RNP-IP (Modified Method)-Getting Majority RNA from RNA Binding Protein in the Cytoplasm

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 modified method will get more RNA existing in cytoplasm. This method does not require a pre-clear step and getting the supernatant for western blot is different from the original method.

Materials and Reagents

1. Normal rabbit IgG
2. RIP-certified antibody (NBL, catalog number depends on what do you want to target)
3. NaCl
4. $MgCl_2$
5. Nonidet P40
6. NaoAc
7. Protein A beads (GE Healthcare Dharmacon, catalog number: 17-0780-01) or Protein G beads (Thermo Fisher Scientific, catalog number: 22852)
8. Ethanol (molecular biology grade)
9. 2-Propanol (Molecular Biology)

10. Nuclease-free PBS
11. Nuclease-free water
12. Isotype control IgG (if necessary)
13. Digitornin
14. Aprotinin (final concentration 10 μg/ml)
15. Leupeptin (5 μg/ml)
16. Phenylmethylsulfonyl fluoride (PMSF) (final concentration 0.5 mM)
17. RNase inhibitor (Life Technologies, Invitrogen™, catalog number: 10777-019)
18. Dithiothreitol (DTT) (reducing agent)
19. Lysis buffer (see Recipes)
20. Wash buffer (NT2) (see Recipes)
21. Precaution: Additional buffer preparation (see Recipes)

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

Day 1

1. The following components in DEPEC treated water are final concentrations.
   50 mM Tris (pH 7.5)
   50 mM NaCl

1 mM $MgCl_2$

0.05% Nonidet P40

2. Wash beads with NT2 buffer. If you have 4 samples, you need to take 100 µl beads to each Eppendorf tube, total 4 tubes. 3,600 rpm 1 min at 4 °C, remove supernatant. Wash 3 times with 200 µl NT2 buffer.
3. Add 30 µg target antibody (such as HuR 1 µg/µl from NBL) to each tube.
4. Add 320 µl NT2 buffer to each tube. Overnight rotate at 4 °C.

Day 2

5. Centrifuge the tubes (Ab+ beads) 5,000 *x g* 5 min at 4 °C. Discard supernatant wash twice with freshly made NT2 buffer 1 ml. After last wash, temporarily keep supernatant and put on ice until cell lysate is ready.

B. Lysis of mammalian cells

6. Prepare cell pellet:
   a. Detach the cells from the culture dish by pipetting or using a cell scraper, if necessary. Collect the cell suspension into centrifuge tube.
   b. Centrifuge the cell suspension at 300 *x g* for 5 min at 4 °C to pellet the cells. Carefully remove and discard the supernatant.
   c. Wash the cells by resuspending the cell pellet with ice-cold PBS.
   d. Centrifuge the cell suspension at 300 *x g* for 5 min at 4 °C to pellet the cells. Carefully remove and discard the supernatant.
   e. Wash the cells once again using steps 3-4.
   f. Wash the cells by resuspending the cell pellet with ice-cold nuclease-free PBS.
   g. Centrifuge the cell suspension at 300 *x g* for 5 min at 4 °C to pellet the cells. Carefully remove and discard the supernatant.
   h. Wash the cells by resuspending the cell pellet with ice-cold nuclease-free PBS.
   i. Aliquot the cell suspension to each new microcentrifuge tube.
   j. Centrifuge the cell suspension at 300 *x g* for 5 min at 4 °C to pellet the cells. Carefully remove and discard the supernatant.
7. Lysis cell pellet
   a. Make RSB buffer in DEPEC treated water with final following concentrations.
      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 freshly), 3 µl RNase inhibitor/ml buffer, 40 µl proteinase inhibitor cocktail/ml.

b. Add 200 µl of RSB buffer to each cell pellet. On ice 2 min. Centrifuge 4,400 rpm 4 °C, 8 min.

c. Make 753 µl master mix for each tube (Ab + beads). For 4 tubes, you can make 4.5 times more.
   700 µl NT2 buffer
   10 µl 0.1 M DTT
   10 µl RNase inhibitor
   33 µl 0.5 M EDTA (pH 8)

d. Then add each supernatant of cell lysate to tube (Ab+ beads). Rotate at 4 °C 2 h.

e. Take 20 µl for western blot from the mixture. The centrifuge the tube at 5,000 *x g* 4 °C 2 min.

f. Discard supernatant. Wash pellet with 1 ml NT2 buffer twice at 5,000 *x g* 4 °C 2 min.

g. Further process the mixture to get mRNAs. Make DNAase master mix.
   Now we have 4 tubes, so make 4.5 times master mix.
   100 µl of NT2 buffer x 4.5=450 µl
   5 µl of DNAase (2U/µl) x 4.5=22.5 µl
   Add 105 µl of DNAase master mix to each tube and 37 °C, 10 min incubation.

h. Then add 1ml NT2 buffer centrifuge 5,000 *x g* 5 min 4 °C. Remove supernatant.

i. Add proteinase K (20 mg/µl) master mix 103 µl to each tube. 55 °C, 15 min plus mixing.
   Master mix: 2.5 µl proteinase K x 4.5=11.25 µl
   0.5 µl 20% SDS x 4.5=2.25 µl
   100 µl NT2 buffer x 4.5=450 µl

j. Centrifuge 5,000 *x g* 4 °C 5 min.

k. Collect 100 µl supernatant to a new Eppendorf tube. Add 200 µl of NT2 buffer to old tube, centrifuge 5,000 *x g* 4 °C, 5 min again. Collect 200 µl of supernatant and pool to the new tube, which becomes 300 µl of supernatant.

l. Add equal volume of acid phenol/chloroform to above supernatant.
   Vertex 1 min, then centrifuge 1 min RT, 13k rpm.

m. Collect about 250 µl of aqueous phase (top phase)
   Add: 25 µl of 3 M NaoAc (pH 5.5)
   625 µl of 100% ethanol
   3 µl of glycogen blue
   Mix well and put it at -20 °C or below for 20 min (or for overnight, if necessary).

n. Centrifuge the tube at 12,000 *x g* for 10 min at 4 °C, then aspirate the supernatant carefully.
o. Rinse with 1 ml 70% Ethanol, 13,000 *x g* 2 min at 4 °C. Discard supernatant. Invert the tube, air dry 5 min. The pellet is mRNAs.

Recipes

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.
   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 freshly), 3 µl RNase inhibitor/ml buffer, 40 µl proteinase inhibitor cocktail/ml.
2. Wash buffer
   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.
   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
3. 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).

Acknowledgments

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

References

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

