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TITLE:

Capture and Identification of RNA-binding Proteins by Using Click Chemistry-assisted RNA-interactome Capture (CARIC) Strategy

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SHORT ABSTRACT:

A detailed protocol for applying the click chemistry-assisted RNA-interactome capture (CARIC) strategy to identify proteins binding to both coding and noncoding RNAs is presented.

LONG ABSTRACT:

A comprehensive identification of RNA-binding proteins (RBPs) is key to understanding the posttranscriptional regulatory network in cells. A widely used strategy for RBP capture exploits the polyadenylation [poly(A)] of target RNAs, which mostly occurs on eukaryotic mature mRNAs, leaving most binding proteins of non-poly(A) RNAs unidentified. Here we describe the detailed procedures of a recently reported method termed click chemistry-assisted RNA-interactome capture (CARIC), which enables the transcriptome-wide capture of both poly(A) and non-poly(A) RBPs by combining the metabolic labeling of RNAs, *in vivo* UV cross-linking, and bioorthogonal tagging.

INTRODUCTION:

The human genome is transcribed into various types of coding and noncoding RNAs (ncRNAs), including mRNAs, rRNAs, tRNAs, small nuclear RNAs (snRNAs), amall nucleolar RNAs (snoRNAs),

and long non-coding RNAs (lncRNAs)[1]. Most of these RNAs possess clothing of RBPs and function as ribonucleoprotein particles (RNPs)[2]. Therefore, a comprehensive identification of RBPs is a prerequisite for understanding the regulatory network between RNAs and RBPs, which is implicated in various human diseases[3-5].

The past a few years have witnessed a great boost of RBPs discovered in various eukaryotic systems[2,6], including human[7-11], mouse[12-14], yeast[9,15,16], zebrafish[17], *Drosophila melanogaster*[18,19], *Caenorhabditis elegans*[16], *Arabidopsis thaliana*[20-22], and human parasites[23-25]. These advances have been facilitated by an RBP capture strategy developed by Castello *et al.*[7] and Baltz *et al.*[8] in 2012, which combines *in vivo* UV cross-linking of RNA and its interacting proteins, oligo(dT) capture of poly(A) RNAs, and mass spectrometry (MS)-based proteomic profiling. However, given the fact that poly(A) mostly exists on mature mRNAs, which account for only ~3% - 5% of the eukaryotic transcriptome[26], this widely used strategy is not capable of capturing RBPs interacting with non-poly(A) RNAs, including most ncRNAs and pre-mRNAs.

Here, we report the detailed procedures of a recently developed strategy for the transcriptome-wide capture of both poly(A) and non-poly(A) RBPs[27]. Termed CARIC, this strategy combines *in vivo* UV cross-linking and metabolic labeling of RNAs with photoactivatable and "clickable" nucleoside analogs (which contain a bioorthogonal functional group that can participate in click reaction), 4-thiouridine (4SU), and 5-ethynyluridine (EU). Steps that are key to get ideal results with the CARIC strategy are efficient metabolic labeling, UV cross-linking and click reaction, and the maintenance of RNA integrity. Because Cu(I) used as the catalyst in click reaction can cause the fragmentation of RNAs, a Cu(I) ligand that can reduce RNA fragmentation is essential. We describe how to perform efficient click reactions in cell lysates without causing severe RNA degradation.

Although RBP capture and identification in HeLa cells only is described in this protocol, the CARIC strategy can be applied to various cell types and possibly to living organisms. Besides RBP capture, this protocol also provides streamlined step-by-step procedures for MS sample preparation and protein identification and quantification, which can be helpful for those who are not familiar with proteomic experiments.

PROTOCOL:

CAUTION: When applicable, the reagents used should be purchased in the form of RNase-free, or dissolved in RNase-free, solvents (for most cases, in diethyl pyrocarbonate (DEPC)-treated water). When handling RNA samples and RNase-free reagents, always wear gloves and masks, and change them frequently to avoid RNase contamination.

1. Preparation of Lysate of Metabolically Labeled and UV Cross-linked Cells

1.1. Metabolic incorporation of EU and 4SU

1.1.1. Culture HeLa cells in Dulbecco's Modified Eagle's Medium (DMEM) supplemented with 10%

fetal bovine serum (FBS), 100 U/mL penicillin, and 100 µg/mL streptomycin at 37 °C in a 5% CO_2 atmosphere. Culture ~4 x 10^7 HeLa cells (in two 15-cm dishes) for preparing one experimental or control sample for one standard MS run.

1.1.2. When the cultured HeLa cells reach ~80% confluence, remove the culture medium and add 15-mL of prewarmed fresh medium per 15-cm dish.

1.1.3. Add 15 µL per dish of 100 mM EU (dissolved in phosphate-buffered saline (PBS)) to a final concentration of 1 mM, and 7.5 µL per dish of 100 mM 4SU (dissolved in PBS) to a final concentration of 0.5 mM for experimental and noUV-control samples. Add 15 µL per dish of 100 mM EU (dissolved in PBS) to a final concentration of 1 mM for no4SU-control samples.

Note: 4SU is photo-activatable; thus, protection from light after adding 4SU is required.

1.1.4. Cover the dishes with foil and culture the cells for 16 h. Add half of the amount of the EU and 4SU or only EU from step 1.1.3 to the experimental, noUV-, and no4SU-control samples, respectively, and continue culturing for another 2 h.

1.2. *In vivo* UV cross-linking

1.2.1. Remove the culture medium, wash the cells 3x with 5 mL of PBS per dish, and remove residual PBS as much as possible.

Note: Residue liquid will significantly reduce cross-linking efficiency.

1.2.2. For experimental and no4SU-control samples, place the dishes on ice with the lid removed and irradiate the cells with 365-nm UV light at 2 J/cm^2 by a UV cross-linker.

1.2.3. For noUV-control samples, place the dishes on ice and protect them from light.

Note: All following steps for noUV-control samples should be performed in a darkened room.

1.3. Cell lysis and homogenization

1.3.1. Add 1 mL per dish of pre-lysis buffer (10 mM Tris·HCl, pH 7.5, 50 mM LiCl, 0.02% Nonidet P-40, and ethylenediaminetetraacetic acid (EDTA)-free protease inhibitor cocktail) to the cells. Scrape the cells using a rubber cell lifter and collect the pre-lysis suspension in a 15-mL tube.

Note: This step will break the cell membrane and release soluble cytoplasmic proteins and RNAs. **DO NOT** centrifuge the tube and remove the supernatant.

1.3.2. For the suspension from two 15-cm dishes, adjust the volume to 6 mL by adding pre-lysis buffer. Add to the pre-lysis suspension an equal volume of R-lysis buffer (200 mM Tris·HCl, pH 7.5, 500 mM LiCl, 2% lithium dodecyl sulfate [LDS]).

1.3.3. Homogenize the cell lysate by passing it through a syringe with a narrow needle (27-G) several times till the lysate is clear and homogenous. Incubate the lysate at 4 °C with gentle rotation (~15 rpm) for 1 h.

Note: This last step will allow the complete denaturing of proteins. The lysate can be safely stored at -70 °C for up to one month.

1.4. Preparation for click reaction

1.4.1. Dilute the lysate by adding 20 volumes of dilution buffer (50 mM Tris·HCl, pH 7.5) and divide it into 15-mL fractions.

Note: Solutions containing a high concentration of salt and detergent will compromise the efficiency of the Cu(I)-catalyzed click reaction; thus, the buffer of the lysate must be changed.

1.4.2. Concentrate each fraction by using a 15-mL ultrafiltration tube (with a molecular weight cutoff of 10 kDa) till the volume is smaller than 1 mL. Use a swinging-bucket rotor to spin the ultrafiltration tube at 4,000 x *g* at 4 °C for ~15 min.

1.4.3. Add 14 mL of dilution buffer to the concentrated lysate fraction and repeat step 1.4.2. Combine the fractions and concentrate them to a volume of 6 mL by ultrafiltration (4,000 x *g* at 4 °C for ~15 min).

Note: Most of the salt and LDS will now be removed, so the lysate is ready for the click reaction. The lysate can be stored at -70 °C for up to one week. Avoid multiple freeze-thaw cycles, because they will result in significant RNA degradation. Aliquot the lysate if small-scale characterizations are required.

2. Preparation of Samples for RNA-interactome Capture

2.1. Preclearing of the lysate

2.1.1. Add 100-µL streptavidin magnetic beads per 6 mL of lysate, and gently rotate (~15 rpm) for 30 min at room temperature to eliminate naturally biotinylated proteins.

2.1.2. Pellet the beads using a magnet (for ~20 min at 4 °C) and transfer the precleared lysate to a new tube.

2.2. Performance of the click reaction

2.2.1. Prepare the reaction mix: 6.5 µL of biotin stock (100 mM azide-biotin dissolved in dimethyl sulfoxide [DMSO] at a final concentration of 100 µM), 3.25 µL of copper stock (make it fresh; 1 M $CuSO_4$ dissolved in water at a final concentration of 500 µM), 65 µL of ligand stock (200 mM

THPTA dissolved in water at a final concentration of 2 mM), and 262.75 µL of H_2O.

Note: THPTA stands for Tris[(1-hydroxypropyl-1H-1,2,3-triazol-4-yl)methyl]amine.

2.2.2. Add the reaction mix to 6 mL of precleared lysate and mix well. Then, add 162.5 µL of reducing reagent (make it fresh; 40 mg/mL sodium ascorbate at a final concentration 5 mM) to the lysate and mix well. The final volume should be 6.5 mL.

2.2.3. Incubate the reaction mixture for 2 h at room temperature on an orbital shaker (800 rpm). Add 5 mM EDTA to the reaction mixture and incubate it for 5 min to quench the reaction.

2.3. Small-scale characterizations

2.3.1. Prepare the reaction mix as in step 2.2.1 with the biotin stock replaced by dye stock (*e.g.*, 100 mM azide-Cy5 dissolved in DMSO).

Note: The reagent amount should be adjusted according to the volume of lysate. Typically, a 20-µL aliquot of the lysate is enough for characterizations such as an in-gel fluorescence analysis.

2.3.2. Add the reaction mix to the lysate and incubate it for 2 h at room temperature. Then, add one-third of the volume of the LDS sample buffer (4x), denature it at 55 °C for 5 min, and resolve the sample on a 10% bis-Tris gel.

Note: To confirm the fluorescence signal is presented on RNAs, include controls with RNase A digestion after the click reaction.

2.4. Cleaning up of the reaction mixture

2.4.1. Add eight volumes of prechilled methanol (100%) to the quenched reaction mixture and incubate it for 30 min at -30 °C for precipitation. Perform the precipitation in 50-mL conical centrifuge tubes.

Note: If the total volume is greater than 50 mL, divide the reaction mixture into two 50-mL conical centrifuge tubes.

2.4.2. Prepare reconstitution buffer: combine one volume of buffer A (4% sodium dodecyl sulfate [SDS] and 10 mM EDTA) with eight volumes of buffer B (1% Brij-97, 150 mM NaCl, and 50 mM triethanolamine, pH 7.4).

2.4.3. Centrifuge at 4,000 x *g* for 15 min at 4 °C and discard the supernatant. Add ~1 - 2 mL of prechilled methanol to the pellet. Pipette up and down to break the pellet and make sure the pellet is completely suspended with no visible chunks. Fill the tube with prechilled methanol. Repeat this step 2x.

2.4.4. Centrifuge at 4,000 x *g* for 15 min at 4 °C and discard the supernatant. Put back the tubes and centrifuge again at 4,000 x *g* for 5 min. Carefully draw out the residual methanol as much as possible without disturbing the pellet.

2.4.5. Add 10 mL of reconstitution buffer to the pellet. Pipette up and down to dissolve the pellet. Centrifuge at 4,000 x *g* for 10 min at 4 °C.

2.4.6. Transfer the supernatant to a new tube. Collect 20 µL of the sample for quality control (see section 4).

Note: Now, the sample is ready for RNA-interactome capture. The reconstituted sample can be stored at -70 °C for up to one week.

3. RNA-interactome Capture

3.1. Preparation of the streptavidin-agarose beads

3.1.1. Take 1,600 µL of streptavidin-agarose slurry (800 µL of settled beads) per 10 mL of reconstituted sample into a 15-mL conical centrifuge tube.

3.1.2. Spin down the beads at 4000 x *g* for 5 min. Carefully remove the supernatant without disturbing the settled beads.

3.1.3. Wash the beads with 10 mL of 50 mM Tris·HCl (pH 7.5). Spin down the beads (4,000 x *g* for 5 min) and remove the supernatant. Repeat this step 2x.

3.2. Affinity pulldown

3.2.1. Transfer the cleaned-up and reconstituted sample from step 2.4.6 to the streptavidin-agarose beads (see step 3.1). Incubate overnight with gentle rotation at 4 °C.

3.3. Washing of the streptavidin beads

3.3.1. Spin down the beads (4,000 x *g* for 5 min) and transfer the supernatant to a new tube. Collect 20 µL of the sample for quality control.

3.3.2. Wash the beads with 10 mL of wash buffer A (2% SDS in PBS, pH 7.4). Incubate for 10 min with gentle rotation (~15 rpm) at room temperature. Spin down the beads (4,000 x *g* for 5 min) and remove the supernatant. Repeat 1x.

3.3.3. Repeat step 3.3.2 with wash buffer B (8 M urea and 250 mM NH_4HCO_3 dissolved in water). Repeat step 3.3.2 with wash buffer C (2.5 M NaCl in PBS, pH 7.4). Then, wash the beads with 10 mL of 50 mM Tris·HCl (pH 7.5). Spin down the beads (4,000 x *g* for 5 min) and remove the supernatant.

3.3.4. Split the beads evenly and transfer them to two 1.5-mL microcentrifuge tubes.

3.4. Elution of the captured RNPs

3.4.1. Prepare biotin elution buffer: 12.5 mM biotin, 75 mM NaCl, 7.5 mM Tris·HCl (pH 7.5), 1.5 mM EDTA, 0.15% SDS, 0.075% sarkosyl, and 0.02% sodium deoxycholate.

Note: Store the buffer at room temperature, for biotin may precipitate at 4 °C.

3.4.2. To 400 µL of washed settled beads, add 400 µL of biotin elution buffer.

3.4.3. Incubate them on an orbital shaker (1,500 rpm) at room temperature for 20 min. Then, incubate on an orbital shaker with a heat block (1,500 rpm, 65 °C) for 10 min. Spin down the beads (7,800 x *g* for 1 min) and collect the eluted RNP.

3.4.4. To the beads, add 400 µL of fresh biotin elution buffer and repeat step 3.4.3. Combine the two elutes into one 15-mL tube.

3.5. RNase digestion

3.5.1. Add three volumes of dilution buffer to the eluted RNP to decrease the concentration of SDS. Concentrate the diluted sample by using a 0.5-mL ultrafiltration tube (with a molecular weight cutoff of 10 kDa; spin at 12,000 x *g* at 4 °C for ~30 min) to ~40 µL.

3.5.2. Add 0.5 µg/µL RNase A and incubate it for 2 h at 37 °C to release RBPs from cross-linked RNAs. Collect 2 µL of RBPs for quality control (see section 4).

4. Quality Control

4.1. Control of the efficiency of the affinity pulldown

4.1.1. Take 10 µL of the “before-pulldown” sample from step 2.4.6 and 10 µL of the “after-pulldown” sample from step 3.3.1.

4.1.2. Analyze the samples using standard western blot procedures (10% bis-Tris gel).

4.1.3. Stain the polyvinylidene fluoride (PVDF) membrane with streptavidin-HRP conjugate to monitor the residue biotin signals of the “after-pulldown” sample.

Note: If the biotin signal of the “after-pulldown” sample is greater than one-fifth of the signal of the “before-pulldown” sample, increase the amount of streptavidin-agarose beads used in step 3.1.1.

4.2. Control of the total capture efficiency

4.2.1. Take 2 µL of the released RBP sample from step 3.5.2 and 0.5 µL of the "before-pulldown" sample (as 0.1% input) from step 2.4.6.

4.2.2. Analyze the samples using standard silver-staining procedures.

4.2.2.1. Fix the gel with fixation buffer (40% ethanol, 10% acetic acid) for 20 min followed by sensitization (13 mM $Na_2S_2O_3$, 83 mM sodium acetate, 30% ethanol) for 30 min.

4.2.2.2. Wash the gel 3x with water for 5 min and, then, stain it with a 15 mM $AgNO_3$ solution for 20 min. Wash the gel 2x with water for 1 min, develop it in 0.24 M Na_2CO_3 and 0.012% formaldehyde, and terminate with 45 mM EDTA when the staining is sufficient.

Note: The silver-staining intensity of the captured RBPs should be similar to that of the 0.1% input.

5. Preparation of the Samples for MS

5.1. In-gel trypsin digestion of captured RBPs[28]

5.1.1. Add one-fourth volume of SDS sample buffer (5x) to the released RBP samples from step 3.5.2. Denature the sample at 95 °C for 10 min.

5.1.2. Resolve the RBPs on a 1.5-mm 10% SDS-polyacrylamide gel.

5.1.3. Stain the gel with silver, following standard protocols.

5.1.4. Excise the lane of experimental sample or control sample with stacking gel and the major band of RNase A (~15 kDa) removed.

5.1.5. Cut the excised lane into small pieces (~1 - 1.5 mm x ~1 - 1.5 mm).

Note: The shortest edge of the gel piece should be no shorter than 1 mm to prevent clogging in pipette tips.

5.1.6. Transfer the gel pieces to a microcentrifuge tube and destain with destaining buffer (a mixture of equal volumes of 100 mM $Na_2S_2O_3$ and 30 mM $K_3[Fe(CN)_6]$).

5.1.7. Wash the gel pieces with 200 mM ammonium bicarbonate (ABC) till the gel pieces are totally colorless.

5.1.8. Dehydrate the gel pieces in 1 mL of neat acetonitrile (ACN). Rehydrate with 200 µL of 10 mM dithiothreitol (dissolved in 50 mM ABC) and incubate at 56 °C for 45 min.

Note: Completely dehydrated gel pieces should be very hard and opaque. If the gel pieces are still soft after dehydration, remove the ACN and add 1 mL of neat ACN to dehydrate again.

5.1.9. Cool down the gel pieces to room temperature. Add 200 µL of 58 mM iodoacetamide (dissolved in 50 mM ABC) and incubate at room temperature for 45 min in the dark.

5.1.10. After a brief wash with water, dehydrate the gel pieces in 1 mL of neat ACN.

Note: The gel pieces must be completely dehydrated.

5.1.11. Rehydrate the gel pieces with the appropriate amount of 10 ng/µL trypsin (dissolved in 50 mM ABC) and incubate at 37 °C for 12 - 16 h.

Note: The gel pieces should be completely rehydrated with no opaque cores. Remove any excess liquid.

5.2. Stable isotope dimethyl labeling of the digested peptides[29]

5.2.1. Extract the digested peptides from the gel pieces by adding 200 µL of extraction buffer (5% formic acid and 50% ACN in water) and incubate at 37 °C for 30 min with vortexing (at 1,200 rpm).

5.2.2. Repeat step 5.2.1 2x. Combine the extracts into one microcentrifuge tube.

5.2.3. Dry the extracted peptides by vacuum centrifugation.

5.2.4. Reconstitute the peptides in 200 µL of 100 mM triethylammonium bicarbonate (TEAB, pH 8.5).

CAUTION: Steps 5.2.4 - 5.2.6 should be performed on ice in a fume hood.

5.2.5. Add 8 µL of 4% CH_2O and 8 µL of 4% $^{13}CD_2O$ to the experimental and control samples, respectively.

Note: To control the bias of stable isotopic labeling, swap the stable isotope for experimental and control samples of the other biologically independent replicate.

5.2.6. Add 8 µL of 0.6 M $NaBH_3CN$ (make it fresh) and mix well.

5.2.7. Incubate the samples at room temperature for 1 h with vortexing.

5.2.8. Cool down the samples on ice. Quench the reaction by adding 32 µL of 1% ammonia aqueous solution. Then, further quench the reaction by adding 16 µL of formic acid.

5.2.9. Combine the experimental sample with the corresponding control sample into one

microcentrifuge tube. Dry the samples by vacuum centrifugation.

5.3. Fractionation of dimethyl-labeled peptides

5.3.1. Prepare the stop-and-go-extraction tips (StageTips)[30].

5.3.1.1. Insert a C18 membrane into an extended-length, 10-µL tip.

5.3.1.2. Add 300 µg of high-pH C18 beads suspended in ACN to the tip.

5.3.1.3. Place the tip upright in a microcentrifuge tube with a home-made rack which can stabilize the tip and lift the tip off the bottom.

5.3.1.4. Spin the tip at 1,400 x *g* for 2 min. Discard the flow-through.

5.3.1.5. Wash the tip with 50 µL of 80% ACN in 10 mM ABC (pH 10.0). Repeat 1x.

Note: Adjust the pH of 10 mM ABC solution by adding 28% ammonium hydroxide.

5.3.1.6. Wash the tip with 50 µL of 50% ACN in 10 mM ABC (pH 10.0). Repeat 1x.

5.3.1.7. Wash the tip with 50 µL of 10 mM ABC (pH 10.0). Repeat 1x.

5.3.2. Reconstitute the peptides in 50 µL of 10 mM ABC (pH 10.0).

5.3.3. Add the reconstituted sample to the prepared tip. Reload the flow-through to the tip to ensure efficient peptide binding.

5.3.4. Wash the tip with 50 µL of 10 mM ABC (pH 10.0). Repeat 1x.

5.3.5. Elute the peptide stepwise for 12 fractions with 50 µL of 6%, 9%, 12%, 15%, 18%, 21%, 25%, 30%, 35%, 40%, 80%, and 6% ACN in 10 mM ABC (pH 10.0).

5.3.6. Combine two fractions with an equal interval (fraction 1 with 7, 2 with 8, and so on) to get six combined fractions.

5.3.7. Dry the samples by vacuum centrifugation. The dried peptides can be stored at -30 °C.

6. Performance of the MS and Data Analysis

6.1. Peptide analysis by liquid chromatography-tandem mass spectrometry

6.1.1. Reconstitute the dried peptide fractions from step 5.3.7 in 15 µL of water containing 0.1% formic acid. Check the pH of reconstituted peptides by spotting 1 µL of the solution on a pH strip

(the pH should be under 3).

6.1.2. Inject the reconstitute sample into the liquid chromatography (LC) column. Apply an appropriate gradient of solvent (solvent A is water containing 0.1% formic acid, solvent B is ACN containing 0.1% formic acid) in high-performance liquid chromatography (HPLC). A typical gradient of solvent B is as follows: 5% - 35% in 40 min; 35% - 70% in 4 min; and held at 75% for 10 min.

6.1.3. Ionize the eluted peptides by electrospray and operate the mass spectrometer in data-dependent mode. Select 15 most abundant ions (multiply charged: 2+, 3+, or higher) in the initial MS scan for a tandem mass spectrometry (MS/MS) scan (collision-induced dissociation, CID). Set the dynamic exclusion size to 500 with a maximum duration time of 25 s.

6.2. Protein identification and quantification using MaxQuant[31]

6.2.1. Set the false discovery rate (FDR) of protein identification to 0.01 and set the number of unique peptides to 2 in order to increase accuracy and reliability.

6.2.2. Set the minimal required ratio counts (unique + razor) for protein quantification to 2, and enable the **Re-quantify** and **Match between runs** functions.

6.3. Enrichment significance evaluation using the R/Bioconductor package limma[32]

6.3.1. Perform a moderated *t*-test implemented in limma to test the Log2-fold change against zero from at least three biological replicates. Use the **read.table** function to read the data table. Then, use the **lmFit** and **eBayes** functions for data fitting. Use the **topTable** function to export the calculation results (including the averaged Log2-fold change and *P* values).

6.3.2. Correct the *P* values using the Benjamini–Hochberg method for controlling the FDR.

6.3.3. Apply an FDR of 0.01 to generate a list of proteins significantly enriched in the experimental samples. Set a cutoff of two- or threefold change to further control the false positives.

REPRESENTATIVE RESULTS:

The representative results of quality control steps are presented. The results include figures of the in-gel fluorescence analysis described in step 2.3.2 (**Figure 1**), the western blot analysis described in step 4.1.3 (**Figure 2A**), and the silver-staining analysis described in step 4.2.2 (**Figure 2B**). The quality control steps are critical for the optimization of CARIC protocols. Always include quality controls in the preparation of large-scale RBP identification experiments.

FIGURE AND TABLE LEGENDS:

Figure 1: In-gel fluorescence analysis of the click-labeled samples described in step 2.3.2. (**A**) This panel shows a typical in-gel fluorescence pattern of click-labeled samples. Only the doubly

labeled sample shows a strong smear band at a high molecular weight (> 250 kDa), which represents the signal of cross-linked RNPs. To abolish the RNP signal, omit either 4SU or EU or digest with RNase A. The background sharp bands at a lower molecular weight represent the signals of non-specific labeled proteins. (**B**) In some occasions, a strong smeared band (~130 - 250 kDa) can be observed in the no4SU-control sample. This band represents the signal of labeled uncross-linked RNAs, which will be degraded during the heat denaturation, for most cases. It will not interfere with the subsequent procedures. CBB = Coomassie brilliant blue.

Figure 2: Quality control of affinity pulldown efficiency and the captured RBPs. (**A**) This panel shows a western blot analysis of the biotin signals in samples before pulldown (input) and in samples after pulldown (supernatant). Estimate the ratio of the remaining signals and optimize the bead amount used in step 3.1.1. (**B**) This panel shows a silver-staining analysis of captured RBPs compared to 0.1% input total proteins. For HeLa cells, the general total capture efficiency is ~0.05% - 0.1% of input proteins. This value can vary significantly due to the variance of the metabolic labeling efficiency of different cell types.

Figure 3: Representative MS results of CARIC. (**A**) This panel shows a volcano plot displaying the averaged Log2-fold change and adjusted *P* values of quantified proteins, calculated by the limma package. 597 of proteins with a Log2-fold change of > 2 and an adjusted *P* value of < 0.01 were classified as "CARIC RBPs". (**B**) This panel shows the overlap of the CARIC proteins with previously identified human poly(A) RBPs[7-11]. The overlapped proteins are mostly coding RBPs, while the rest of the CARIC RBPs are more likely to be non-coding RBPs. This figure is a reprinted from previously published work with permission from the National Academy of Sciences[27].

DISCUSSION:

The maintenance of fair RNA integrity is one of the keys to successful CARIC experiments. With appropriate ligands of Cu(I) and careful operation, RNA degradation can be significantly reduced, although partial degradation was observed. The substitution ratios of EU and 4SU in experimental samples are 1.18% and 0.46%, respectively (data not shown). For intact RNAs with a length of 2,000 nt, ~90% of RNAs contain at least one EU and one 4SU. For partially degraded RNAs with a length of 1,000 nt, ~70% of RNAs contain at least one EU and one 4SU. Therefore, partial degradation of RNAs does not dramatically decrease the efficiency of CARIC, while severe degradation is not acceptable.

Another critical step is step 1.4, the preparation for the click reaction. The Cu(I)-catalyzed click reaction on RNAs is sensitive to LDS concentration. A high concentration (> 0.1%) of LDS will lead to a decrease of labeling signals on EU-containing RNAs and an increase of background signals on proteins (data not shown).

In addition to EU, CARIC is also compatible with other clickable nucleosides, such as alkynyl and azido analogs of adenosine[33-36]. However, the application of CARIC is significantly limited by the metabolic efficiency of unnatural clickable nucleosides in a biological system of interest. Therefore, before performing CARIC using conditions other than those demonstrated in this protocol, always check the metabolic labeling efficiency (*e.g.*, by fluorescent imaging).

Recently, a similar strategy called RICK (capture of the newly transcribed RNA interactome using click chemistry), which incorporates only EU to label total RNAs and uses 254-nm UV to cross-link RNAs and proteins, was reported[37]. Notably, 254-nm UV can activate all four natural nucleosides, as well as EU. Thus, 254-nm UV irradiation may cross-link free EU and its metabolites (*e.g.*, EU phosphates) with corresponding binding proteins, which should be taken into consideration as possible false positives.

One intriguing application of CARIC is to identify RBPs in bacteria whose RNAs are mostly non-polyadenylated. The large-scale identification of RBPs will provide invaluable resources to understand the molecular basis of posttranscriptional regulations in bacteria[38].

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DISCLOSURES:

The authors have nothing to disclose.

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Figure1

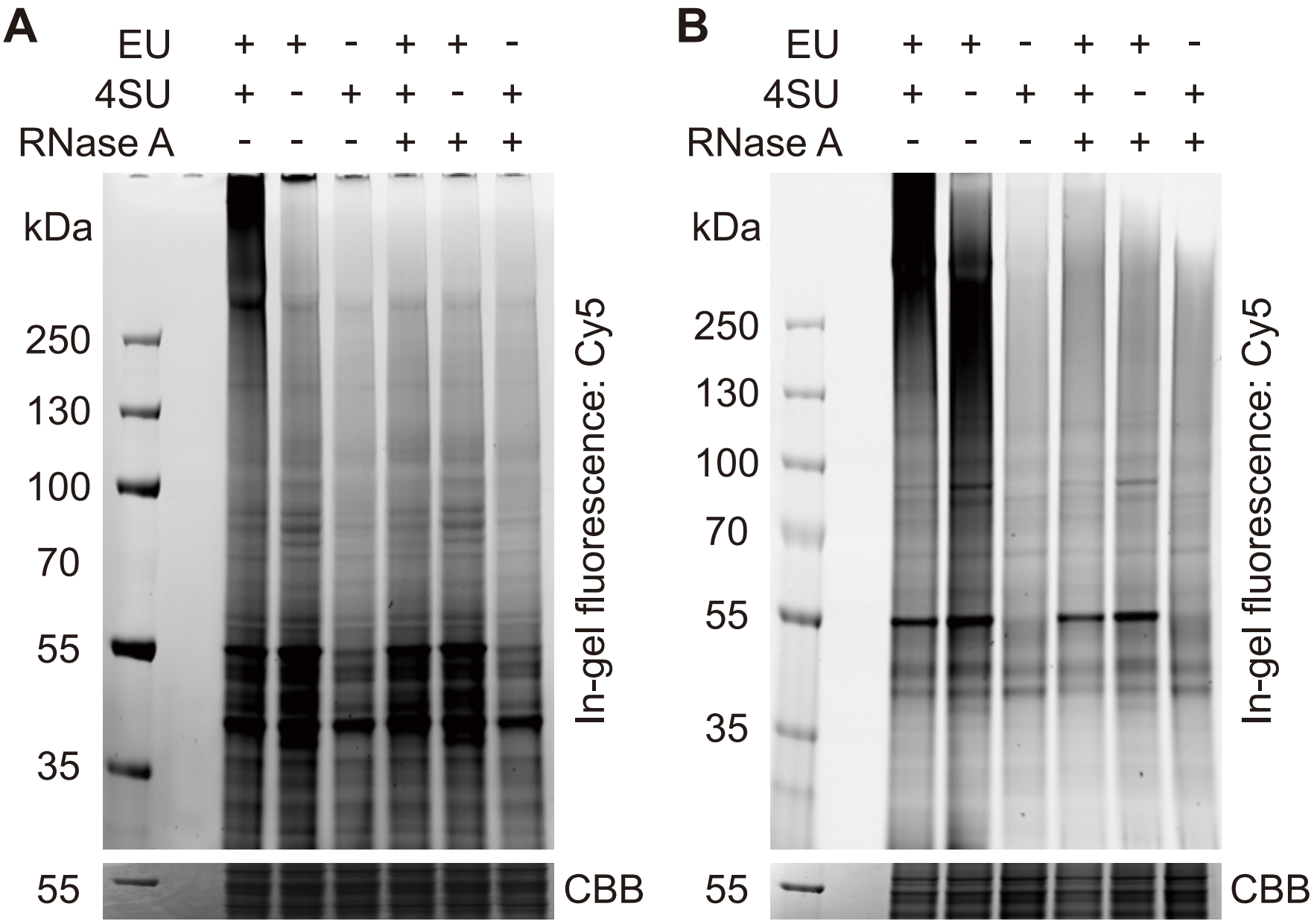
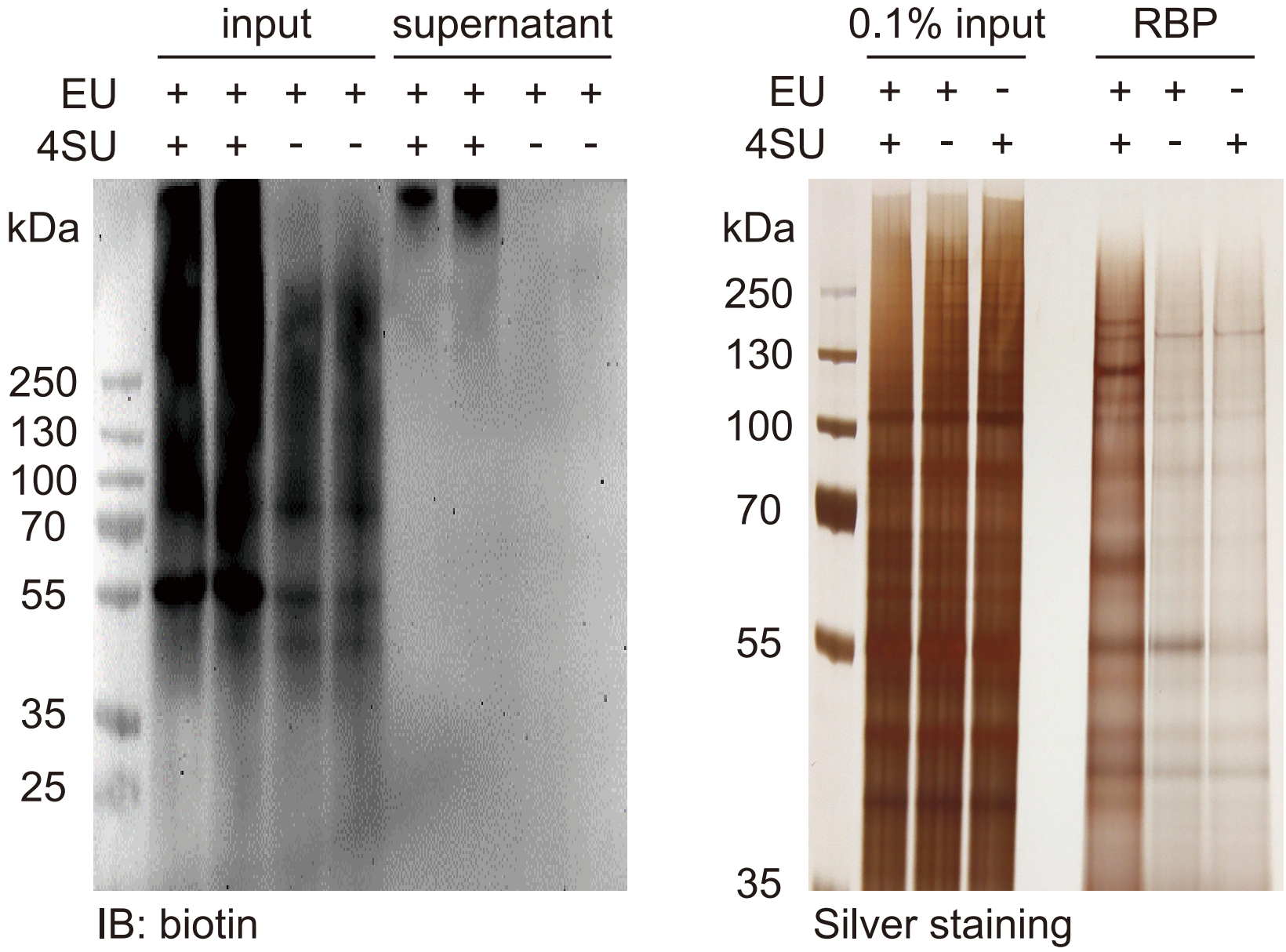
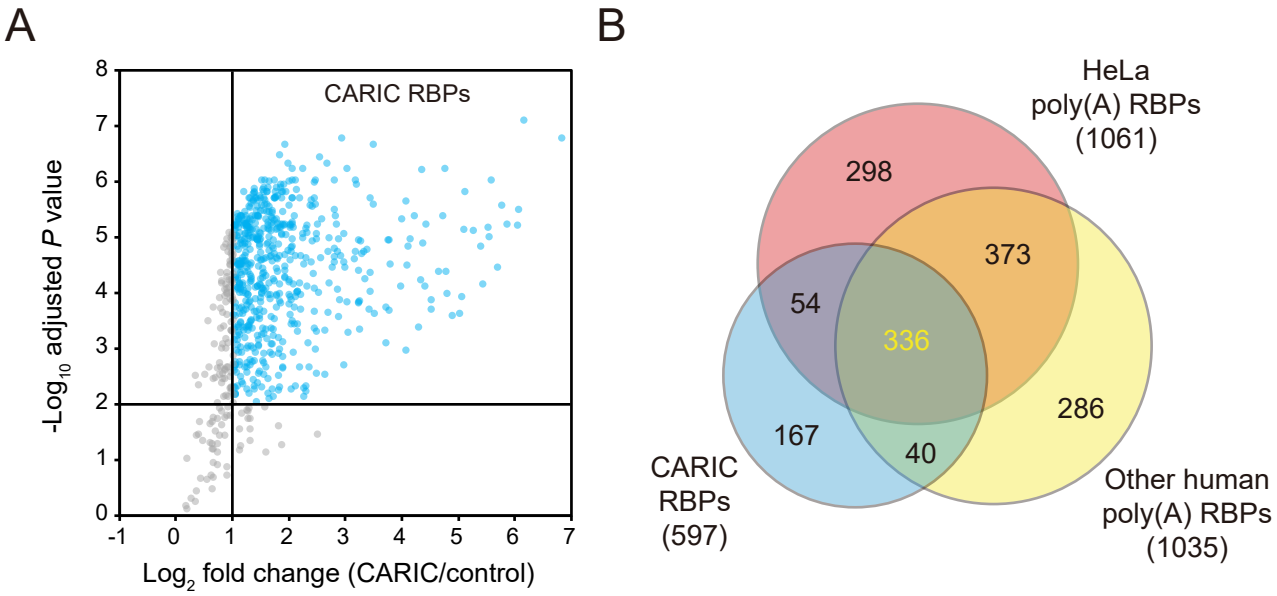


Figure2





Name of Material/ Equipment	Company	Catalog Number
HeLa	ATCC	
DMEM (Dulbecco's Modified Eagle Medium)	Thermo Fisher Scientific	11995065
FBS (Fetal Bovine Serum)	Thermo Fisher Scientific	10099141
Penicillin & Streptomycin	Thermo Fisher Scientific	15140122
EU (5-ethynyl uridine)	Wuhu Huaren Co.	CAS:69075-42-9
4SU (4-thiouridine)	Sigma Aldrich	T4509
10×PBS (Phosphate-Buffered Saline)	Thermo Fisher Scientific	AM9625
UV cross-linker	UVP	CL-1000
DEPC (Diethyl pyrocarbonate)	Sigma Aldrich	D5758
Tris·HCl, pH 7.5	Thermo Fisher Scientific	15567027
LiCl	Sigma Aldrich	62476
Nonidet P-40	Biodee	74385
EDTA-free protease inhibitor cocktail	Thermo Fisher Scientific	88265
LDS (Lithium dodecyl sulfate)	Sigma Aldrich	L9781
15-mL ultrafiltration tube (10 kDa cutoff)	Millipore	UFC901024
0.5-mL ultrafiltration tube (10 kDa cutoff)	Millipore	UFC501096
Streptavidin magnetic beads	Thermo Fisher Scientific	88816
DMSO (Dimethyl sulfoxide)	Sigma Aldrich	41639
Azide-biotin	Click Chemistry Tools	AZ104
Copper(II) sulfate	Sigma Aldrich	C1297
THPTA [Tris(3-hydroxypropyltriazolylmethyl)amine]	Sigma Aldrich	762342
Sodium ascorbate	Sigma Aldrich	11140
Azide-Cy5	Click Chemistry Tools	AZ118
LDS sample buffer (4×)	Thermo Fisher Scientific	NP0008
10% bis-Tris gel	Thermo Fisher Scientific	NP0301BOX
EDTA	Thermo Fisher Scientific	AM9260G
RNase A	Sigma Aldrich	R6513
SDS (Sodium dodecyl sulfate)	Thermo Fisher Scientific	15525017
NaCl	Sigma Aldrich	S3014
Brij-97 [Polyoxyethylene (20) oleyl ether]	J&K	315442
Triethanolamine	Sigma Aldrich	V900257
Streptavidin agarose	Thermo Fisher Scientific	20353

Urea	Sigma Aldrich	U5378
Sarkosyl (*N* -Lauroylsarcosine sodium salt)	Sigma Aldrich	61743
Biotin	Sigma Aldrich	B4501
Sodium deoxycholate	Sigma Aldrich	30970
MaxQuant		

Comments/Description

Equiped with 365-nm UV lamp
To treat water. Highly toxic!

One tablet for 50 mL lysis buffer.

Version: 1.5.5.1

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Dear Editor,

Thank you for providing us with the editorial and peer review comments on our manuscript entitled “Identification of coding and noncoding RNA-binding proteins by using click chemistry-assisted RNA-interactome capture (CARIC)”. We have added more details to the critical steps and reorganized the highlighted steps according to the comments. Several steps have been combined or reorganized in order to meet the page limit. The revisions are summarized in the following line-by-line responses.

Editorial comments:

“1.3.1: What is the concentration of EDTA-free protease inhibitor cocktail?”

Response: The EDTA-free protease inhibitor cocktail is purchased in the form of tablets. We added one tablets for 50 mL Pre-Lysis buffer. We’ve added this note to the Table of Materials.

“1.3.5: Mention needle gauge.”

(The step number 1.3.5 has been changed to 1.3.4)

Response: We thank the editor for pointing this out. The needle gauge has been added.

“1.3.6,2.1.1: Mention rotation speed.”

(The step number 1.3.6 has been changed to 1.3.5)

Response: We thank the editor for pointing this out. The rotation speed has been noted in these

two steps.

“1.4.2,1.4.4,: Do you centrifuge? If so, mention speed in g and duration.”

Response: Yes, we centrifuge the tubes. We’ve added more details, including rotation speed and duration time.

“2.1.2: Specify the duration on the magnet.”

Response: The duration on the magnet is specified.

“2.3.1: Methanol %?”

(The step number 2.3.1 has been changed to 2.4.1)

Response: The methanol concentration is added in the text.

“3.3.1,3.3.4,3.3.5,3.4.5,: Mention centrifuge speed and duration.”

(The step number 3.3.4, 3.3.5, and 3.4.5 have been changed to 3.3.2, 3.3.3, and 3.4.3, respectively)

Response: We thank the editor for pointing this out. The centrifuge speed and duration have been added in these steps.

“3.3.4: Mention rotation speed.”

(The step number 3.3.4 has been changed to 3.3.3)

Response: Step 3.3.3 is a repetitive step of 3.3.2. The rotation speed is added in step 3.3.2.

“4.1.2: Mention gel %.”

Response: The acrylamide percentage of the gel is added.

“4.2.2: For filming, please describe the steps (in brief is okay). What is the silver concentration /percentage?”

Response: We thank the editor for this advice. We have added a brief workflow of the silver staining step.

“6.1.1, 6.2.2: Please rewrite in the imperative voice.”

(We believe “6.1.1” is a typo for “6.2.1” because step 6.1.1 was already written in imperative voice.)

Response: We thank the editor for pointing this out. These two steps have been rewritten in imperative voice.

“The highlighting must include all relevant details that are required to perform the step. For example, if step 2.5 is highlighted for filming and the details of how to perform the step are given in steps 2.5.1 and 2.5.2, then the sub-steps where the details are provided must be included in the highlighting.”

Response: We thank the editor for the kind advice. We have reorganized the highlighting protocols so that all sub-steps of the highlighted steps are included in the highlighting.

“Some of your shorter protocol steps can be combined so that individual steps contain 2-3 actions and maximum of 4 sentences per step.”

Response: We have combined some short or repetitive steps to shorten the manuscript length. The combined and reorganized steps are (old step number to new step number): 1.3.3 and 1.3.4 to 1.3.3; 1.3.6 and 1.3.7 to 1.3.5; 1.4.4 and 1.4.5 to 1.4.4; 2.2.2 and 2.2.3 to 2.2.2; 2.2.4 and 2.2.6 to 2.2.3; 2.2.5 to 2.3.1 and 2.3.2; 2.3.7 and 2.3.8 to 2.4.7; 3.3.3 and 3.3.4 to 3.3.3; 3.4.3, 3.4.4, and 3.4.5 to 3.4.3; 3.5.1 and 3.5.2 to 3.5.1.

"The highlighted steps should form a cohesive narrative, that is, there must be a logical flow from one highlighted step to the next."

Response: We thank the editor for the kind advice. We have carefully reorganized the highlighted steps in a logical way.

"Please highlight complete sentences (not parts of sentences). Include sub-headings and spaces when calculating the final highlighted length."

Response: The length of highlighted steps has been re-calculated in the right way.

"Notes cannot be filmed and should be excluded from highlighting."

Response: The notes have been excluded from highlighting.

"Please bear in mind that software steps without a graphical user interface/calculations/ command line scripting cannot be filmed."

Response: We thank the editor for the reminder. We didn't include any software steps in highlighting.

"Please ensure that the manuscript title best reflects the filmable content (i.e. the portions you highlight)."

Response: We thank the editor for the reminder. We have checked the filmable content.

"JoVE articles are focused on the methods and the protocol, thus the discussion should be similarly focused. Please ensure that the discussion covers the following in detail and in paragraph form: 1) modifications and troubleshooting, 2) limitations of the technique, 3) significance with respect to existing methods, 4) future applications and 5) critical steps within the protocol."

Response: We have added paragraphs in the Discussion section regarding the demanding contents, including the detail description of a critical step, the limitation of our technique, and the future applications.

"JoVE is unable to publish manuscripts containing commercial sounding language, including trademark or registered trademark symbols (TM/R) and the mention of company brand names before an instrument or reagent. Examples of commercial sounding language in your manuscript are StageTips, MaxQuant,"

Response: We thank the editor for pointing this out. However, StageTips and MaxQuant are not commercial names. The StageTip (stop-and-go-extraction tip) is a technology developed by Professor Mann and colleagues for micro-purification, enrichment, pre-fractionation and storage of peptides for proteomics (see reference 30 of the manuscript). The MaxQuant is a software developed by Professor Mann and colleagues for proteomic data analysis and quantification (see

reference 31 of the manuscript).

"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/software in separate columns in an xls/xlsx file. Please include items such as software used, cell lines, etc."

Response: The information of the cell line and software is included in the table of materials.

Peer-review comments:

Reviewer #1

"1.3.6. Incubate the lysate at 4 °C with gentle rotation for 1 hr.

** Is the abovementioned step necessary for homogenization?"*

Response: We thank the reviewer for pointing this out. This step allows the complete denaturing of proteins, which is critical for minimizing the background signal from uncross-linked RBPs or non-RNA binders.

"1.4.2. Concentrate each fraction by using a 15-mL ultrafiltration tube 177 (molecular weight cutoff of 10 kDa) till the volume is smaller than 1 mL.

** Further clarification is needed for the following step, how long and what speed is needed for spinning the ultrafiltration columns."*

Response: More details of this step including centrifuge speed and duration have been added to the protocol.

"2.2.4. Incubate the reaction mixture for 2 hr at room temperature with vortex.

** Agitation? Rotation? Or occasional vortexing?"*

(The step number 2.2.4 has been changed to 2.2.3)

Response: We thank the reviewer for pointing this out. We incubate the reaction mixture on an orbital shaker at 800 rpm. This description has been added to the protocol.

"Line 215 volume of lysate. After incubation of 2 hr at room temperature

** After incubating 2hrs at room temperature."*

Response: We thank the reviewer for pointing this out. The description of this step has been reorganized.

"3.4. Elution of the captured RNP.

** RNP ◊ RBPs*

** How efficient is the protocol for eluting biotinylated molecules directly from Streptavidin beads if compared to eluting RBPs off the beads by degrading RNA using RNase?*

** Why is RNase treatment done after elution, rather than as part of the elution?"*

Response: In this step, biotinylated RNAs are eluted along with the cross-linked binding proteins. Thus we believe "RNP" is a more appropriate description than "RBP". We tried RNase A digestion for elution instead of using biotin elution buffer. However, it didn't work well even with very high concentration (~1 mg/mL) of RNase A. It is possible that the RNase is blocked by streptavidin and the cross-linked RBPs. The biotin elution buffer contains high concentration of detergent which will

denature the RNase. Therefore, we need to remove most of the detergent by ultrafiltration after elution to avoid inactivation of RNase.

"4.1.3) … If there are significant biotin signals remains…

** Improve the grammar!"*

Response: We have corrected it in the revised manuscript.

"5.1.8) Dehydrate the gel pieces in neat acetonitrile (ACN) and rehydrate with 200 µl of 10 mM dithiothreitol (dissolved in 50 mM ABC) at 56 °C for 45 min.

** This step needs to be clarified. How much ACN should be added? How many times?*

** Likewise, step 5.1.10"*

Response: We use 1 mL neat ACN for gel pieces of each lane. For most cases, two rounds of dehydration are needed. We've added this note to the protocol.

"5.3.1.5) Wash the tip with 50 µl of 80% ACN in 10 mM ABC (pH 10.0).

** How the pH of ABC should be adjusted to 10 mM? By adding ammonium hydroxide to ammonium formate? It needs to be clarified."*

Response: We thank the reviewer for pointing this out. The pH of ABC is adjusted by adding ammonium hydroxide. We've added this note to the protocol.

"6.1.1) Reconstitute the dried peptide fractions from step 5.3.7 in 15 µl water containing 0.1% formic acid

** The acidity of the samples should be check on a pH strip."*

Response: We thank the reviewer for pointing this out. We've added this note to the protocol.

"6.1. Peptide analysis by LC-MS/MS.

** The MS method needs to be described in detail here."*

Response: Detailed description of MS method has been added to the protocol.

"6.3.1) Perform a moderated t-test implemented in limma to test the Log2-fold change against zero from at least three biological replicates.

** What does zero stand for? The negative control? If so, explain.*

** Describe in detail how to use the limma package."*

Response: The data used in the moderated t-test analysis is the Log2-fold change value of the protein abundance in the experimental sample compared to that in the control sample. If one protein's Log2-fold change is zero, the abundance of this protein is equal in the experimental sample and the control sample, indicating this protein is not enriched at all.

The detailed description of how to use the limma package for moderated t-test has been added to the protocol.

Reviewer #2

"In Figure 1A, the difference between samples with or without RNaseA treatment is not so obvious."

Response: The main signal of cross-linked RNPs are located at high molecular weight region (>250

kDa) in the form of smeared bands as described in the figure legend. This signal is completely abolished by RNase digestion. The sharp bands at lower molecular weight region are non-specific background signals.

"In line 88, it's better to add a reference for '3~5% of the eukaryotic transcriptome'."

Response: We thank the reviewer for pointing this out. A reference has been added for this statement.

"In line 89, change 'on' to 'interacting with'."

Response: We thank the reviewer for pointing this out. We have corrected this in current manuscript.

"In line 254, it's better to keep consistence between '10 mL' and '10-mL' in line 260."

Response: We thank the reviewer for the kind advice. Now the description is consistent.

"How much time does it take to concentrate the lysate? This is not mentioned in the protocol. And, could this affect the integrity of RNA?"

Response: The centrifuge speed and duration have been added to the protocol. This step normally takes 2-3 hr. The RNAs will be partially degraded after this step. However, as we mentioned in the discussion section, the ultrafiltration step is critical for this method and partial degradation of RNAs will not significantly affect the results.

“THPTA should be spelled out on first use.”

Response: We thank the reviewer for pointing this out. The full name of THPTA is now presented on first use.

We thank the editor and reviewers again for their comments. We hope that you find the paper is now acceptable for publication. Of course, we would be happy to address any additional concerns, or make any other amendments that you deem necessary.

Thank you for considering our revised paper.

Sincerely,

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