RIP-ChIP Protocol

Adapted from Keene, J. et at.(2006) *Nature Protocols*. Vol 1. No 1. pg 302

REAGENT SETUP

1. Polysome lysis buffer

100 mM KCl
5 mM MgCl2
10 mM HEPES (pH 7.0)
0.5% NP40
1 mM DTT
100 units ml−1 RNase Out
400 μM VRC
Protease inhibitor cocktail

***** To make 5 ml of polysome lysis buffer, add 50 μl of 1 M HEPES (pH 7.0), 500 μl of 1 M KCl, 25 μl of 1 M MgCl2 and 25 μl of NP40 to 4.7 ml of RNase-DNase−free H2O. Add 50 μl of 1 M DTT, 12.5 μl of 100 U/ml RNase Out, 200 μl of Protease inhibitor cocktail (dissolve tablets according to the manufacturer's instructions) and 10 μl of 200 mM VRCs at the time of use.

2. NT2 buffer

50 mM Tris-HCl (pH 7.4)
150 mM NaCl
1 mM MgCl2
0.05% IGEPAL

*To make 500 ml of NT2 buffer, add 25 ml of 1 M Tris (pH 7.4), 15 ml of 5 M NaCl, 500 μl of 1 M MgCl2 and 1.25ml of 20% IGEPAL, Q.S. to 500ml with RNase-DNase−free H2O.

PROCEDURE

Preparation of mRNP lysate

1. Collect enough tissue culture cells to generate 2−5 mg of total protein per RIP. Typically, this is comparable to 5−20 × 10_6 mammalian cells. Pellet by centrifugation (~1,000*g*) for 10 min at 4 °C, washing several times with 10 ml of ice cold phosphate buffered saline (PBS) in a conical tube. Alternatively, whole tissue may be ground using a mechanical homogenizer. Additionally, individual cells derived by micro-dissection may be used. The total amount of protein used per RIP must be optimized based upon the abundance of the RNA-binding protein being investigated as well as the planned method of RNA detection.

2. Resuspend final cell pellet with an approximately equal volume of polysome lysis buffer supplemented with RNase inhibitors and protease inhibitors (see REAGENT SETUP). Clumps of cells should be broken up by pipetting up and down several times. Allow mRNP lysate to incubate on ice for 5 min and store at −100 °C. Lysate may be stored for several months at −100 °C. The lysis of certain cell types can be enhanced by pumping the lysate through a small gauge syringe needle.

▲ **CRITICAL STEP** Immediate freezing of the lysate is essential to complete the lysis process as well as preventing adventitious binding. Additional freeze-thaw cycles should be avoided to prevent protein and RNA degradation.

Antibody coating of protein A/G beads

3. At 4 °C, pre-swell protein-A Sepharose beads in NT2 supplemented with 5% BSA to a final ratio of 1:5 for at least 1 h before use. Protein G or A/G Sepharose beads may be substituted depending upon the isotype of the antibody being used.

■ **PAUSE POINT** Pre-swollen beads may be stored for several months at 4 °C when supplemented with 0.02% sodium azide.

4. In a 1.5 ml microcentrifuge tube, add 250–500 µl of protein A–BSA slurry. After a pulse centrifugation this should yield a pelleted bead volume of ~50 µl at the bottom of the microcentrifuge tube.

5. Add antibody to bead slurry and incubate for 2–18 hours, tumbling end over end at 4 °C. The volume of antibody added to the beads is dependent upon antibody titer, but this amount should be more than enough to pull down all available protein being investigated.

***** 10-15ug of Antibody is typical used

***** Ab and bead slurry is tumbled overnight at 4°C in 4.5ml NT2 buffer (15ml conical tube)

■ **PAUSE POINT** This mixture may be stored for several weeks at 4 °C when supplemented with 0.02% sodium azide.

▲ **CRITICAL STEP** In parallel, a control antibody must be used to assess background RNA levels. Typically, this is an isotype matched antibody or whole normal sera from a matched species. The amount of control antibody should be equal to the amount of immunoprecipitating antibody being used.

6. Immediately before use, wash antibody-coated beads with 1 ml of ice-cold NT2 buffer 4–5 times. To wash,

- Spin down beads 2000 rpm for 2min at 4 °C, remove liquid with hand pipettor or aspirator
- Resuspend in ice-cold NT2 buffer by flicking the tube several times with a finger.
- Repeat

* If one IP is being prepared (50ul beads/ Ab) then the next steps can be done in a 1.5ml microcentrifuge tube.

*This washing removes unbound antibody as well as contaminants such as RNases, which may be present in the antibody mixture, and is one of the reasons we pre-bind the antibody to beads.

Immunoprecipitation reaction and RNA precipitation

7. After the final wash, resuspend Ab coated beads in ~850ul of the following solution:

For 50ul beads:

- 200 units of an RNase inhibitor (5 µl RNase Out)
- 2 µl VRC (final concentration of 400 µM) Vanadyl ribonucleoside complexes
- 10 µl of 100 mM DTT
- 30 µl of 0.5M EDTA
- 800ul Cold NT2 buffer

*For preparation of more then one IP at a time → prepare a mastermix, then aliquot 850ul solution to resuspend 50ul Ab coated beads

8. Thaw mRNP lysate on ice and centrifuge at 15,000*g* for 15 min to clear lysate of large particles. Transfer cleared supernatant to microfuge tube and store on ice. Additionally,

pre-clearing of lysate with beads may be used to reduce background, if necessary. This may, however, reduce signal.

9. Add 150 µl of cleared lysate (20mg/ml) to antibody mixture prepared in Step 7.
* A typical IP uses 3mg of protein lysate

10. Immediately flick tube several times with a finger to mix, and centrifuge briefly at 8,000−10,000*g* to pellet beads. Remove 100 µl of supernatant to represent total cellular mRNA. OPTIONAL

11. Incubate for 4 h at 4 °C tumbling end over end. Alternatively, incubate 2 h at room temperature (18−25 °C) and times as short as 15 min have worked well in some cases.
* Incubate for 2 hours at room temp

12. Pellet beads and save supernatant for later analysis if desired. Supernatant may be stored at −20 °C for several months. OPTIONAL

13. Pellet beads by centrifugation at 2000 rpm for 2min and removing supernatant with a hand pipettor or an aspirator. Wash beads 4−5 times with 1 ml of ice-cold NT2 buffer
* 3 washes with 1% Urea-NT2 buffer
* 4 washes with NT2 buffer

▲ **CRITICAL STEP** Thorough washing is critical for reducing background. NT2 buffer may be supplemented with urea, sodium deoxycholate or SDS to increase stringency depending upon the RNA-binding protein being investigated. All tubes should be kept on ice as much as possible while working quickly during the washing process to reduce degradation.

14. Resuspend the beads in 100 µl of NT2 buffer supplemented with 30 µg of proteinase K to release the RNP components. Incubate mixture for 30 min at 55 °C, flicking the tube occasionally. (SKIP step if using TriZol Method of RNA Isolation)

15. Release the RNP components and isolate the RNA from the immunoprecipitated pellet by adding either Trizol reagent (Invitrogen) or phenol-chloroform-isoamyl alcohol directly to the beads. Precipitate RNA and resuspend in a volume appropriate for subsequent detection method.

- Addition of glycogen (20 µg) as a carrier to the precipitation reaction aids in making the RNA pellet more readily visible and aids in recovery of RNA.

*We use the TriZol reagent as follows:

1. Add 1ml TRIzol to beads and vortex
2. Set at room temp for 5 min
3. Add 200ul Chloroform
4. Shake vigorously by hand for 15 sec
5. Incubate for 2 min at room temp
6. Centrifuge 12k x g for 15 min at 4°C
7. Transfer aqueous layer to a new tube
8. Add 500ul chloroform (back extraction)
9. Centrifuge 12k x g for 5 min at 4°C
10. Transfer aqueous phase to new tube (Do Not get chloroform in sample)
11. Add 1ul of 20mg/ml glycogen and finger vortex
12. Add 500ul Isopropanol
13. Incubate 10min at room temp

14. Centrifuge 12k x g 10min at 4°C
15. Remove and discard supernatant from pellet
16. Add 1ml 75% Ethanol
17. Store -20°C overnight
18. RNA pellet visible on bottom of tube
19. Inverted several times to dislodge pellet
20. Centrifuge at 7.5k x g 5min at 4°C
21. Remove supernatant with pipette , leaving a little at bottom and spin down contents
22. Remove all ethanol with p20, place pipette tip at bottom away from pellet and aspirate
23. Dry pellet (a dry RNA pellet becomes a clear film) Do Not over dry
24. Resuspend in 15ul ddH_2O
25. Triturate
26. Incubate 50°C for 10 min
27. Transferred to a clean tube (do not spin contents down, precipitation contains contaminants)