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

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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.

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).

Keywords

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

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DISCLOSURES:
The authors have nothing to disclose.

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 proteins[3]. 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 disadvantages[4–7].

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 RNA[8]. 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)[9, 10]. 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 complexes[11].

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, PYM[12] 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 EJC[13], UPF1[14]), 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[15, 16] (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% CO_2 (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% CO_2.

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 × *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 μL 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 × *g* for 10 min at 4 °C. Collect 20 μL 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 µL pre-washed FLAG agarose beads in a 5 mL tube (bed volume of washed FLAG agarose beads will be 375 µL). Incubate FLAG agarose beads and cell extract for 1−3 h at 4 °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 × *g* for 1 min at 4 °C. Collect 20 µL 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 × *g* for 1 min at 4 °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 µL 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 °C for 10 min.

5.2. Pellet beads at 400 × *g* for 1 min at 4 °C. Collect 20 µL 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 µL 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 µL 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 µL magnetic beads (i.e., Dynabeads; Table of Materials) 3x in 1 mL IsoWB in 1.5 mL tube. Resuspend magnetic beads in 100 µL 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 μL 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 μL 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 μL 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 μL 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 ddH_2O, 400 μL of phenol- chloroform isoamyl alcohol (PCIAA, pH 4.5), and vortex for 30 s and spin at room temperature at 12,000 × *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 $MgCl_2$, 10 μg of glycogen, and 1 mL of 100% ethanol. Incubate overnight at −20 °C.

10.2. To pellet RNA, centrifuge at 12,000 × *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 ddH_2O, 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 6[17]. 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 ddH_2O and 400 μL of PCIAA pH 4.5 to the tube. Vortex for 30 s, centrifuge at 12,000 × *g* for 5 min. Collect aqueous phase and add 35 μL of 3 M sodium acetate, 1 μL of 1 M $MgCl_2$, 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 g^{32}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 a size and quantity standards.

11.2. Resolve labeled RNA/DNA on 26% urea-PAGE gel (20 × 27 × 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 × 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 $MgCl_2$ (Table 1), 2.25 μL of 100 mM DTT, 1.2 μL of reverse transcriptase enzyme to a final volume of 45 μL (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 μL of 2x urea load buffer (Table 1) to RT reaction. Dilute 1 μg of a low range DNA Ladder in 45 μL and add 45 μL of 2x urea load buffer.

14.2. Prepare 10% urea-PAGE gel (20 × 28 × 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 μL of DNA elution buffer (Table 1).

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

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

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

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

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

15. Circularization of RT product

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

15.2. Incubate the circularization reaction on a thermal cycler at 60 °C for 4 h. Heat inactivate the ssDNA ligase I by heating at 80 °C for 10 min then hold at 4 °C.

16. Test PCR

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

16.2. Prepare a 45 μL PCR reaction with 4.0−6.0 μL of the circularized product from step 15.2, 9.0 μL of 5x reaction buffer, 0.9 μL 10 M dNTPs, 2.25 μL of 10 μM PE1.0 primer (Table of Materials), 2.25 μL of 10 μM PE2.0 primer (Table of Materials), and 0.045 μL of high-fidelity DNA polymerase (Table of Materials), and water.

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

16.4. Use the following PCR conditions: 98 °C—30 s; 98 °C—5 s; 65 °C—10 s; 72 °C—15 s; 72 °C—2 min; 12 °C—hold.

16.5. Add 3 μL 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 μL 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 μL 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 × *g* for 3 min, collecting supernatant into a fresh tube.

17.5. Add another 400 μL 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 μL each. Precipitate DNA by adding 1 mL of 100% ethanol and 10 μg of glycogen. Vortex and incubate a least 2 h at −20 °C.

17.7. Pellet DNA at 12,000 × *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 μL 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).

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 itself[18]. 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 approach[19]. 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 RIPiT[20]. 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[21–23], 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.

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REFERENCES:

1. Karousis ED, Nasif S, Mühlemann O Nonsense-mediated mRNA decay: novel mechanistic insights and biological impact. Wiley Interdisciplinary Reviews: RNA. n/a-n/a, doi: 10.1002/wrna.1357 (2016).

2. Ivanov PV, Gehring NH, Kunz JB, Hentze MW, Kulozik AE Interactions between UPF1, eRFs, PABP and the exon junction complex suggest an integrated model for mammalian NMD pathways. The EMBO Journal. 27 (5), 736–747, doi: 10.1038/emboj.2008.17 (2008). [PubMed: 18256688]

3. Papasaikas P, Valcárcel J The Spliceosome: The Ultimate RNA Chaperone and Sculptor. Trends in Biochemical Sciences. 41 (1), 33–45, doi: 10.1016/j.tibs.2015.11.003 (2016). [PubMed: 26682498]

4. Jensen KB, Darnell RB CLIP: Crosslinking and ImmunoPrecipitation of In Vivo RNA Targets of RNA-Binding Proteins. Methods in molecular biology (Clifton, N.J.). 488, 85–98, doi: 10.1007/978-1-60327-475-3_6 (2008).

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

6. Konig J et al. iCLIP - Transcriptome-wide Mapping of Protein-RNA Interactions with Individual Nucleotide Resolution. Journal of Visualized Experiments: JoVE. (50), doi: 10.3791/2638 (2011).

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

8. Wheeler EC, Van Nostrand EL, Yeo GW Advances and challenges in the detection of transcriptome-wide protein–RNA interactions. Wiley Interdisciplinary Reviews. RNA 9 (1), doi: 10.1002/wrna.1436 (2018).

9. Singh G et al. The Cellular EJC Interactome Reveals Higher-Order mRNP Structure and an EJC-SR Protein Nexus. Cell. 151 (4), 750–764, doi: 10.1016/j.cell.2012.10.007 (2012). [PubMed: 23084401]

10. Singh G, Ricci EP, Moore MJ RIPiT-Seq: A high-throughput approach for footprinting RNA:protein complexes. Methods. 65 (3), 320–332, doi: 10.1016/j.ymeth.2013.09.013 (2014). [PubMed: 24096052]

11. Mabin JW et al. The Exon Junction Complex Undergoes a Compositional Switch that Alters mRNP Structure and Nonsense-Mediated mRNA Decay Activity. Cell Reports. 25 (9), 2431–2446.e7, doi: 10.1016/j.celrep.2018.11.046 (2018). [PubMed: 30466796]

12. Gehring NH, Lamprinaki S, Kulozik AE, Hentze MW Disassembly of Exon Junction Complexes by PYM. Cell. 137 (3), 536–548, doi: 10.1016/j.cell.2009.02.042 (2009). [PubMed: 19410547]

13. Dostie J, Dreyfuss G Translation Is Required to Remove Y14 from mRNAs in the Cytoplasm. Current Biology. 12 (13), 1060–1067, doi: 10.1016/S0960-9822(02)00902-8 (2002). [PubMed: 12121612]

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

15. Gangras P, Dayeh DM, Mabin JW, Nakanishi K, Singh G Cloning and Identification of Recombinant Argonaute-Bound Small RNAs Using Next-Generation Sequencing. Argonaute Proteins: Methods and Protocols. 1–28, doi: 10.1007/978-1-4939-7339-2_1 (2018).

16. Heyer EE, Ozadam H, Ricci EP, Cenik C, Moore MJ An optimized kit-free method for making strand-specific deep sequencing libraries from RNA fragments. Nucleic Acids Research. 43 (1), e2, doi: 10.1093/nar/gku1235 (2015). [PubMed: 25505164]

17. Cameron V, Uhlenbeck OC 3'-Phosphatase activity in T4 polynucleotide kinase. Biochemistry. 16 (23), 5120–5126 (1977). [PubMed: 199248]

18. Ricci EP et al. Staufen1 senses overall transcript secondary structure to regulate translation. Nature Structural & Molecular Biology. 21 (1), 26–35, doi: 10.1038/nsmb.2739 (2014).

19. Lackner DH et al. A generic strategy for CRISPR-Cas9-mediated gene tagging. Nature Communications. 6, 10237, doi: 10.1038/ncomms10237 (2015).

20. 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). [PubMed: 30415953]

21. 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).

22. 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). [PubMed: 24829458]

23. 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). [PubMed: 26990993]

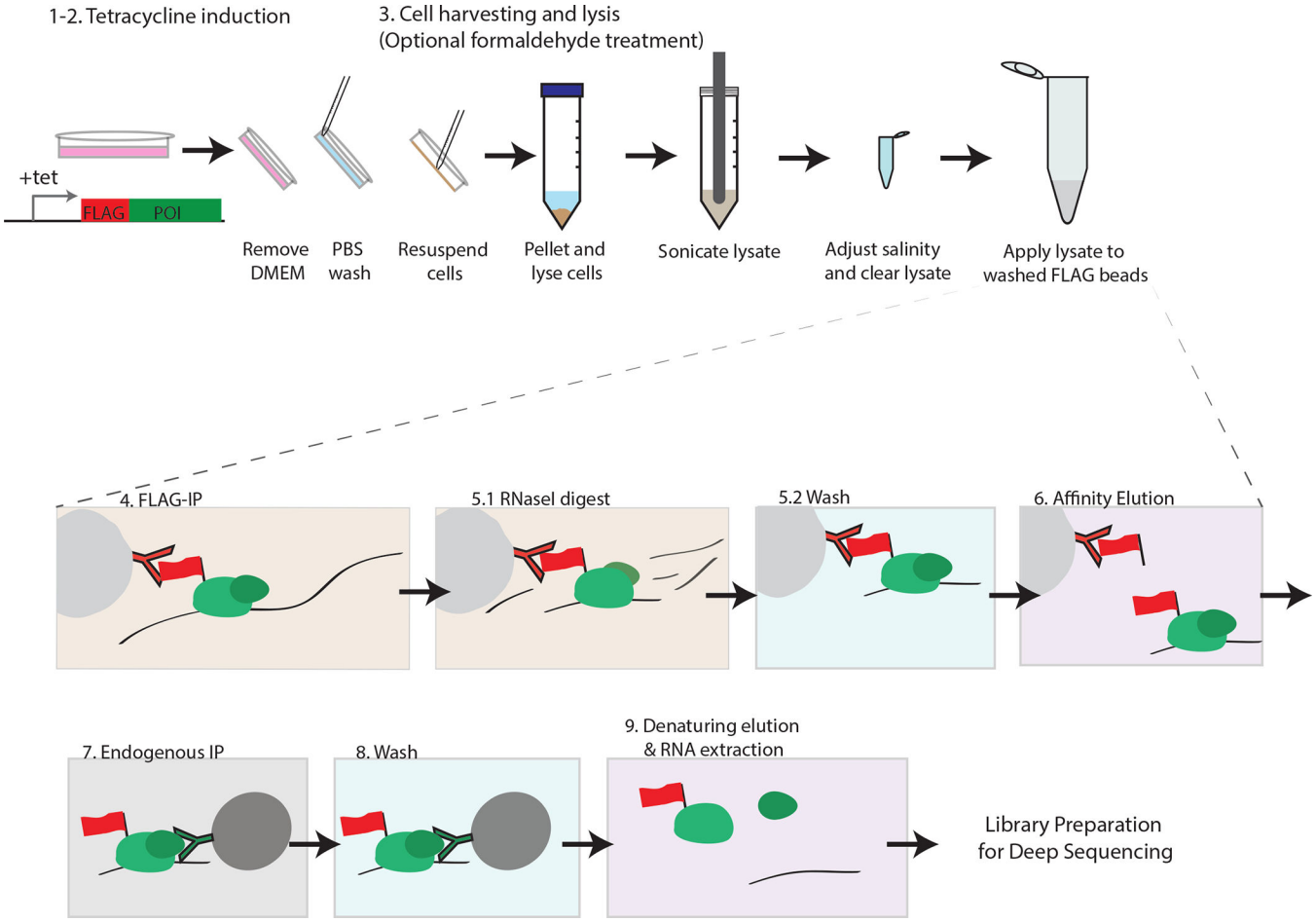

Figure 1:
Schematic outlining the main steps in RIPiT.

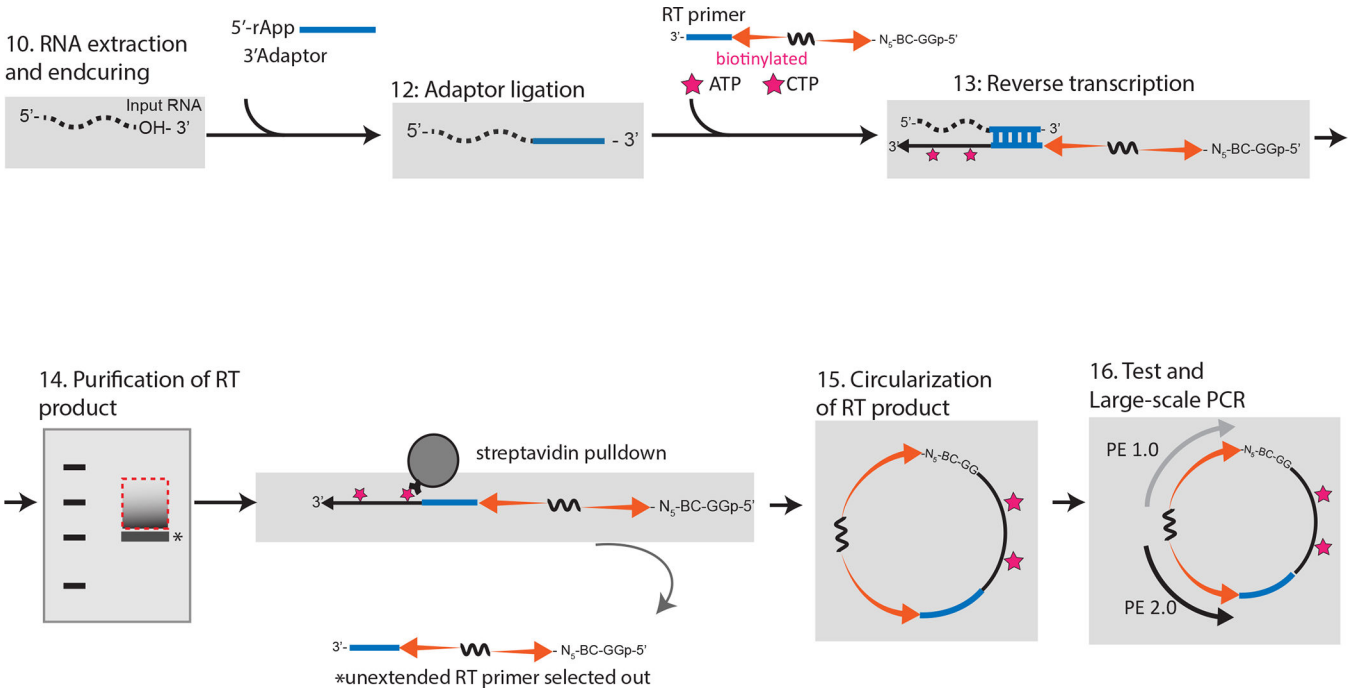

Figure 2:

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

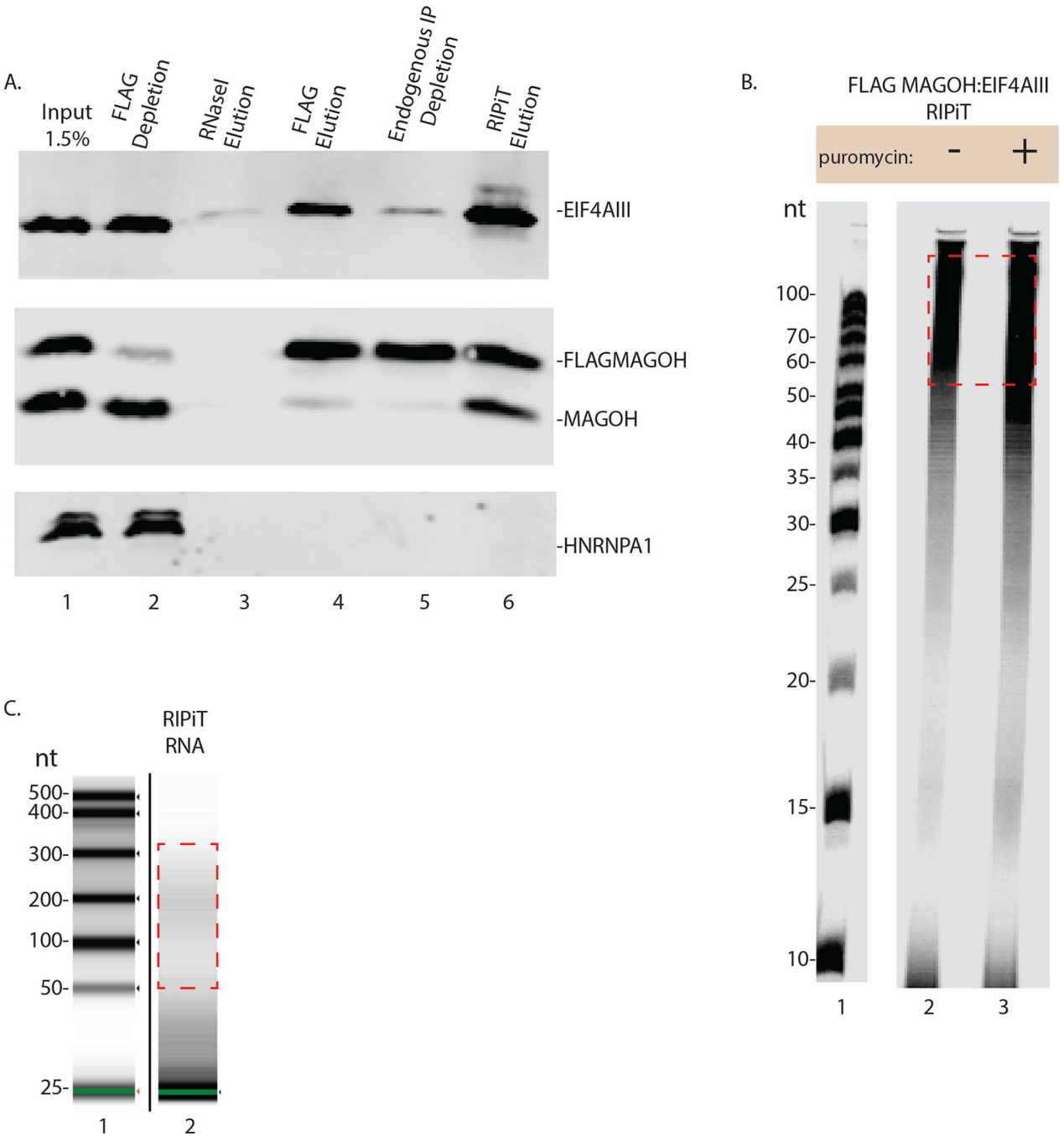

Figure 3: Estimation of RNA and protein yields from RIPiT.

(**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.

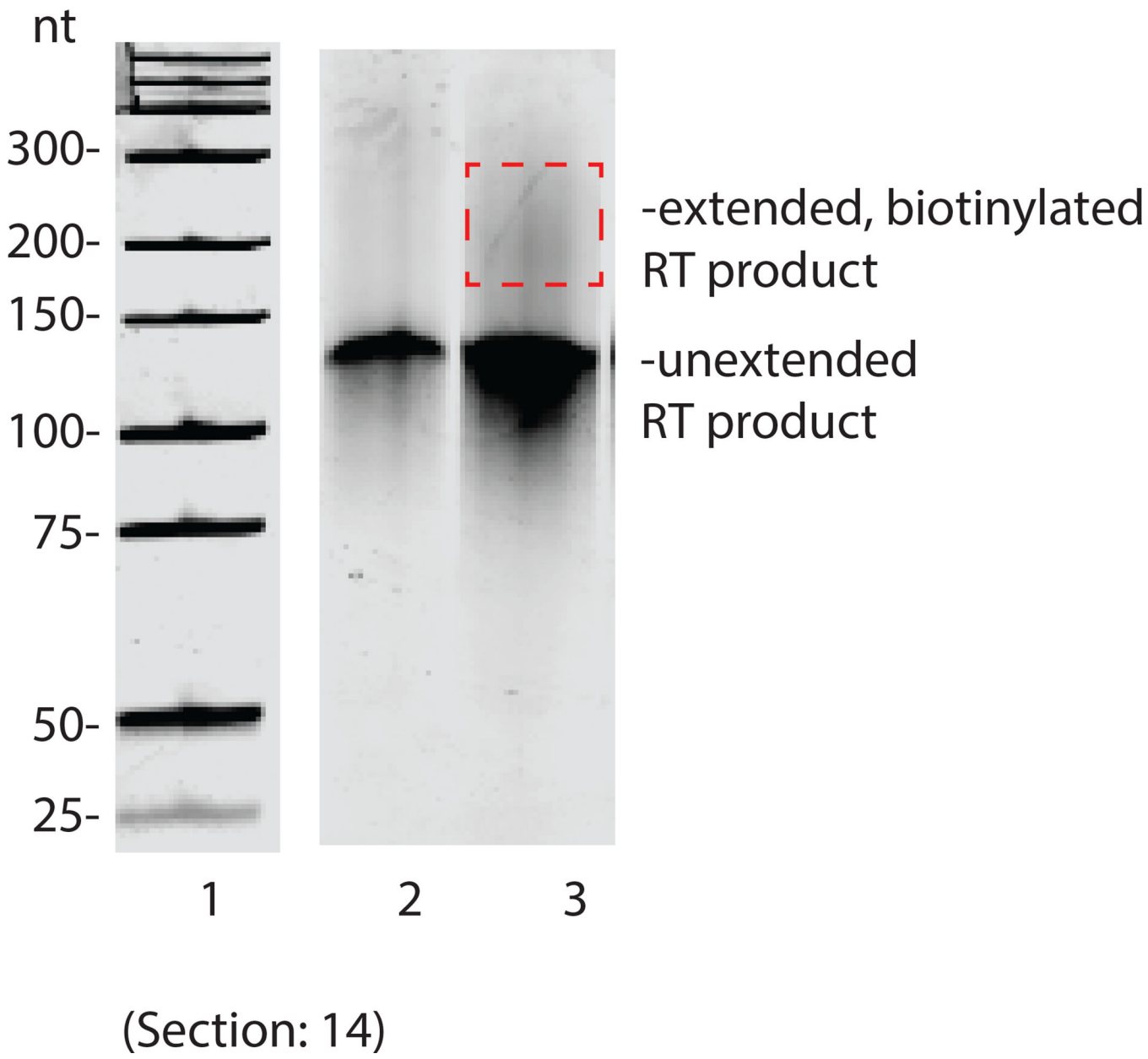

Figure 4: Reverse transcription (RT) product resolved on a 10% urea-PAGE gel and stained with gold nucleic acid gel stain.

Red box indicates gel region excised for gel purification of extended RT products.

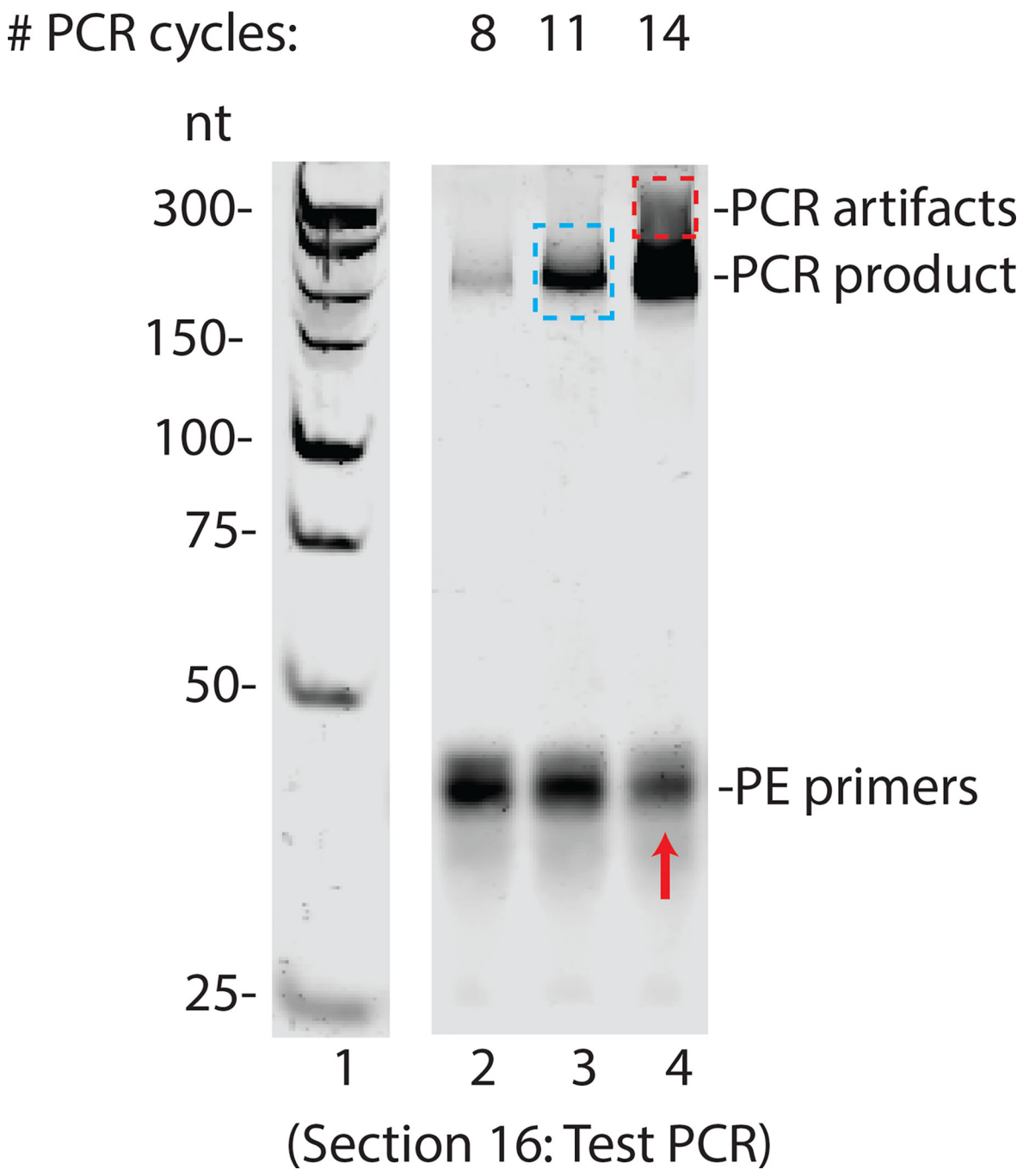

Figure 5: Test PCR resolved on 8% non-denaturing PAGE.
Note the aberrantly large PCR products which appear at 14 cycles (red box) and the parallel depletion of primers (red arrow) indicative of overamplification. For this sample, 11 cycles was chosen for the large-scale PCR (blue box).

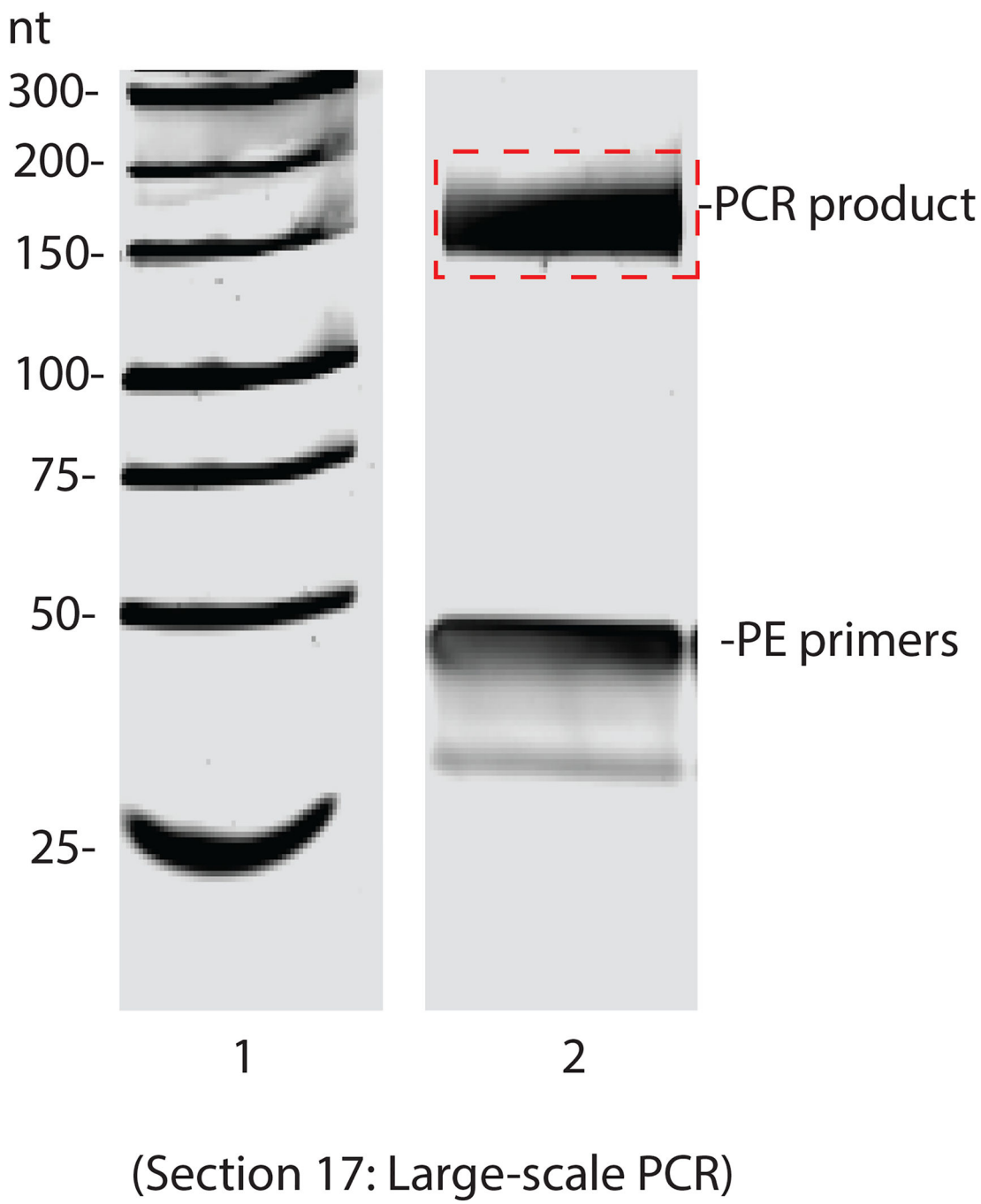

Figure 6: Large-scale PCR resolved on 8% non-denaturing PAGE.
Red box indicates the gel piece excised for gel purification.

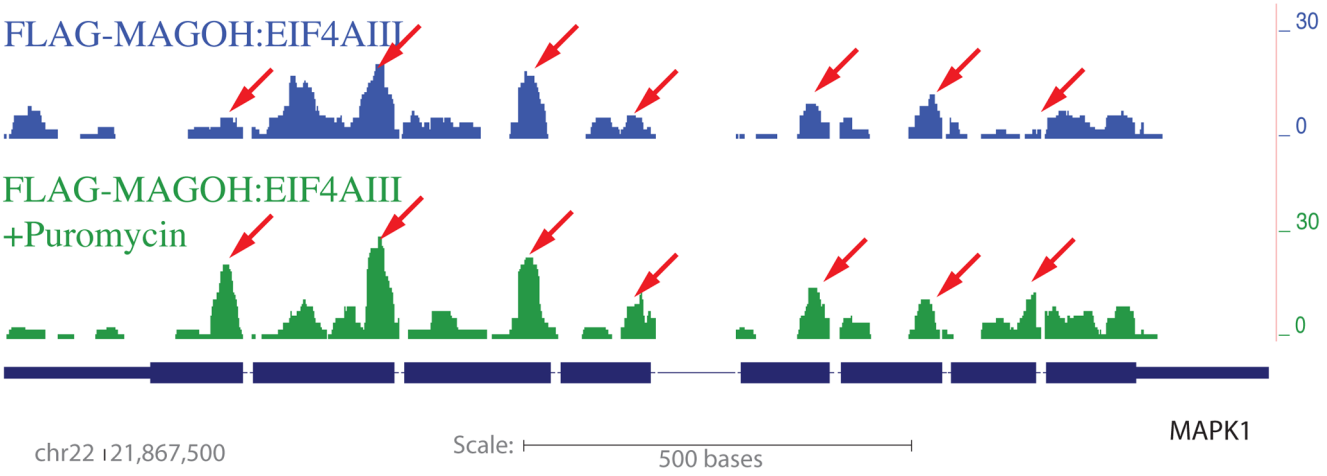

Figure 7: Genome browser screenshot of the MAPK1 gene showing distribution of FLAG-MAGOH:EIF4AIII footprints as representative results from a RIPiT.

Red arrows denote the expected canonical EJC binding sites.

Table 1:

Buffers.

PBS	137 mM NaCl	
	2.7 mM KCl	
	10 mM $Na_2HPO_4{\cdot}7H_2O$ KH_2PO_4	
	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*	*must be added fresh every time
	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*	*must be added fresh every time
	1x Leupeptin*	
	1x Pepstatin*	
	1 mM PMSF*	
2x Denaturing Load Buffer	3 mL 5x TBE	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
	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 percentage	
	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	

Name of Material/ Equipment	Company	Catalog Number	Comments/ Description
Anti-FLAG Affinity Gel	Sigma	A2220	
ATP, [γ−32P]- 3000Ci/mmol 10mCi/ml EasyTide, 250μCi	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		this protocol is only compatible with the Illumina sequencing platform
Mirus transIT-X2 transfection reagent	Mirus	MIR 6004	
Mth RNA ligase	NEB	E2610S	
PE1.0 5′-AATGATACGGCGACCACCGAGATCTACACTCTTTCCCTACACGACGCTCTTCCGATC*T-3′	Any		this protocol is only compatible with the Illumina sequencing platform
PE2.0 5′-CAAGCAGAAGACGGCATACGAGATCGGTCTCGGCATTCCTGCTGAACCGCTCTTCCGATC*T-3′	Any		this protocol is only compatible with the Illumina sequencing platform
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	

Name of Material/ Equipment	Company	Catalog Number	Comments/ Description
SybrGold	ThermoFisher	S11494	
T4 Polynucleotide Kinase-2500U	NEB	M0201L	
T_4RNL2 Tr. K227Q	NEB	M0351S	
Tetracycline	Sigma	87128	
Thermostable 5′ App DNA/RNA Ligase	NEB	M0319S	
TruSeq_SE1 5′-pGGCACTANNNNNAGATCGGAAGAGCGTCGTGTAGGGAAAGAGTGT-SPACER 18-CTCGGCATTCCTGCTGAACCGCTCTTCCGATCTCCTTGGCACCCGAGAATTCCA-3′	Any		this protocol is only compatible with the Illumina sequencing platform
TruSeq_SE10 5′-pGGTGTTCNNNNNAGATCGGAAGAGCGTCGTGTAGGGAAAGAGTGT-SPACER 18-CTCGGCATTCCTGCTGAACCGCTCTTCCGATCTCCTTGGCACCCGAGAATTCCA-3′	Any		this protocol is only compatible with the Illumina sequencing platform
TruSeq_SE11 5′-pGGTAAGTNNNNNAGATCGGAAGAGCGTCGTGTAGGGAAAGAGTGT-SPACER 18-CTCGGCATTCCTGCTGAACCGCTCTTCCGATCTCCTTGGCACCCGAGAATTCCA-3′	Any		this protocol is only compatible with the Illumina sequencing platform
TruSeq_SE12 5′-pGGAGATGNNNNNAGATCGGAAGAGCGTCGTGTAGGGAAAGAGTGT-SPACER 18-CTCGGCATTCCTGCTGAACCGCTCTTCCGATCTCCTTGGCACCCGAGAATTCCA-3′	Any		this protocol is only compatible with the Illumina sequencing platform
TruSeq_SE2 5′-pGGGTAGCNNNNNAGATCGGAAGAGCGTCGTGTAGGGAAAGAGTGT-SPACER 18-CTCGGCATTCCTGCTGAACCGCTCTTCCGATCTCCTTGGCACCCGAGAATTCCA-3′	Any		this protocol is only compatible with the Illumina sequencing platform
TruSeq_SE35′-pGGTCGATNNNNNAGATCGGAAGAGCGTCGTGTAGGGAAAGAGTGT-SPACER 18-CTCGGCATTCCTGCTGAACCGCTCTTCCGATCTCCTTGGCACCCGAGAATTCCA-3′	Any		this protocol is only compatible with the Illumina sequencing platform
TruSeq_SE4 5′-pGGCCTCGNNNNNAGATCGGAAGAGCGTCGTGTAGGGAAAGAGTGT-SPACER 18-CTCGGCATTCCTGCTGAACCGCTCTTCCGATCTCCTTGGCACCCGAGAATTCCA-3′	Any		this protocol is only compatible with the Illumina sequencing platform
TruSeq_SE5 5′-pGGTGACANNNNNAGATCGGAAGAGCGTCGTGTAGGGAAAGAGTGT-SPACER 18-CTCGGCATTCCTGCTGAACCGCTCTTCCGATCTCCTTGGCACCCGAGAATTCCA-3′	Any		this protocol is only

Name of Material/ Equipment	Company	Catalog Number	Comments/ Description
			compatible with the Illumina sequencing platform
TruSeq_SE6 5′-pGGTAGACNNNNNAGATCGGAAGAGCGTCGTGTAGGGAAAGAGTGT-SPACER 18-CTCGGCATTCCTGCTGAACCGCTCTTCCGATCTCCTTGGCACCCGAGAATTCCA-3′	Any		this protocol is only compatible with the Illumina sequencing platform
TruSeq_SE7 5′-pGGGCCCTNNNNNAGATCGGAAGAGCGTCGTGTAGGGAAAGAGTGT-SPACER 18-CTCGGCATTCCTGCTGAACCGCTCTTCCGATCTCCTTGGCACCCGAGAATTCCA-3′	Any		this protocol is only compatible with the Illumina sequencing platform
TruSeq_SE8 5′-pGGATCGGNNNNNAGATCGGAAGAGCGTCGTGTAGGGAAAGAGTGT-SPACER 18-CTCGGCATTCCTGCTGAACCGCTCTTCCGATCTCCTTGGCACCCGAGAATTCCA-3′	Any		this protocol is only compatible with the Illumina sequencing platform
TruSeq_SE9 5′-pGGACTGANNNNNAGATCGGAAGAGCGTCGTGTAGGGAAAGAGTGT-SPACER 18-CTCGGCATTCCTGCTGAACCGCTCTTCCGATCTCCTTGGCACCCGAGAATTCCA-3′	Any		this protocol is only compatible with the Illumina sequencing platform
Typhoon 5 Bimolecular Imager	GE Healthcare Life Science	29187191	