Cross-linked RNA Immunoprecipitation

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[Abstract] This method is for the immunoprecipitation of Flag-Tagged RNA binding proteins from mammalian cell lines and isolation of the bound RNAs for analysis by quantitative real-time PCR. The RNA binding protein of interest should be tagged with the M2 Flag-tag and expressed in the mammalian cell line of interest (Knuckles *et al.*, 2012). However, specific antibodies for the protein of interest can be used in conjunction with Sepharose G-beads.

Materials and Reagents

1. Anti-Flag M2 Affinity gel (Sigma Aldrich, catalog number: A2220)
2. RNase inhibitor (Life Technologies, Invitrogen™, catalog number: N8080119 or Bioline, catalog number: BIO-65028)
3. Complete Protease Inhibitor Cocktail Tablets (F. Hoffmann-La Roche, catalog number: 05 892 970 001)
4. RnaseZap (Life Technologies, Ambion®, catalog number: AM9780)
5. Trizol reagent (Life Technologies, Invitrogen™, catalog number: 15596-026)
6. General chemicals (Sigma Aldrich)
7. DNase I recombinant, RNase-free inc. buffer (F. Hoffmann-La Roche, catalog number: 04716728001)
8. BioScript™ (Bioline, catalog number: BIO-27036)
9. Formaldehyde
10. Fetal calf serum
11. Glycine
12. SDS
13. HEPES
14. TritonX-100
15. EDTA
16. DTT
17. NaCl
18. IP lysis buffer (see Recipes)
19. RIP buffer (see Recipes)

Equipment

1. Sonicator (the model is not critical but preferably a devise with a probe ≤ 5 mm in diameter)
2. Tube Rotator
3. Shaker
4. Centrifuge
5. PCR machine
6. Heating block
7. 15 ml conical tubes

Procedure

A. Preparation of beads and cells

1. Blocking of the beads:
   Wash 40 µl Anti-Flag M2 Affinity Gel twice with 900 µl of pure H_2O (cold); add 400 µl IP lysis buffer +1% BSA; incubate at 4 °C (on a rotating wheel) overnight.
   Alternatively use 30 µl SepharoseG-Beads + specific antibody. The amount of antibody and beads to be used in the precoupling will need to be determined in preliminary experiments. Typically using 10 µg of Ig for coupling is a reasonable starting point.
2. Use 2 x 10 cm^2 tissue culture plate per condition (this might vary depending on the cell type and the level of expression of the protein of interest). Do not process more than 10 plates at a time.
3. Transfect cells and incubate them for 48 h to express the tagged protein.

B. Harvesting the cells

4. Remove medium, wash the cells once with phosphate buffered saline and add 1 ml 0.25% Trypsin (pre-heated to 37 °C). Incubate at 37 °C until the cells start to detach.
5. Add 5 ml DMEM including 10% fetal calf serum (pre-warmed to 37 °C) to inhibit the trypsin.
6. Remove the cells from the plate by pipetting.
7. Transfer the cells to a 15 ml conical tube and incubate on ice for 5 min.
8. Harvest cells 2 min at 100 *x g* at room temperature, decant the supernatant and resuspend the cell pellet in 5 ml ice-cold phosphate buffered saline. Keep a 250 µl aliquot for Western blot analysis to be used as a transfection control.
9. Add 143 µl 37% formaldehyde (over a period of approximately 10 sec, treat all of the samples in the same way).

10. Place the 15 ml conical tubes on a rocking plate and shake for 10 min at room temperature.
11. Add 685 µl 2 M glycine (over a period of approximately 10 sec, treat all of the samples in the same way) to block the formaldehyde.
12. Place the 15 ml conical tubes on a rocking plate and shake for 5 min at room temperature.
13. Harvest cells by centrifugation for 2 min at 100 *x g* at room temperature.
14. Decant the supernatant and transfer the cell pellet to ice.
15. Wash the cells twice with 5 ml ice-cold phosphate buffered saline and harvest the cells 2 min at 100 *x g* at 4 °C.
16. Remove the supernatant from the cells after the last wash and add 1 ml of IP lysis buffer + 20 µl 0.1 M phenylmethylsulfonyl fluoride + 20 µl complete protease inhibitor (50x) + 5 µl RNase inhibitor (40 U/ul) to each sample.
17. Sonicate the cells, keeping them on ice at all times, 10x (10 sec on, 10 sec off, Amplitude 15 µm) until the lysate is clear. Clean probe sonicator with RNaseZap between the samples to avoid contamination with RNases.
18. Transfer the cells to a 1.5 ml snap-cap tube and keep on ice.
19. Centrifuge the lysates for 3 min at 14,000 *x g* at room temperature and keep 50 µl of the supernatant as an INPUT control (used to standardize the qPCR analysis).

C. Immunoprecipitation

20. Add the rest of the lysate to the blocked beads and incubate overnight at 4 °C on a rotating wheel.
21. Wash the beads 5 times with 900 µl of IP lysis buffer collecting the beads by centrifugation for 1 min at 400 *x g* at room temperature.
22. Remove all of the supernatant after the last wash and add 100 µl of RIP buffer + 1 µl RNase inhibitor.

    Add 50 µl of RIP buffer to the INPUT control (step 18).
23. Incubate the samples and INPUT controls for 1 h at 70 °C to reverse the cross-link.
24. Centrifuge for 1 min at 400 *x g* at room temperature to sediment the beads and collect 100 µl of supernatant.
25. Extract the RNA with Trizol reagent (proceed according to the manufacturer'sinstruction (Invitrogen)). *Optional:* Add 1 µl of Glycogen blue before precipitation of the RNA with Isopropanol to make the pellet visible. Precipitation can also be performed at -20 °C for 1 h to increase the amount of precipitated RNA.
26. DNAse treatment of the RNA to avoid contamination with genomic DNA.

Dilute the RNA pellet directly in DNase mastermix (per sample: 16 µl DEPC treated H_2O, 2 µl DNaase buffer, 2 µl DNAse) in order to use all RNA in the following RT reaction and proceed after the manufacturer's instruction (Roche).

D. Reverse transcription of the RNA into cDNA

27. BioScript™ (Bioline) kit works well but other reverse transcriptase kits may also be used. 9 µl of the DNAse treated RNA was used in a reverse transcriptase-containing and a reverse transcriptase-minus (negative control) reaction for each sample. cDNA was primed with random hexamer primers and the reaction performed according to the manufacturer's instructions.
28. Dilute the cDNA 1:4 and continue with quantitative real-time PCR analysis of target gene to identify specific changes in target mRNA level. Alternatively the cDNA can be used to generate a library.

Recipes

1. IP lysis buffer
   50 mM Hepes (pH 7.5)
   0.4 M NaCl
   1 mM EDTA
   1 mM DTT
   0.5% TritonX-100
   10% Glycerol
2. RIP buffer
   50 mM Hepes (pH 7.5)
   0.1 M NaCl
   5 mM EDTA
   10 mM DTT
   0.5% TritonX-100
   10% Glycerol
   1% SDS
   All solutions should be prepared RNAse free with Diethylpyrocarbonate (DEPC)-treated water and autoclaved before use.

Acknowledgments

This protocol was previously used in Knuckles *et al.* (2012).

References

1. Knuckles, P., M. A. Vogt, S. Lugert, M. Milo, M. M. Chong, G. M. Hautbergue, S. A. Wilson, D. R. Littman and V. Taylor (2012). Drosha regulates neurogenesis by controlling neurogenin 2 expression independent of microRNAs. *Nat Neurosci* 15(7): 962-969.

