RNA pull-down assay

Chang Lab's protocol with modification

Day 1

1. DIG-labeled RNAs were in vitro transcribed with the DIG RNA Labeling Mix (Roche) and T7 RNA polymerase (Ambion), treated with RNase-free DNase I (Ambion) and precipitated with LiCl (Roche).

2. Northern blot was followed by manufacture's manual (NorthernMax kit, Ambion) to demonstrate that all the RNAs are DIG labeled and transcribed at the right size.

Day 2

3. 3ug of DIG labeled RNA was heated to 90°C for 2 min put on ice for 2 min, supplied with RNA structure buffer (10 mM Tris pH 7, 0.1 M KCl, 10 mM MgCl2) (make a 5X stock), and then shifted to RT for 20 min to allow proper secondary structure formation.

4. 2g of staged embryo were homogenized in equal volume of embryo homogenization buffer (100 mM potassium acetate; 30 mM HEPES-KOH at pH 7.6; 2 mM magnesium acetate; 5 mM DTT and 1 tablet of protease inhibitor (Roche), 40 units/ml RNaseOUT (Ambion)) with a Dounce homogenizer with 15–20 strokes by hand on ice without introducing air bubbles into the homogenate.

5. The homogenate is centrifuged at 14,500g for 30 min at 4°C. The fat layer is removed and the supernatant solution is transferred to a new tube without touching the pellet. The extract can be used immediately or added dropwise to liquid nitrogen and stored at -80°C.

6. 3ug of folded RNA was then mixed with 1mg of cytoplasmic extract in extraction buffer (typically 100-1000ul total binding reaction) and incubated at RT for 1h.

7. Add 5 ug of anti-DIG Ab into mixture of DIG labeled RNA and cytolasmic extract, and further incubate at 4°C O/N. The optimal amount of Ab needed depends upon the individual Ab used. Dilute antibody (typically 1-10 ug) in 200 ul PBS with Tween-20. The incubation time depends on the affinity of the antibody.

Day 3

8. Prepare Dynabeads:

- Resuspend Dynabeads in the vial (vortex >30 sec or tilt and rotate 5 min).
- Transfer 50 ul (1.5 mg) Dynabeads to a tube.
- Place the tube on the magnet to separate the beads from the solution and remove the supernatant.
- Remove the tube from the magnet.

9. Binding of antibody:

- Add RNA/extract/Ab mixture to the Dynabeads.
- Incubate with rotation for 10 min at RT.

- Place the tube on the magnet and transfer the supernatant to a clean tube for further analysis if desired (loading on SDS-PAGE gel for control).
- Remove the tube from the magnet and gently pipette to resuspend the RNA/extract/Ab/Dynabeads complex in 200 ul PBS with Tween-20. Wash by gentle pipetting.
- Wash the RNA/extract/Ab/Dynabeads complex 3 times using 200 ul washing buffer (PBS+0.1% Tween-20) for each wash. Separate on the magnet between each wash, remove supernatant and resuspend by gentle pipetting.
- Resuspend the Dynabeads-Ab-Ag complex in 100 ul washing buffer and transfer the bead suspension to a clean tube. This is recommended to avoid contamination of proteins bound to the tube wall.

* For storage, freeze the RNA/extract/Ab/Dynabeads complex after adding elution buffer and sample buffer. For analysis of the sample, thaw and continue with SDS-PAGE gel analysis.

Day 4

10. Elution and detection

- Place the tube on the magnet and remove the supernatant.
- Add 20 ul of Elution buffer and 10ul premixed NuPAGE LDS Sample Buffer and 2-mercaptoethanol (NuPAGE LDS Sample Buffer:2-mercaptoethanol 9:1).
- Gently pipette to resuspend the RNA/extract/Ab/Dynabeads complex.
- Heat for 10 min at 70°C.
- Place the tube on the magnet and load the supernatant/sample onto a SDS-PAGE gel.

* If don't run gel immediately, store the sample at -80°C. Thaw RNA/extract/Ab/Dynabeads complexs and boil for 5 min when you want to run gel.