RNA-Immunoprecipitation (RNA-IP)

Kathrin Leppek, Georg Stoecklin lab, 2013

Small scale RNA-IP to confirm mRNA targets of RNA-binding proteins by RT-qPCR. In this case RAW264.7 macrophages were used to IP RNA-BP targets. HA-antibody was used as a negative control. Keep in mind that some cells, in particular primary cells, contain high concentrations of RNases, which will make it difficult to carry out RNA-IP since the RNA will be severely degraded after the IP procedure.

Preparation of beads (for 4 RNA-IP samples)
- 320 µL of 50% ProtA/G-agarose beads (*Pierce*)
- 3x wash in 1 mL of 1x lysis buffer
- spin 500 rpm, 1 min, 4°C
- remove supernatant
- make 1:1 slurry in lysis buffer

Lysis (using a mixermill, will allows excellent preservation of frozen RNP complexes)
- collect cells, typically from one subconfluent 10-cm dish per RNA-IP sample
- place cell pellet on ice
- resuspend cells in 1 mL PBS and transfer to 2 mL tube
- spin 1'000 rpm, 2 min, 4°C, take off PBS completely with pipette
- add iron ball, transfer the tube to liquid N_2 and lyse with a mixermill (TissueLyzer II, Qiagen, settings: 15 sec, 25 Hz, the tube holder is kept at -70ºC until use so that the cell material remains frozen during millling)
- transfer tube to ice and add 400 µL ice-cold lysis buffer, let thaw
- tumble for 10 min at 4°C
- spin 5 min, 2'000 rpm (tabletop centrifuge) at 4°C to pellet debris
- transfer supernatants into fresh tubes = cytoplasmic lysates (400 µL), keep on ice
- <u>input protein</u>: save a 10 µl aliquot of the lysate and add 10 µL 2x SDS-sample buffer
- <u>input RNA</u>: extract RNA from a 30 µl aliquot of each lysate using 500 µL of TriFast (*PeqLab*)

Preclear
- add 30 µL 50% pre-washed ProtA/G-agarose beads to each lysate (~400 µl)
- tumble for 1 h at 4°C
- spin at 5'000 rpm for 2 min at 4°C
- transfer the precleared supernatants to a fresh tube
- discard beads

IP
- add 2-4 µg antibody to each precleared lysate (~400 µl)
- tumble 1.5 h at 4°C
- add 50 µL 50% pre-washed Prot A/G-agarose beads (to 400 µl, 4x)
- tumble 1.5 h at 4°C
- spin 1 min, 500 rpm, 4°C
- remove supernatant. If you want to analyse the unbound material, extract protein and RNA from this supernatant as you did for the input material.
- wash beads once with 1 mL 1x lysis buffer and 4x with 1 mL wash buffer. Each time, tumble 5 min at 4°C and spin 1 min, 500 rpm, 4°C.
- after the last wash, add 1 mL wash buffer and resuspend the beads. Split the bead suspension into two tubes by transferring 200 µl (for protein) and 800 µl (for RNA). Spin tubes, remove supernatants completely with a thin loading tip.
- <u>elute protein</u> bound to the beads by adding 30 µL 2x SDS-sample buffer to beads. For Western blot analysis, load 10 µL of the input samples (total 20 µL) and 15 µL of the IP eluate (total 30 µL) onto an appropriate SDS-polyacrylamide gel.
- <u>elute RNA</u> bound to the beads by adding 1 µL GlycoBlue (Ambion) and RNA extraction using 500 µl of TriFast

RNA Extraction using TRIzol or TriFast

- *(pre-cool centrifuge to 4°C)*
- Add 500 µL TRIzol or TriFast to the beads under the hood, mix by vortexing
- Let stand 5 min at RT
- Add 100 µL chloroform
- Shake 15 sec by hand
- Let stand 2-3 min at RT
- Centrifuge 10 min at 12.000 g at 4°C
- Transfer the aqueous (upper) phase into a fresh tube
- add 500 µL isopropanol and 1 µL GlycoBlue/Glycogen (5-10 µg), vortex to mix
- precipitate RNA: 10 min at RT, 1 h or o/n at -20°C, or 30 min at -80°C
- centrifuge 20 min at 12'000 g at 4°C to pellet RNA
- remove supernatant, wash RNA pellet with 1 mL ice-cold 75% EtOH (vortex shortly)
- centrifuge 5 min at 10'000 rpm (7'500 g, not more) at 4°C
- discard supernatant (WATCH slippery pellet), dry pellet 10-20 min with tube upside down under hood
- resuspend RNA pellet in 10-20 µL ddH_2O, (vortex; 10 min, 65°C; vortex, spin)
- keep RNA on ice if you use it the same day, otherwise store at -80°C

qPCR

- Use 4 µL of RNA (input and elution) for DNase treatment in 10 µL (RQ1 Dnase, Promega)
- Perform RT reaction using the 10 µl of DNase-treated RNA in 20 µL with Transcriptor RT (Roche)
- Dilute the cDNA 1:12 for usage in the qPCR reaction to obtain reasonable Ct-values. This might vary depending on the expression level of the mRNA.

Things to consider:

- always use DNA/RNA LoBind tubes (*Eppendorf*) when working with beads or lysates, for initial bead washing and lysate preparation this is not so critical but the elution of RNA is essential to occur in LoBind tubes otherwise the Core Facitlity is not able to recover enough RNA for deep seq for example.
- change to fresh tubes after the last wash step to minimize contamination by protein/RNA sticking to the tube wall
- work on ice all the time, keep centrifuges at 4°C
- use thin loading tips to remove residual fluid from beads before elution
- if you don't use the mixermill, you can add final 1% NP-40 to the lysis buffer for detergent lysis
- extensive washing might reduce background binding to such an extend that you will not pick up the negative control mRNAs in the qPCR

Solutions

lysis buffer (for 20 ml)

400 µl	1 M Tris pH 7.5 (20 mM)
3 ml	1 M NaCl (150 mM)
300 µl	0.1 M $MgCl_2$ (1.5 mM)
20 µl	1 M DTT (1 mM)
2 tablets	Mini complete protease inhibitors, EDTA-free, (Roche)
16.1 ml	milliQ-H_2O

wash buffer (for 40 ml)

800 µl	1 M Tris pH 7.5 (20 mM)
12 ml	1 M NaCl (300 mM)
1 ml	0.1 M $MgCl_2$ (2.5 mM)
40 µl	1 M DTT (1 mM)
26 ml	milliQ- H_2O