Pull-down of RNA binding proteins with immobilized biotinylated transcripts

Buffers

- Cell lysis buffer:
    - 85 mM M KCl
    - 0.5% NP-40 (Pierce)
    - 5 mM HEPES, pH 7.4
    -
- Nuclei lysis buffer:
    - 300 mM NaCl
    - 20 mM Tris-HCl, pH 8.0
    - 0.2 % Tween-20
    - 1 mM EDTA
    - 1 mM EGTA
- 2x Binding/Washing buffer:
    - 10 mM Tris-HCl, pH 7.5
    - 1 mM EDTA
    - 2 M NaCl
    - 0.1 % Tween-20
- Streptavidin beads (Dynabeads)

1. Preparation of biotinylated transcripts

Materials

- Ambion MEGAscript T7 RNA-Polymerase Kit
- Bio-UTP (10 mM)
- Linearized template
- PCR machine
- RNeasy Kit (or Tri/TRIzol for purification of transcript).

Specific comment

- Original protocol from the Ambion Kit was changed to reduce the amount of biotinylated UTP.

Protocol

- Reaction mix (pipette in the order below):
    - 2 mM ATP, CTP and TTP

- 1 mM UTP
- 1 mM bio-UTP
- 2µl 10x buffer
- 1µg linearized vector (use less in case of PCR products, see protocol from the kit).
- 2 µl enzyme mix
- H_2O ad 20 µl

- Incubate overnight at 37°C ??? (have to look up)
- Add 1 µl DNase I from Ambion MEGAscript Kit, incubate at 37°C for 15 min.
- Purify RNA with RNeasy Kit according to protocol supplied with the kit
- Elute RNA with 50 µl H_2O

2. Immobilization of the biotinylated RNA to Streptavidin Dynabeads

Materials

- Streptavidin beads (Dynabeads)
- 2x Binding/Washing buffer:
    - 10 mM Tris-HCl, pH 7.5
    - 1 mM EDTA
    - 2 M NaCl
    - 0.1 % Tween-20

Protocol

Preparation of beads

- Wash Streptavidin beads (Dynabeads) 3x with Binding/Washing buffer (use 50 µl of bead suspension in a final assay with 1 ml of cell lysate)
- Wash beads 2x for 2 min with 50 µl of 0.1 M NaOH/0.05 M NaCl
- Wash once with the same volume of 0.1 M NaCl
- Resuspend beads in 50 µl of 0.1 M NaCl
- Transfer equal amounts of beads to DNA Low Binding tubes (0.5 ml sized)

Binding of RNA to beads

- Dilute biotinylated transcripts with H_2O (should have the same volume as the bead suspension)
- Denature RNA at 65°C for 10 min
- Cool down to RT over 20 min
- Add transcripts to beads; add water or unrelated RNA to control beads
- Incubate on a turning wheel at RT for 15 min
- Remove supernatant
- Measure OD_{260} of the supernatant to verify efficient binding of the RNA to the beads
- Wash beads 3x with 1x Binding/Washing buffer

3. Preparation of cell lysates and pull down with immobilized RNA

Materials

- Mammalian cells growing adherently on 10 cm or 15 cm dishes
- Cell lysis buffer:
    - 85 mM M KCl
    - 0.5% NP-40 (Pierce)
    - 5 mM HEPES, pH 7.4
    -
- Nuclei lysis buffer:
    - 300 mM NaCl
    - 20 mM Tris-HCl, pH 8.0
    - 0.2 % Tween-20
    - 1 mM EDTA
    - 1 mM EGTA

Protocol

- *Extract preparation*
    - Remove medium, harvest cells with 1x PBS, transfer cells into a tube
    - Spin for 5 min at 800 rcf
    - Wash cell pellet once with 1x PBS
    - Resuspend pellet in 2-3 volumes of cell lysis buffer (at least 100 µl)
    - Incubate on ice for 10 min
    - Centrifuge for 5 min at 5,000 rpm at 4°C, discard supernatant (cytoplasmic extract)
    - Resuspend nuclear pellet in 3-4 volumes of nuclei lysis buffer (at least 200 µl, better more). If the pellet is difficult to resuspend use a syringe or sonify samples in a bioruptor (setting: 15 cycles, high, 30s on/off)
    - Incubate lysate for 30 min on a turning wheel at 4°C
    - Centrifuge for 10 min at 13,200 rpm and 4°C
    - Transfer supernatant to a fresh tube
    - Adjust lysate to 200 mM NaCl
    - Measure protein concentration (Bradford assay), do not use more then 2-3 mg of protein/experiment. If the protein is overexpressed 1 mg of lysate is enough
- *Capture of proteins by bead-bound RNA*
    - Add equal amounts of lysate to each aliquot of beads
    - Incubate on a turning wheel at 4 °C for 3-4 h
    - Wash beads 3x with nuclei lysis buffer
    - After the final wash add 20 µl of 1x Laemmli buffer and heat samples for 10 min at to 75 °C.
    - Load samples onto a SDS PAA gel and detect proteins by Western blotting