between UPF1, eRFs, PABP and the exon junction complex suggest an integrated model

617 for mammalian NMD pathways. *The EMBO Journal*. **27** (5), 736–747, doi:
618 10.1038/emboj.2008.17 (2008).

619 3. Papasaïkas, P., Valcárcel, J. The Spliceosome: The Ultimate RNA Chaperone and
620 Sculptor. *Trends in Biochemical Sciences*. **41** (1), 33–45, doi: 10.1016/j.tibs.2015.11.003
621 (2016).

622 4. Jensen, K.B., Darnell, R.B. CLIP: Crosslinking and ImmunoPrecipitation of In Vivo RNA
623 Targets of RNA-Binding Proteins. *Methods in Molecular Biology (Clifton, N.J.)*. **488**, 85–98,
624 doi: 10.1007/978-1-60327-475-3_6 (2008).

625 5. Garzia, A., Morozov, P., Sajek, M., Meyer, C., Tuschl, T. PAR-CLIP for Discovering Target
626 Sites of RNA-Binding Proteins. *mRNA Decay: Methods and Protocols*. **1720**, 55–75, doi:
627 10.1007/978-1-4939-7540-2_5 (2018).

628 6. König, J. et al. iCLIP - Transcriptome-wide Mapping of Protein-RNA Interactions with
629 Individual Nucleotide Resolution. *Journal of Visualized Experiments*. (50), e2638, doi:
630 10.3791/2638 (2011).

631 7. Sibley, C.R. Individual Nucleotide Resolution UV Cross-Linking and
632 Immunoprecipitation (iCLIP) to Determine Protein–RNA Interactions. *RNA Detection:
633 Methods and Protocols*. **1649**, 427–454, doi: 10.1007/978-1-4939-7213-5_29 (2018).

634 8. Wheeler, E.C., Van Nostrand, E.L., Yeo, G.W. Advances and challenges in the detection
635 of transcriptome-wide protein–RNA interactions. *Wiley Interdisciplinary Reviews: RNA*. **9**
636 (1), doi: 10.1002/wrna.1436 (2018).

637 9. Mabin, J.W. et al. The Exon Junction Complex Undergoes a Compositional Switch that
638 Alters mRNP Structure and Nonsense-Mediated mRNA Decay Activity. *Cell Reports*. **25** (9),
639 2431–2446., doi: 10.1016/j.celrep.2018.11.046 (2018).

640 10. Gehring, N.H., Lamprinakì, S., Kulozik, A.E., Hentze, M.W. Disassembly of Exon
641 Junction Complexes by PYM. *Cell*. **137** (3), 536–548, doi: 10.1016/j.cell.2009.02.042
642 (2009).

643 11. Dostie, J., Dreyfuss, G. Translation Is Required to Remove Y14 from mRNAs in the
644 Cytoplasm. *Current Biology*. **12** (13), 1060–1067, doi: 10.1016/S0960-9822(02)00902-8
645 (2002).

646 12. Zünd, D., Gruber, A.R., Zavolan, M., Mühlemann, O. Translation-dependent
647 displacement of UPF1 from coding sequences causes its enrichment in 3' UTRs. *Nature
648 Structural & Molecular Biology*. **20** (8), 936–943, doi: 10.1038/nsmb.2635 (2013).

649 13. Gangras, P., Dayeh, D.M., Mabin, J.W., Nakanishi, K., Singh, G. Cloning and
650 Identification of Recombinant Argonaute-Bound Small RNAs Using Next-Generation
651 Sequencing. *Argonaute Proteins: Methods and Protocols*. **1680**, 1–28, doi: 10.1007/978-
652 1-4939-7339-2_1 (2018).

653 14. Heyer, E.E., Ozadam, H., Ricci, E.P., Cenik, C., Moore, M.J. An optimized kit-free
654 method for making strand-specific deep sequencing libraries from RNA fragments. *Nucleic
655 Acids Research*. **43** (1), e2, doi: 10.1093/nar/gku1235 (2015).

656 15. Cameron, V., Uhlenbeck, O.C. 3'-Phosphatase activity in T4 polynucleotide kinase.
657 *Biochemistry*. **16** (23), 5120–5126 (1977).

658 16. Ricci, E.P. et al. Staufen1 senses overall transcript secondary structure to regulate
659 translation. *Nature Structural & Molecular Biology*. **21** (1), 26–35, doi:
660 10.1038/nsmb.2739 (2014).

17. Lackner, D.H. et al. A generic strategy for CRISPR-Cas9-mediated gene tagging. *Nature Communications*. **6**, 10237, doi: 10.1038/ncomms10237 (2015).
18. Metkar, M. et al. Higher-Order Organization Principles of Pre-translational mRNPs. *Molecular Cell*. **72** (4), 715–726.e3, doi: 10.1016/j.molcel.2018.09.012 (2018).
19. Giudice, G., Sánchez-Cabo, F., Torroja, C., Lara-Pezzi, E. ATtRACT-a database of RNA-binding proteins and associated motifs. *Database: The Journal of Biological Databases and Curation*. **2016**, doi: 10.1093/database/baw035 (2016).
20. Paz, I., Kosti, I., Ares, M., Cline, M., Mandel-Gutfreund, Y. RBPmap: a web server for mapping binding sites of RNA-binding proteins. *Nucleic Acids Research*. **42** (Web Server issue), W361–367, doi: 10.1093/nar/gku406 (2014).
21. Sundararaman, B. et al. Resources for the Comprehensive Discovery of Functional RNA Elements. *Molecular Cell*. **61** (6), 903–913, doi: 10.1016/j.molcel.2016.02.012 (2016).

Figure 1.

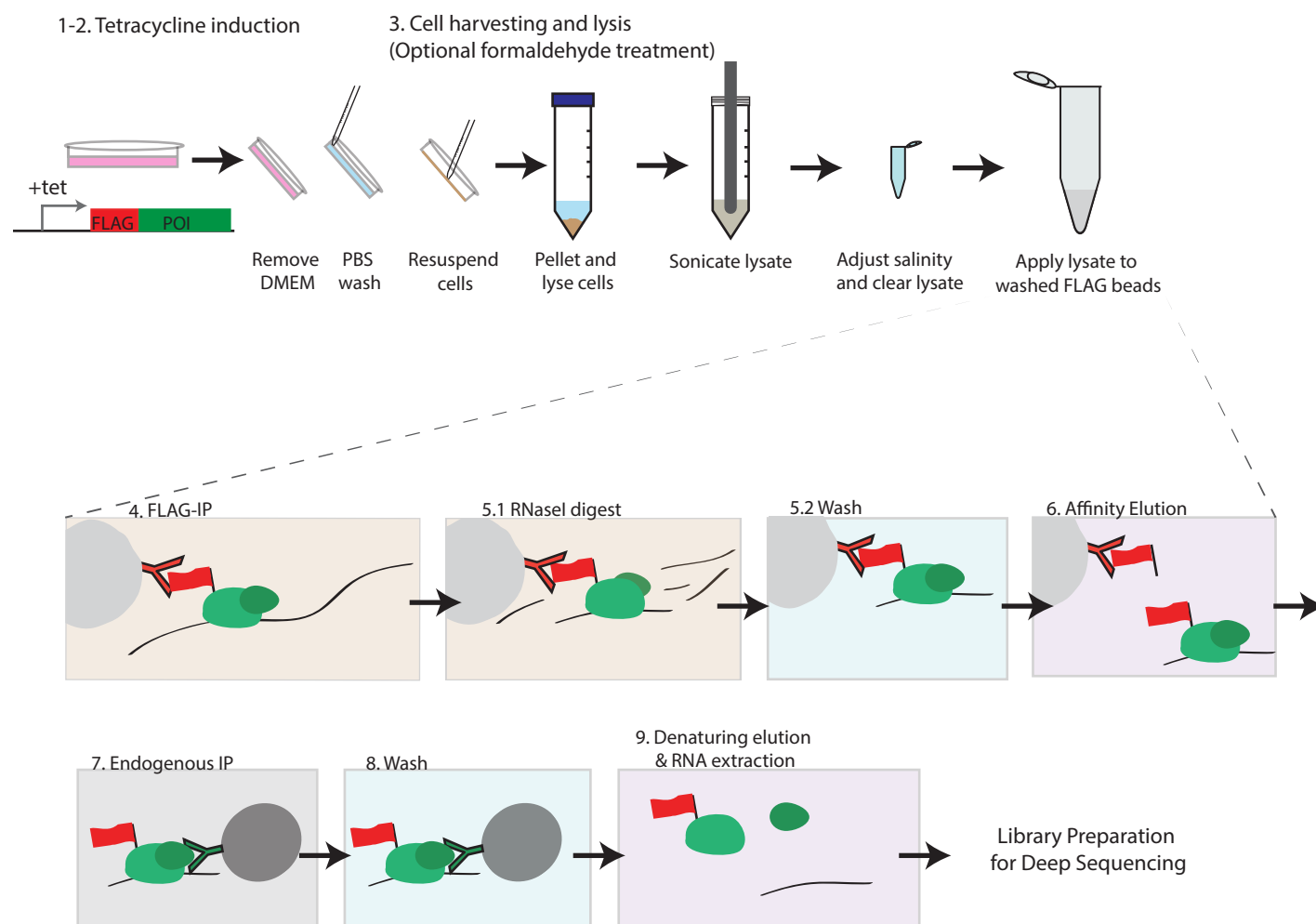


Figure2.

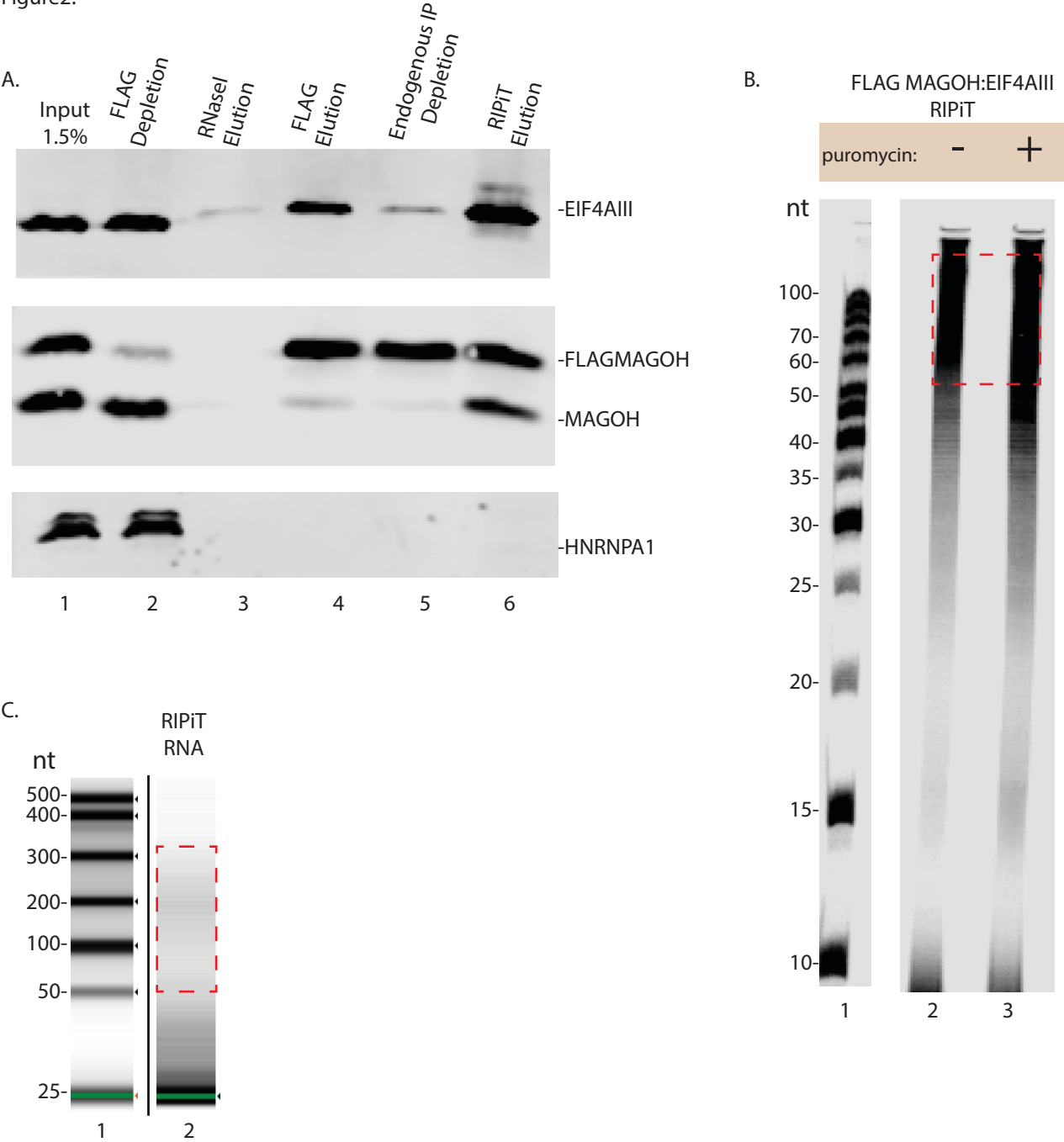


Figure 3.

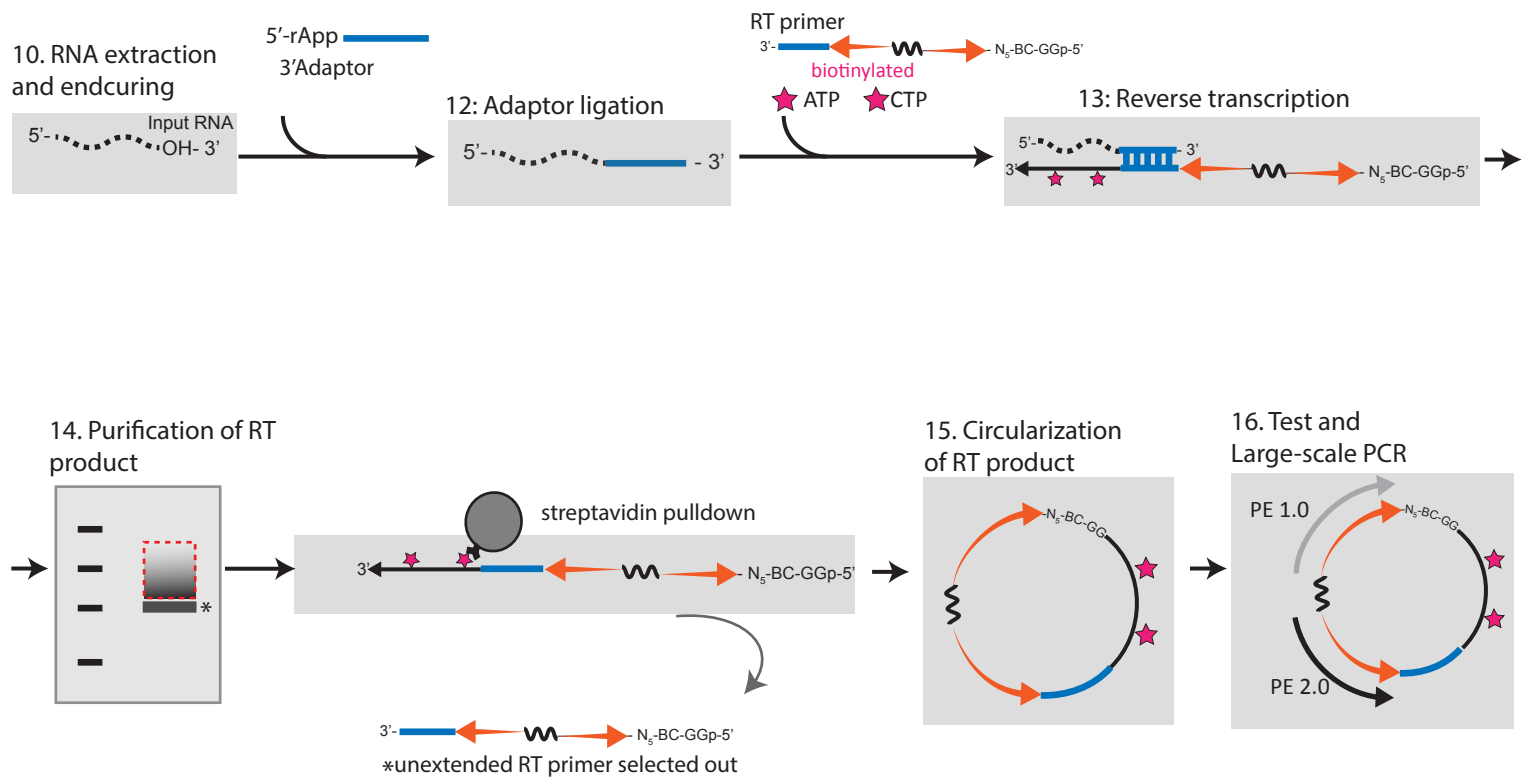
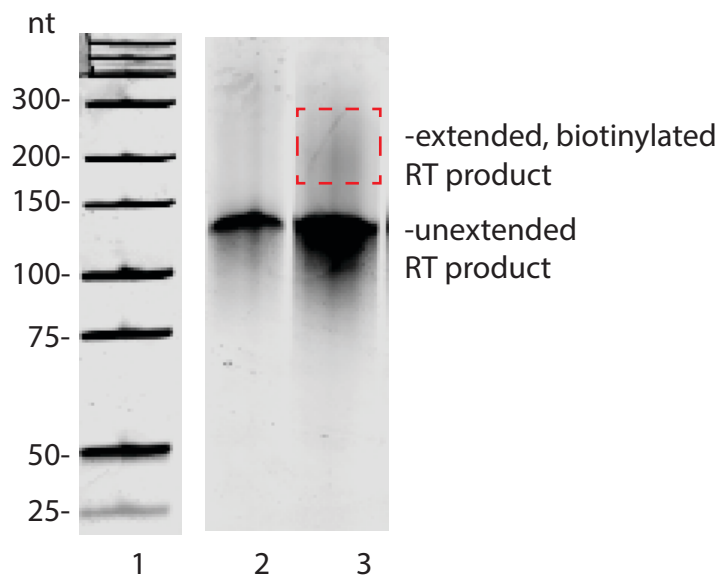


Figure 4.



(Section: 14)

Figure 5.

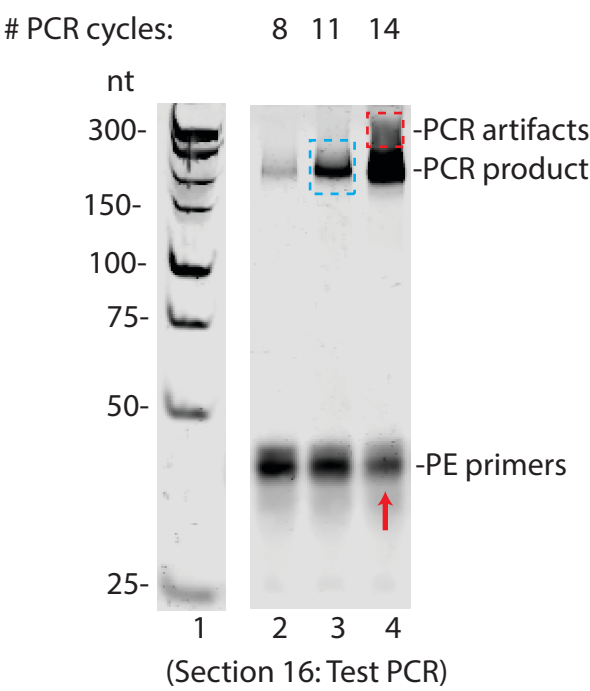
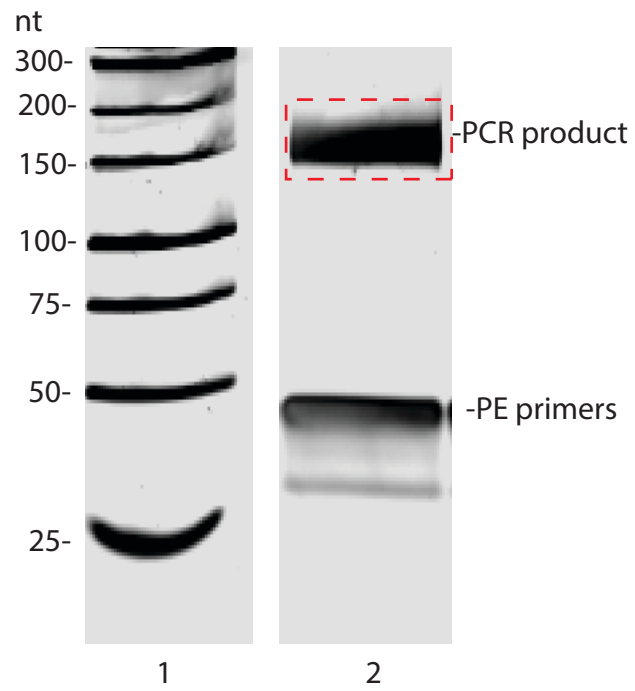
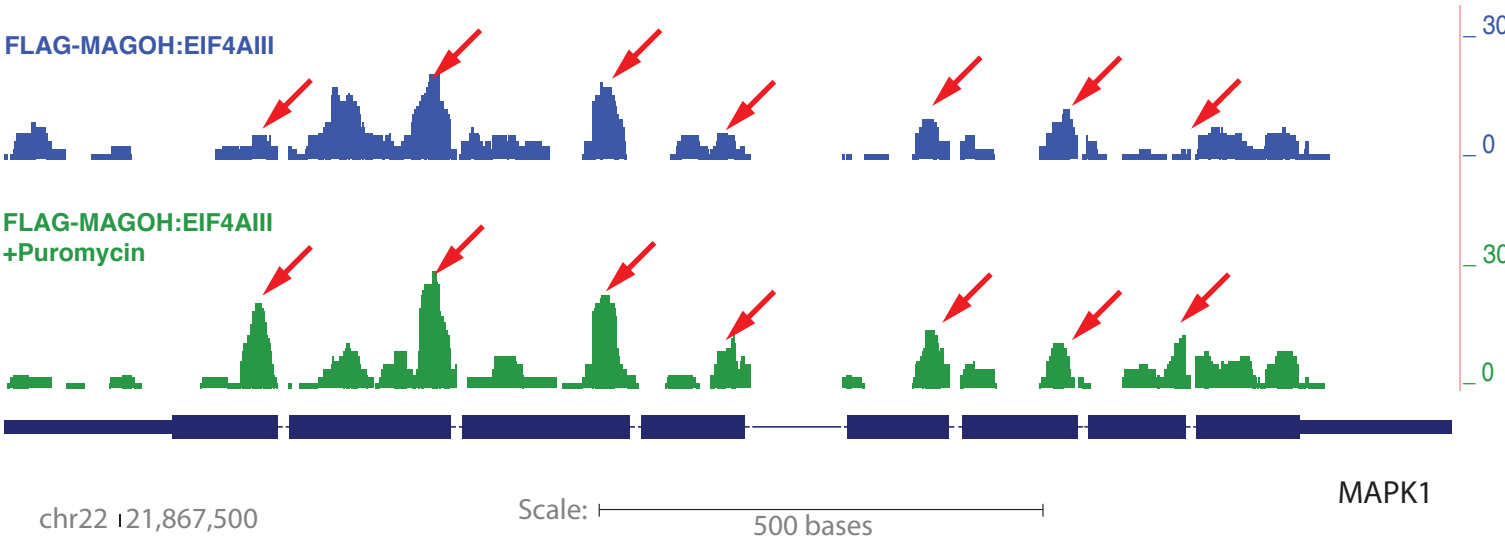


Figure 6.



(Section 17: Large-scale PCR)

Figure 7.



Buffers

PBS

137 mM NaCl
 2.7 mM KCl
 10 mM Na₂HPO₄·7H₂O
 KH₂PO₄
 pH 7.4

Quenching Buffer

2.5 M Glycine
 2.5 mM Tris-Base

Hypotonic Lysis Buffer (HLB)

20 mM Tris-HCl pH 7.5
 15 mM NaCl
 10 mM EDTA
 0.5% IGEPAL
 0.1% Triton-X-100
 1x Aprotinin*
 1x Leupeptin*
 1x Pepstatin*
 1 mM PMSF (phenylmethylsulfonyl fluoride)*

Isotonic Wash Buffer (IsoWB)

20 mM Tris-HCl pH 7.5
 150 mM NaCl
 0.1% IGEPAL

Conjugation Buffer

0.02% Polysorbate-20
 1x PBS

Clear Sample Buffer

100 mM Tris-HCl pH 6.8
 4% SDS
 10 mM EDTA

4xdNTPmix

0.25 mM dGTP
 0.25 mM dTTP
 0.175 mM dATP
 0.1625 mM dCTP
 0.075 mM biotin-dATP

0.0875 mM biotin-dCTP

5x First-Strand Buffer w/o MgCl₂ 250 mM Tris-HCl pH 8
375 mM KCl

RIPiT dilution buffer (1 mL) 1 mL IsoWB
5 µL 200x BSA
20 µL 10% Triton-X-100
20 µL 0.5M EDTA
1x Aprotinin*
1x Leupeptin*
1x Pepstatin*
1 mM PMSF*

2x Denaturing Load Buffer 3 mL 5x TBE
1.8 g Ficoll Type 400
6.3 g urea
3 mg bromophenol blue
3 mg xylene cyanol
Adjust volume to 15 mL ddH₂O

Urea-PAGE gel 6 M Urea
Acrylamide:bisacrylamide (40% [w/v]) to appropriate p
0.5x TBE
0.01% TEMED

DNA Elution Buffer 300 mM NaCl
1 mM EDTA

Streptavidin Bead Wash Buffer 0.5 M NaOH

20 mM Tris-HCl pH 7.5
1 mM EDTA

*must be added fresh every time

*must be added fresh every time

To get into solution, place tube in water in a beaker and boil on hot plate for 10–15 min. Add dyes after adjusting the volume to 15 mL

percentage

Name of Material/ Equipment	Company	Catalog Number
Anti-FLAG Affinity Gel	Sigma	A2220
ATP, [γ -32P]- 3000Ci/mmol 10mCi/ml EasyTide, 250 μ Ci	PerkinElmer	BLU502A250UC
BD Disposable Syringes with Luer-Lok Tips (200)	Fisher	14-823-435
Betaine 5M	Sigma	B0300
biotin-dATP	TriLink	N-5002
biotin-dCTP	Perkin Elmer	NEL540001EA
Branson Sonifier, Model SSE-1	Branson	
CircLigase I	VWR	76081-606
DMEM, High Glucose	ThermoFisher	11995-065
DNA load buffer NEB	NEB	
Dynabeads Protein A	LifeTech	10002D
Flp-In-T-REx 293 Cell Line	ThermoFisher	R78007
GeneRuler Low Range DNA Ladder	ThermoScientific	FERSM1203
Hygromycin B	ThermoFisher	10687010
Mini-PROTEAN TBE Gel 10 well	Bio-Rad	4565013
Mini-PROTEAN TBE-Urea Gel	Bio-Rad	4566033
miRCAT-33 adapter 5'-TGGAATTCTCGGGTGCCAAGGddC-3'	Any	
Mirus transIT-X2 transfection reagent	Mirus	MIR 6004
Mth RNA ligase	NEB	E2610S
PE1.0 5'-AATGATACGGCGACCAACCGAGATCTACACTCTTTCCC'	Any	
PE2.0 5'-CAAGCAGAAGACGGCATACGAGATCGGTCTCGGCAT	Any	
Phenol/Chloroform/Isoamyl Alcohol (25:24:1, pH 6.7, 100ml)	Fisher	BP1752I-100
Purple Gel Loading Dye (6x)	NEB	NEB #7025
Q5 DNA Polymerase	NEB	M0491S/L
RNase I, E. coli, 1000 units	Eppicenter	N6901K
SPIN-X column	Corning	CLS8160-24EA
Streptavidin beads	ThermoFisher	60210

Superscript III (SSIII)	ThermoScientific	18080044
SybrGold	ThermoFisher	S11494
T4 Polynucleotide Kinase-2500U	NEB	M0201L
T ₄ RNL2 Tr. K227Q	NEB	M0351S
Tetracycline	Sigma	87128
Thermostable 5' App DNA/RNA Ligase	NEB	M0319S
TruSeq_SE1 5'-pGGCACTANNNNNAGATCGGAAGAGCGTCGT Any		
TruSeq_SE10 5'-pGGTGTTCCNNNNNAGATCGGAAGAGCGTCG Any		
TruSeq_SE11 5'-pGGTAAGTNNNNNAGATCGGAAGAGCGTCG Any		
TruSeq_SE12 5'-pGGAGATGNNNNNAGATCGGAAGAGCGTCC Any		
TruSeq_SE2 5'-pGGGTAGCNNNNNAGATCGGAAGAGCGTCGT Any		
TruSeq_SE35'-pGGTCGATNNNNNAGATCGGAAGAGCGTCGT Any		
TruSeq_SE4 5'-pGGCCTCGNNNNNAGATCGGAAGAGCGTCGT Any		
TruSeq_SE5 5'-pGGTGACANNNNNNAGATCGGAAGAGCGTCGT Any		
TruSeq_SE6 5'-pGGTAGACNNNNNAGATCGGAAGAGCGTCGT Any		
TruSeq_SE7 5'-pGGGCCCTNNNNNAGATCGGAAGAGCGTCGT Any		
TruSeq_SE8 5'-pGGATCGGNNNNNAGATCGGAAGAGCGTCGT Any		
TruSeq_SE9 5'-pGGACTGANNNNNNAGATCGGAAGAGCGTCGT Any		
	GE Healthcare	
Typhoon 5 Bimolecular Imager	Life Science	29187191

Comments/Description

ssDNA ligase I

this protocol is only compatible with the Illumina sequencing platform

this protocol is only compatible with the Illumina sequencing platform
this protocol is only compatible with the Illumina sequencing platform

reverse transcriptase enzyme
gold nucleic acid gel stain

[illegible]



1 Alewife Center #200
Cambridge, MA 02140
tel. 617 945.9051
www.jove.com

ARTICLE AND VIDEO LICENSE AGREEMENT

Title of Article: Identification of footprints of RNA:protein complexes via RNA immunoprecipitation in Tandem followed by sequencing (RIPIT-seq)
Author(s): Lauren Woodward, Pooja Gangras, Guramrit Singh

Item 1: The Author elects to have the Materials be made available (as described at <http://www.jove.com/publish>) via:

☒ Standard Access

☐ Open Access

Item 2: Please select one of the following items:

☒ The Author is **NOT** a United States government employee.

☐ The Author is a United States government employee and the Materials were prepared in the course of his or her duties as a United States government employee.

☐ The Author is a United States government employee but the Materials were **NOT** prepared in the course of his or her duties as a United States government employee.

ARTICLE AND VIDEO LICENSE AGREEMENT

1. **Defined Terms.** As used in this Article and Video License Agreement, the following terms shall have the following meanings: "Agreement" means this Article and Video License Agreement; "Article" means the article specified on the last page of this Agreement, including any associated materials such as texts, figures, tables, artwork, abstracts, or summaries contained therein; "Author" means the author who is a signatory to this Agreement; "Collective Work" means a work, such as a periodical issue, anthology or encyclopedia, in which the Materials in their entirety in unmodified form, along with a number of other contributions, constituting separate and independent works in themselves, are assembled into a collective whole; "CRC License" means the Creative Commons Attribution-Non Commercial-No Derivs 3.0 Unported Agreement, the terms and conditions of which can be found at: <http://creativecommons.org/licenses/by-nc-nd/3.0/legalcode>; "Derivative Work" means a work based upon the Materials or upon the Materials and other pre-existing works, such as a translation, musical arrangement, dramatization, fictionalization, motion picture version, sound recording, art reproduction, abridgment, condensation, or any other form in which the Materials may be recast, transformed, or adapted; "Institution" means the institution, listed on the last page of this Agreement, by which the Author was employed at the time of the creation of the Materials; "JoVE" means MyJoVE Corporation, a Massachusetts corporation and the publisher of The Journal of Visualized Experiments; "Materials" means the Article and / or the Video; "Parties" means the Author and JoVE; "Video" means any video(s) made by the Author, alone or in conjunction with any other parties, or by JoVE or its affiliates or agents, individually or in collaboration with the Author or any other parties, incorporating all or any portion

of the Article, and in which the Author may or may not appear.

2. **Background.** The Author, who is the author of the Article, in order to ensure the dissemination and protection of the Article, desires to have the JoVE publish the Article and create and transmit videos based on the Article. In furtherance of such goals, the Parties desire to memorialize in this Agreement the respective rights of each Party in and to the Article and the Video.

3. **Grant of Rights in Article.** In consideration of JoVE agreeing to publish the Article, the Author hereby grants to JoVE, subject to Sections 4 and 7 below, the exclusive, royalty-free, perpetual (for the full term of copyright in the Article, including any extensions thereto) license (a) to publish, reproduce, distribute, display and store the Article in all forms, formats and media whether now known or hereafter developed (including without limitation in print, digital and electronic form) throughout the world, (b) to translate the Article into other languages, create adaptations, summaries or extracts of the Article or other Derivative Works (including, without limitation, the Video) or Collective Works based on all or any portion of the Article and exercise all of the rights set forth in (a) above in such translations, adaptations, summaries, extracts, Derivative Works or Collective Works and (c) to license others to do any or all of the above. The foregoing rights may be exercised in all media and formats, whether now known or hereafter devised, and include the right to make such modifications as are technically necessary to exercise the rights in other media and formats. If the "Open Access" box has been checked in Item 1 above, JoVE and the Author hereby grant to the public all such rights in the Article as provided in, but subject to all limitations and requirements set forth in, the CRC License.

ARTICLE AND VIDEO LICENSE AGREEMENT

4. **Retention of Rights in Article.** Notwithstanding the exclusive license granted to JoVE in Section 3 above, the Author shall, with respect to the Article, retain the non-exclusive right to use all or part of the Article for the non-commercial purpose of giving lectures, presentations or teaching classes, and to post a copy of the Article on the Institution's website or the Author's personal website, in each case provided that a link to the Article on the JoVE website is provided and notice of JoVE's copyright in the Article is included. All non-copyright intellectual property rights in and to the Article, such as patent rights, shall remain with the Author.

5. **Grant of Rights in Video – Standard Access.** This Section 5 applies if the "Standard Access" box has been checked in Item 1 above or if no box has been checked in Item 1 above. In consideration of JoVE agreeing to produce, display or otherwise assist with the Video, the Author hereby acknowledges and agrees that, Subject to Section 7 below, JoVE is and shall be the sole and exclusive owner of all rights of any nature, including, without limitation, all copyrights, in and to the Video. To the extent that, by law, the Author is deemed, now or at any time in the future, to have any rights of any nature in or to the Video, the Author hereby disclaims all such rights and transfers all such rights to JoVE.

6. **Grant of Rights in Video – Open Access.** This Section 6 applies only if the "Open Access" box has been checked in Item 1 above. In consideration of JoVE agreeing to produce, display or otherwise assist with the Video, the Author hereby grants to JoVE, subject to Section 7 below, the exclusive, royalty-free, perpetual (for the full term of copyright in the Article, including any extensions thereto) license (a) to publish, reproduce, distribute, display and store the Video in all forms, formats and media whether now known or hereafter developed (including without limitation in print, digital and electronic form) throughout the world, (b) to translate the Video into other languages, create adaptations, summaries or extracts of the Video or other Derivative Works or Collective Works based on all or any portion of the Video and exercise all of the rights set forth in (a) above in such translations, adaptations, summaries, extracts, Derivative Works or Collective Works and (c) to license others to do any or all of the above. The foregoing rights may be exercised in all media and formats, whether now known or hereafter devised, and include the right to make such modifications as are technically necessary to exercise the rights in other media and formats. For any Video to which this Section 6 is applicable, JoVE and the Author hereby grant to the public all such rights in the Video as provided in, but subject to all limitations and requirements set forth in, the CRC License.

7. **Government Employees.** If the Author is a United States government employee and the Article was prepared in the course of his or her duties as a United States government employee, as indicated in Item 2 above, and any of the licenses or grants granted by the Author hereunder exceed the scope of the 17 U.S.C. 403, then the rights granted hereunder shall be limited to the maximum

rights permitted under such statute. In such case, all provisions contained herein that are not in conflict with such statute shall remain in full force and effect, and all provisions contained herein that do so conflict shall be deemed to be amended so as to provide to JoVE the maximum rights permissible within such statute.

8. **Protection of the Work.** The Author(s) authorize JoVE to take steps in the Author(s) name and on their behalf if JoVE believes some third party could be infringing or might infringe the copyright of either the Author's Article and/or Video.

9. **Likeness, Privacy, Personality.** The Author hereby grants JoVE the right to use the Author's name, voice, likeness, picture, photograph, image, biography and performance in any way, commercial or otherwise, in connection with the Materials and the sale, promotion and distribution thereof. The Author hereby waives any and all rights he or she may have, relating to his or her appearance in the Video or otherwise relating to the Materials, under all applicable privacy, likeness, personality or similar laws.

10. **Author Warranties.** The Author represents and warrants that the Article is original, that it has not been published, that the copyright interest is owned by the Author (or, if more than one author is listed at the beginning of this Agreement, by such authors collectively) and has not been assigned, licensed, or otherwise transferred to any other party. The Author represents and warrants that the author(s) listed at the top of this Agreement are the only authors of the Materials. If more than one author is listed at the top of this Agreement and if any such author has not entered into a separate Article and Video License Agreement with JoVE relating to the Materials, the Author represents and warrants that the Author has been authorized by each of the other such authors to execute this Agreement on his or her behalf and to bind him or her with respect to the terms of this Agreement as if each of them had been a party hereto as an Author. The Author warrants that the use, reproduction, distribution, public or private performance or display, and/or modification of all or any portion of the Materials does not and will not violate, infringe and/or misappropriate the patent, trademark, intellectual property or other rights of any third party. The Author represents and warrants that it has and will continue to comply with all government, institutional and other regulations, including, without limitation all institutional, laboratory, hospital, ethical, human and animal treatment, privacy, and all other rules, regulations, laws, procedures or guidelines, applicable to the Materials, and that all research involving human and animal subjects has been approved by the Author's relevant institutional review board.

11. **JoVE Discretion.** If the Author requests the assistance of JoVE in producing the Video in the Author's facility, the Author shall ensure that the presence of JoVE employees, agents or independent contractors is in accordance with the relevant regulations of the Author's institution. If more than one author is listed at the beginning of this Agreement, JoVE may, in its sole

ARTICLE AND VIDEO LICENSE AGREEMENT

discretion, elect not take any action with respect to the Article until such time as it has received complete, executed Article and Video License Agreements from each such author. JoVE reserves the right, in its absolute and sole discretion and without giving any reason therefore, to accept or decline any work submitted to JoVE. JoVE and its employees, agents and independent contractors shall have full, unfettered access to the facilities of the Author or of the Author's institution as necessary to make the Video, whether actually published or not. JoVE has sole discretion as to the method of making and publishing the Materials, including, without limitation, to all decisions regarding editing, lighting, filming, timing of publication, if any, length, quality, content and the like.

12. **Indemnification.** The Author agrees to indemnify JoVE and/or its successors and assigns from and against any and all claims, costs, and expenses, including attorney's fees, arising out of any breach of any warranty or other representations contained herein. The Author further agrees to indemnify and hold harmless JoVE from and against any and all claims, costs, and expenses, including attorney's fees, resulting from the breach by the Author of any representation or warranty contained herein or from allegations or instances of violation of intellectual property rights, damage to the Author's or the Author's institution's facilities, fraud, libel, defamation, research, equipment, experiments, property damage, personal injury, violations of institutional, laboratory, hospital, ethical, human and animal treatment, privacy or other rules, regulations, laws, procedures or guidelines, liabilities and other losses or damages related in any way to the submission of work to JoVE, making of videos by JoVE, or publication in JoVE or elsewhere by JoVE. The Author shall be responsible for, and shall hold JoVE harmless from, damages caused by lack of sterilization, lack of cleanliness or by contamination due to


the making of a video by JoVE its employees, agents or independent contractors. All sterilization, cleanliness or decontamination procedures shall be solely the responsibility of the Author and shall be undertaken at the Author's expense. All indemnifications provided herein shall include JoVE's attorney's fees and costs related to said losses or damages. Such indemnification and holding harmless shall include such losses or damages incurred by, or in connection with, acts or omissions of JoVE, its employees, agents or independent contractors.

13. **Fees.** To cover the cost incurred for publication, JoVE must receive payment before production and publication of the Materials. Payment is due in 21 days of invoice. Should the Materials not be published due to an editorial or production decision, these funds will be returned to the Author. Withdrawal by the Author of any submitted Materials after final peer review approval will result in a US\$1,200 fee to cover pre-production expenses incurred by JoVE. If payment is not received by the completion of filming, production and publication of the Materials will be suspended until payment is received.

14. **Transfer, Governing Law.** This Agreement may be assigned by JoVE and shall inure to the benefits of any of JoVE's successors and assignees. This Agreement shall be governed and construed by the internal laws of the Commonwealth of Massachusetts without giving effect to any conflict of law provision thereunder. This Agreement may be executed in counterparts, each of which shall be deemed an original, but all of which together shall be deemed to be one and the same agreement. A signed copy of this Agreement delivered by facsimile, e-mail or other means of electronic transmission shall be deemed to have the same legal effect as delivery of an original signed copy of this Agreement.

A signed copy of this document must be sent with all new submissions. Only one Agreement is required per submission.

CORRESPONDING AUTHOR

Name:	GURAMRIT SINGH	
Department:	MOLECULAR GENETICS	
Institution:	THE OHIO STATE UNIVERSITY	
Title:	ASSISTANT PROFESSOR	
Signature:		Date: 2/27/2019

Please submit a signed and dated copy of this license by one of the following three methods:

1. Upload an electronic version on the JoVE submission site
2. Fax the document to +1.866.381.2236
3. Mail the document to JoVE / Attn: JoVE Editorial / 1 Alewife Center #200 / Cambridge, MA 02140

Editorial comments:

Changes to be made by the Author(s):

1. Please take this opportunity to thoroughly proofread the manuscript to ensure that there are no spelling or grammar issues. The JoVE editor will not copy-edit your manuscript and any errors in the submitted revision may be present in the published version.

Manuscript has been proofread, and any changes have been tracked.

2. Please submit the figures as a vector image file to ensure high resolution throughout production: (.svg, .eps, .ai). If submitting as a .tif or .psd, please ensure that the image is 1920 pixels x 1080 pixels or 300dpi.

All figures have been provided in their original .ai format.

3. Please ensure that the references appear as the following: [Lastname, F.I., LastName, F.I., LastName, F.I. Article Title. Source. Volume (Issue), FirstPage – LastPage (YEAR).] For more than 6 authors, list only the first author then et al.

This has been addressed.

4. Please include volume and issue numbers for all references.

This has been addressed.

5. Please define all abbreviations before use.

This has been addressed.

6. Please revise the table of the essential supplies, reagents, and equipment. The table should include the name, company, and catalog number of all relevant materials in separate columns in an xls/xlsx file. Please sort the Materials Table alphabetically by the name of the material.

This has been addressed.

7. Please rephrase the Summary to clearly describe the protocol and its applications in complete sentences between 10-50 words: "Here, we present a protocol to ..."

The summary has been changed as requested.

8. Please remove the references from the abstract.

All references have been removed.

9. Please include a one line spacer between all protocol steps and substeps.

Space has been added after each step.

10. JoVE cannot publish manuscripts containing commercial language. This includes trademark symbols (™), registered symbols (®), and company names before an instrument or reagent. Please remove all commercial language from your manuscript and use generic terms instead. All commercial products should be sufficiently referenced in the Table of Materials and Reagents.

For example: Dynabeads, RPiT, Typhoon, Spin-X, etc.

All commercial language has been deleted from the manuscript and accompanying Materials Table.

11. Please add more details to your protocol steps. Please ensure you answer the "how" question, i.e., how is the step performed? Alternatively, add references to published material specifying how to perform the protocol action.

Several steps have been reworded to improve clarity.

12. Please specify all volumes and concentrations.

All necessary volumes for reactions and steps are included.

13. For example: 1.6: Trypsinize how? How much of what is added for how long and at what temperature? Plate at what concentration?

These steps are usually common knowledge for those who are acquainted with cell culture, but trypsinization steps have been clarified.

14. 2.1: What is the concentration is absolute numbers?

Addressed.

15. 2.2: How much tetracycline is added?

This amount of tetracycline will depend on the researcher and experiment. This was addressed in an earlier step. That earlier step has been referenced for clarity.

16. Please specify all conditions for the cell culture: temperature, CO₂, etc.

Tis has been addressed in a Note to step 1.1

17. Please provide all of the buffer compositions in a Table uploaded separately.

A separate buffer table has been made and referenced in the representative results section.

18. Please remove the embedded Table from the manuscript. All tables should be uploaded separately to your Editorial Manager account in the form of an .xls or

.xlsx file. Each table must be accompanied by a title and a description after the Representative Results of the manuscript text.

All embedded tables have been removed, put into a separate excel sheet, and referenced in the representative results section.

19. There is a 10 page limit for the Protocol, but there is a 2.75 page limit for filmable content. Please highlight 2.75 pages or less of the Protocol (including headings and spacing) that identifies the essential steps of the protocol for the video, i.e., the steps that should be visualized to tell the most cohesive story of the Protocol. Remember that non-highlighted Protocol steps will remain in the manuscript, and therefore will still be available to the reader.

All page limits have been adhered to. Highlighted steps have been reworded as necessary to improve flow.

20. Please ensure that the highlighted steps form a cohesive narrative with a logical flow from one highlighted step to the next. Please highlight complete sentences (not parts of sentences). Please ensure that the highlighted part of the step includes at least one action that is written in imperative tense.

Highlighted steps have been reworded as necessary to improve flow.

21. Please include at least one paragraph of text to explain the Representative Results in the context of the technique you have described, e.g., how do these results show the technique, suggestions about how to analyze the outcome, etc. The paragraph text should refer to all of the figures. Data from both successful and sub-optimal experiments can be included.

A descriptive paragraph has been added to the representative results section.

Redundant information was removed from the figure legends.

22. Please obtain explicit copyright permission to reuse any figures from a previous publication. Explicit permission can be expressed in the form of a letter from the editor or a link to the editorial policy that allows re-prints. Please upload this information as a .doc or .docx file to your Editorial Manager account. The Figure must be cited appropriately in the Figure Legend, i.e. "This figure has been modified from [citation]."

The image in figure 3 is based on information from a separate publication and contains similar information, but we have created an original image which is

unique from the first.

23. Figure 2/4/5/6: Please label the units of the ladder.

Ladders have been labeled as "nt"

Reviewers' comments:

Reviewer #1:

Manuscript Summary:

In this manuscript, Lauren Woodward, Pooja Gangras and Guramrit Singh provide a detailed protocol to identify footprints of RNA:protein complexes based on the RNA immunoprecipitation in Tandem followed by sequencing method. This method (first described in Singh G et al., 2012 Cell) allows for the purification of endogenous ribonucleoprotein (RNP) complexes following a two-step approach where one protein of a known complex (bearing a Flag-Tag) is immunoprecipitated using anti-Flag affinity resin. Upon RNase treatment, the RNP complex is eluted from the Flag resin using Flag peptides and a second immunoprecipitation is programmed using antibodies directed against a second protein from the complex (usually different from the first protein that was immunoprecipitated). RNA footprints are recovered from the tandem purified RNPs and used as input for cDNA library preparation and sent for high-throughput sequencing.

This method is a good complement to the traditional CLIP-seq protocol as it allows the purification of the native RNP complex instead of a single RNA-binding protein. Furthermore, thanks to the tandem affinity purification procedure, it is possible to discriminate between different subcomplexes containing any given protein (usually the Flag-tagged protein) simply by changing the nature of the protein that is immunoprecipitated at the second step.

Overall, the manuscript is very well written, the introduction is clear and the protocol is well described and easy to follow. I therefore think it is suitable for a publication in JoVE. Below you will find some minor comments:

Minor Concerns:

- Lines 28 and 79 the authors write: "...two tandem immunopurifications...", however the word tandem already means two consecutive actions so authors should remove the word "two".

The word "two" has been removed.

- Line 168: The authors mention "Apply remaining cell extract from step 3.8" but there is no step 3.8 in their protocol. The authors should add the step 3.8 corresponding to the remaining cell extract from step 3.7.

This mistake has been corrected. Instead of step 3.8, step 3.6 has been referenced here.

- Line 168 to 171: The suggested amount of Flag-beads to use appears to be set by authors based on their protein of interest. Although they mention in the discussion that this amount is robust for a wide range of proteins, the authors should add an asterisk in the protocol section pointing to that statement so that it is clear for readers that the suggested amount of Flag-beads is a good start base-line, which can be further optimized if needed.

A note has been added to step 4.1 to inform the reader that this step may be optimized, as necessary.

- Line 211: "Triturate" means "to grind". I think that in this context, "homogenise" or "resuspend" are more suitable than "triturate".

The wording here has been changed to "resuspend" to better reflect the action in this step.

- Line 230-232: The authors suggest to dephosphorylate the 3' ends of RNA footprints using T4 PNK in the provided buffer. However, this buffer was optimized for the 5' kinase activity of PNK and not for the 3' phosphatase activity (Cameron Vicki and Uhlenbeck Olke 1977, Biochemistry). Particularly, the 3' phosphatase has a pH optimum of 6 while the 5' kinase activity reaches a

maximum between a pH of 7 to 8. I think the authors could point to the work of Cameron and Uhlenbeck so that users are aware that the 3' phosphatase activity can be further improved if necessary.

This information has been relayed in a note to step 10.3.

- Line 277: The authors should mention that the 4xdNTP mix contains biotin-dATP. Otherwise, some readers might miss the information (that is currently only provided in the "buffers" section).

This step has been reworded to make it clear that biotinylated dNTPs are included in the mix. The reader is also directed to the buffers table (Table1) for the exact composition of the 4xdNTP mix.

- Line 329: Are authors sure that CirLigase I is provided with betaine? Based on the lucigen website, only CirLigase II is provided with betaine.

This was an oversight. The note has been corrected to show that betaine must be purchased separately. Our commercial source for betain has also been added to Table 3 (Materials and Reagents Table).

- The image from figure 2A appears pixelated both on the screen and when printed. Could the authors add a higher resolution image?

Original .ai figures have been uploaded to improved resolution.

- Figure 3 (step 14): There is an open black box below the dashed rectangle, what does it correspond to?

This black box is meant to illustrate the unextended RT product. To clarify this, an asterisk has been added to the figure to label the box.

Reviewer #2:

Manuscript Summary:

Woodward et al describes a transcriptome-wide mapping of RNA-protein interactions. Several methods are available to obtain the position of RNA-binding proteins in the transcriptome but most of them use UV-crosslinking. The current manuscript describes a method without crosslinking (with an optional formaldehyde crosslinking). The manuscript is thorough and detailed. The following minor revision is required.

Minor Concerns:

Line 168. Step 3.8 does not exist.

This mistake has been corrected. Instead of step 3.8, step 3.6 has been referenced here.

Line 192. Step 7 is a preparation step for another round of IP. It has to be done while previous steps were done. This information has to be described.

A note has been added to step 6.1 to inform the reader that they should use the wait-time during the affinity elution to set up the antibody conjugation (step7).