RNP-IP (Original Method): Obtaining Majority RNA from RNA Binding Protein in the Nucleus

Fengzhi Liu*

School of Biomedical Sciences, Thomas Jefferson University, Philadelphia, USA
*For correspondence: fengzhi6@yahoo.com

[Abstract] Post-transcriptional regulation of gene expression is a ribonucleoprotein (RNP)-driven process, which involves RNA binding proteins (RBPs) and noncoding RNAs that regulate splicing, nuclear export, subcellular localization, mRNA stability and translation. mRNAs encoding proteins that function in a particular cell process or pathway can be found within a unique mRNP complex, which consists of mRNA and RNP. This provides valuable information regarding not only known components of a particular process or pathway, but importantly, leads to the identification of novel components representing potential therapeutic targets and biomarkers. In addition to those targets identified by pathway expansion, the specific RBPs (RNA binding proteina) regulating RNA functions may be potential therapeutic targets in their own right. RNP-IP is a technology that allows the isolation and identification of mRNAs, microRNAs and protein components of RNP complexes from cell extracts using antibodies to RBPs. Once purified, the RNAs present in the complex are analyzed to identify the target mRNAs using various molecular biology tools such as RT-PCR, gene expression analysis based on microarray technology (Chip analysis), or sequencing. Using this method, more RNA that is present in the nucleus can be obtained.

Materials and Reagents

1. Normal Rabbit IgG
2. High-salt solution
3. RIP-certified antibody (NBL, catalog number depends on what do you want to target)
4. Protease inhibitor (Molecular Biology)
5. Aprotinin
6. Leupeptin
7. Phenylmethylsulfonyl fluoride (PMSF)
8. RNase inhibitor (Life Technologies, Invitrogen™, catalog number: 10777-019)
9. Dithiothreitol (DTT) (reducing agent)
10. Protein A beads (GE Healthcare Dharmacon, catalog number: 17-0780-01) or Protein G beads (Thermo Fisher Scientific, catalog number: 22852)
11. Ethanol (Molecular Biology)

12. 2-Propanol (Molecular Biology)
13. Nuclease-free PBS
14. Isotype control IgG (if necessary)
15. Kit components (see Recipes)
16. Commercial reagent (see Recipes)
17. Buffer preparation (see Recipes)
18. Wash buffer (see Recipes)
19. Precaution: Additional buffer preparation (see Recipes)
20. Preparation of antibody-immobilized protein A or protein G agarose beads-RNP complex

Equipment

1. Microcentrifuge capable of 15,000 *x g*
2. Microcentrifuge tube (1.5 ml or 2 ml) (Nuclease-free) (recommendation; use low-adhesion tube for RIP-Assay)
3. Centrifuge capable of 2,000 *x g*
4. Centrifuge tube (15 ml or 50 ml)
5. Pipette (5 ml, 10 ml, 25 ml) (nuclease-free)
6. Pipette tip (10 µl, 20-100 µl , 200 µl , and 1,000 µl) (nuclease-free) (recommendation; use low-adhesion pipette tip for RIP-Assay)
7. Ultra low temperature freezer (-80 °C)
8. Freezer (below -20 °C)
9. End-over-end rotator
10. Vortex mixer
11. Gloves

Procedure

A. Pre-step: Preparation of antibody-immobilized protein A or protein G agarose beads.

1. Wash the protein A or protein G agarose beads 3 times with equal amount of nuclease-free PBS (centrifuge; 2,000 *x g* for 1 min at 4 °C).
2. Aliquot 25 µl of the 50% beads slurry to each new microcentrifuge tube.
3. Add 1 ml of wash buffer (+) to each tube.
4. Add 15 µg of antibody (normal Rabbit IgG as a negative control or RIP-Certified antibody for target RBP, respectively) to each tube.
5. Incubate the tube with rotation for at least 30 min at 4 °C. If necessary, this incubation can be extended to overnight.

B. Pre-step: Preparation of protein A or protein G agarose beads for preclear

1. Wash the protein A or protein G agarose beads 3 times with equal amount of nuclease-free PBS (centrifuge; 2,000 *x g* for 1 min at 4 °C).
2. Aliquot 25 µl of the 50% beads slurry to each new microcentrifuge tube.
3. Add 500 µl of wash buffer (+) to each tube, and mix briefly.
4. Centrifuge the tube at 2,000 *x g* for 1 min at 4 °C.
5. Discard the supernatant carefully.
6. Leave the beads at 4 °C or on ice until starting Preclear step.
7. Just before Preclear step, wash the beads once with 500 µl of lysis buffer (+).
8. Centrifuge the tube at 2,000 *x g* for 1 min at 4 °C.
9. Discard the supernatant carefully. Use these protein A or protein G agarose beads washed once with lysis buffer (+) for preclear step (step 28).

C. Lysis of mammalian cells

Note: In order to obtain "high-quality RNA", freshly cultured cells should be used in RIP-Assay.

1. Detach the cells from the culture dish by pipetting or using a cell scraper, if necessary. Collect the cell suspension into centrifuge tube.
2. Centrifuge the cell suspension at 300 *x g* for 5 min at 4 °C to pellet the cells. Carefully remove and discard the supernatant.
3. Wash the cells by resuspending the cell pellet with ice-cold PBS.
4. Centrifuge the cell suspension at 300 *x g* for 5 min at 4 °C to pellet the cells. Carefully remove and discard the supernatant.
5. Wash the cells once again using steps 17-18.
6. Wash the cells by resuspending the cell pellet with ice-cold nuclease-free PBS.
7. Centrifuge the cell suspension at 300 *x g* for 5 min at 4 °C to pellet the cells. Carefully remove and discard the supernatant.
8. Wash the cells by resuspending the cell pellet with ice-cold nuclease-free PBS.
9. Aliquot the cell suspension to each new microcentrifuge tube.
10. Centrifuge the cell suspension at 300 *x g* for 5 min at 4 °C to pellet the cells. Carefully remove and discard the supernatant.
11. Add 500 µl of lysis buffer (+) to each tube containing the cell pellet, and vortex thoroughly.
12. Incubate the tube for 10 min at 4 °C or on ice.
13. Centrifuge the cell suspension at 12,000 *x g* for 5 min at 4 °C.

D. Preclear step

1. Transfer the supernatant (cell lysate) to the tube (prepared in step 14) containing protein A or protein G agarose beads washed once with lysis buffer (+); that were prepared in steps 6-14.
2. Incubate the tube with rotation for 1 h at 4 °C.

E. Washing the antibody-immobilized protein A or protein G agarose beads

During Preclear step, wash the antibody-immobilized protein A or protein G agarose beads once with 1 mL of lysis buffer (+).

1. Centrifuge the tube (prepared in step 5) containing antibody-immobilized protein A or protein G agarose beads at 2,000 *x g* for 1 min at 4 °C.
2. Discard the supernatant carefully.
3. Add 1 ml of lysis buffer (+), and mix briefly, then centrifuge the tube at 2,000 *x g* for 1 min at 4 °C.
4. Discard the supernatant carefully.

F. Preparation of antibody-immobilized protein A or protein G agarose beads-RNP complex

1. Centrifuge the tube (prepared in step 29) containing cell lysate and protein A or protein G agarose beads at 2,000 *x g* for 1 min at 4 °C.

   Notes:

   a. *Preparation of Quality Check (QC) sample*

      In order to confirm whether RIP-Assay is running properly, we recommend to perform quality check. Collect QC sample and check the protein and RNA expression level at some steps. At least two additional aliquots may be retained for quality check. Use one of the aliquots (10 µl of precleared cell lysate, Input sample) for analysis of RBP expression level by Western Blotting, and use other aliquots (10 µl of precleared cell lysate) for analysis of Total RNA (See Example of RIP-Assay Results).

   b. *Preparation of Input sample (for western blotting)*

      i. *Add 10 µl of Laemmli's sample buffer to 10 µl of precleared cell lysate, boil for 3-5 min, mix well, and centrifuge.*

      ii. *Resolve 20 µl of the prepared sample on SDS-PAGE, and proceed to western blotting analysis.*

   c. *Preparation of total RNA (for quality check of Total RNA)*

      i. *Place 10 µl of precleared cell lysate at -80 °C until beginning of RNA isolation.*

      ii. *After RNP immunoprecipitation, use the lysate to prepare Total RNA sample according to RNA isolation protocol (See below).*

2. Transfer 500 µl of the precleared cell lysate to the tube (prepared in step 33) containing Antibody-immobilized protein A or protein G agarose beads washed once with lysis buffer (+), that was prepared in steps 30-33.
3. Incubate the tube with rotation for 3 h at 4 °C.

G. Wash of antibody-immobilized protein A or protein G agarose beads-RNP complex

1. Centrifuge the tube (prepared in step 36) containing Antibody-immobilized protein A or protein G agarose beads-RNP complex at 2,000 *x g* for 1 min at 4 °C.
2. Discard the supernatant carefully.
3. Add 1 ml of wash buffer (+), mix briefly, and centrifuge the tube at 2,000 *x g* for 1 min at 4 °C.
4. Discard the supernatant carefully.
5. Wash the antibody-immobilized beads-RNP complex twice using steps 39-40.
6. For fourth wash, add 1 ml of wash buffer (+), then mix well and dispense 100 µl of the mixture to new microcentrifuge tube for QC sample (post-IP beads). Use those aliquots for quality check by western blotting (See Example of RIP-Assay Results).
7. Resolve 20 µl of the prepared sample on SDS-PAGE, and proceed to western blotting analysis.
8. Centrifuge the tube containing antibody-immobilized protein A or protein G agarose beads-RNP complex at 2,000 *x g* for 1 min at 4 °C.
9. Discard the supernatant carefully.

   Notes:

   a. *Preparation of QC sample (for post-IP beads).*
   b. *Preparation of post-IP beads sample (for western blotting)*
      i. *Centrifuge the tube containing 100 µl of the mixture at 2,000 x g for 1 min at 4 °C.*
      ii. *Discard the supernatant carefully.*
      iii. *Resuspend the precipitated beads in 20 µl of Laemmli's sample buffer, boil for 3-5 min, mix well and centrifuge the tube at 2,000 x g for 1 min.*

Recipes

A. Kit components

1. Lysis buffer
2. Wash buffer (NT2)
3. Normal rabbit IgG
4. High-salt solution
5. Solution I: Enzyme solution

6. Solution II: Diluent for solution I
7. Solution III: Protein dissolvent. Solution III can dissolve protein and dissociate immunocomplex.
8. Solution IV: Co-precipitator. Solution IV can increase RNA precipitation efficiently.

B. Commercial reagent

1. Aprotinin (final concentration 10 μg/ml)
2. Leupeptin (5 μg/ml)
3. Phenylmethylsulfonyl fluoride (PMSF) (final concentration 0.5 mM)
4. RNase inhibitor (Life Technologies, Invitrogen™, catalog number: 10777-019) (final concentration is 50-200 U/ml)
5. Dithiothreitol (DTT) (reducing agent, final concentration is 1.5 mM)
6. 100% ethanol (molecular biology grade)
7. 100% 2-propanol (molecular bio)

C. Buffer preparation

1. Lysis buffer

   Add appropriate concentrations of protease inhibitors, RNase inhibitor, and DTT to lysis buffer just before use. Lysis buffer containing these reagents is described as *lysis buffer (+)* in the following protocols. The optimal concentration of each reagent for RIP-Assay is shown as follows.
2. Make RSB buffer in DEPEC treated water with final concentrations as follows:

   10 mM Tris (pH 7.5)

   100 mM NaCl

   2.5 mM $MgCl_2$

   Then add other supplements in per ml RSB buffer.

   10 μl digitornin/ml buffer (Digitornin is dissolved in ethanol 4 mg ml^{-1} freshly), 3 μl RNase inhibitor/ml buffer, 40 μl proteinase inhibitor cocktail/ml.

D. Wash buffer

1. Add final 1.5 mM concentration of DTT to wash buffer just before use. Wash buffer containing DTT is described as wash buffer (+) in the following protocols.
2. Make washing buffer in DEPEC treated water with final concentrations as follows:

   50 mM Tris (pH 7.5)

   50 mM NaCl

   1 mM $MgCl_2$

   0.05% Nonidet P40

E. Precaution: Additional buffer preparation

In some cases, both the lysis buffer (+) and wash buffer (+) may require the addition of appropriate volumes of high-salt solution (in these cases, add 30 µl of high-salt solution to each ml of lysis buffer and wash buffer).

F. RNA isolation (from Antibody-immobilized protein A or protein G agarose beads-RNP complex)

Solution II and Solution III should be equilibrated to room temperature before use.

Reagents should be briefly but thoroughly mixed before use.

1. Prepare master mix solution by diluting 10 µl of Solution I with 390 µl of Solution II per sample.
2. Dispense 2 µl of Solution IV to each new microcentrifuge tube for step 5.
3. Add 400µl of Master mix solution to each tube (prepared in RIP-step 44) containing
4. Antibody-immobilized protein A or protein G agarose beads-RNP complex (obtained in previous).
5. RNP Immunoprecipitation, vortex thoroughly, then spin-down.
6. Add 250 µl of Solution III to each tube, vortex thoroughly, then centrifuge the tube at 2,000·*x g* for 2 min at RT.
7. Carefully transfer the supernatant to the tube containing 2 µl of Solution IV prepared in step 2. (Avoid removing the protein A or protein G agarose beads from the pellet. Contamination of the beads may affect following steps.)
8. Add 600 µl of ice-cold 2-propanol to each tube, vortex briefly but thoroughly, then spin-down.
9. Incubate the tube at -20 °C or below for 20 min (or for overnight, if necessary).
10. Centrifuge the tube at 12,000 *x g* for 10 min at 4 °C, then aspirate the supernatant carefully.
11. Rinse the pellet with 500 µl of ice-cold 70% ethanol, and mix briefly.
12. Centrifuge the tube at 12,000 *x g* for 3 min at 4 °C, then aspirate the supernatant carefully.
13. Rinse the pellet once again using steps 9-10.
14. Dry up the pellet by aspirating excess ethanol followed by evaporation for 5-15 min at RT. Avoid RNase contamination (evaporation in clean bench is recommended).
15. Reconstitute the pellet in 20 µl of nuclease-free water.
16. Store at -80 °C until starting following analysis.

Acknowledgments

This protocol was adapted from the NBL RIP-Assay Kit (see Reference 1).

References

1. Protocol from NBL RIP-Assay Kit (catalog number: RN1001).