miR-CATCHv2.0 step-by-step experimental protocol

Buffers

- **Lysis buffer**
  - 50mM Tris-HCl pH 7.0
  - 5mM EDTA
  - 1% SDS
  - Right before use, add 1mM PMSF, 1X protease inhibitor cocktail and 80U/ml RNasin

- **Hybridisation buffer**
  - 750mM NaCl
  - 1% SDS
  - 50mM Tris-HCl pH 7.0
  - 1mM EDTA
  - 15% formamide (store in the dark at 4 °C)
  - Right before use, add 1mM PMSF, 1X protease inhibitor cocktail and 80U/ml RNasin

- **Wash buffer**
  - 2X SSC buffer
  - 0.5% SDS
  - Add 1mM PMSF right before use

- **Proteinase K buffer**
  - 100mM NaCl
  - 10mM Tris-HCl pH 7.0
  - 1mM EDTA
  - 0.5% SDS

Probe preparation

Pool the ODD probes (probe #1, 3, 5, 7, 9, 11) and the EVEN probes (probe #2, 4, 6, 8, 10, 12) at 100µM final concentration.

Cross-linking protocol

1. Harvest 10^7 cells into a 50ml tube and wash once with PBS.
2. Prepare 10ml of 1% formaldehyde in PBS from paraformaldehyde stock.
3. With a pipette, resuspend the pellet in 1ml fixing reagent and then add the remaining 9ml.
4. Incubate on a shaker for 10min at room temperature.
5. Quench the reaction with 1ml of 1.25M glycine for 5min at room temperature.
6. Centrifuge at 2000g for 5min at 4°C.
7. Discard supernatant and resuspend the pellet in 50ml of ice cold PBS. Centrifuge at 2000g for 5min at 4°C.
8. Repeat step 7 once.
9. Resuspend the pellet in 0.6ml of ice cold PBS and transfer into a 1.5ml screw-cap microtube.
10. Resuspend any remaining cells with additional 0.6ml of ice cold PBS and transfer it to the same 1.5ml screw-cap microtube.
11. Centrifuge at 2000g for 5min at 4°C and discard the supernatant. With a 20µl pipette, remove any remaining drop of supernatant.
12. Flash-freeze the cell pellet in liquid nitrogen.

Stopping point: flash-frozen cell pellet can be stored at -80°C until use.

Lysis and sonication protocol

1. Thaw the cell pellet very briefly at room temperature and flick to dislodge.
2. Spin down the pellet at 2000g for 3min at 4°C and remove any supernatant.
3. Add 1ml of Lysis buffer per pellet and resuspend.
4. Move the lysate into a Vbottom 15ml tube and place the tube in an ice water bath with constant convection.
5. Place the sonication probe in the tube and sonicate for 12 rounds at 70% amplitude for 30 second pulses with 45 second cool down pauses in between (n° of rounds of sonication depend on the cell type).
6. Flash freeze the samples in liquid nitrogen and store at -80°C.
   Stopping point: sonicated and flash-frozen lysate can be stored at -80°C until use.

Affinity purification protocol

1. Thaw the tubes of lysate at room temperature.
2. Pool sonicated lysates to have a minimum of 1ml for each pool of probes (ODD/EVEN).
3. Wash 2x 30µl of MyOne Streptavidin C1 Dynabeads three times with 1ml unsupplemented Lysis Buffer and, at the end of the washes, resuspend them in 2x 30µl of complete Lysis Buffer.
4. Add the beads to 1ml of lysate in a 1.5ml tube and rotate in a 37°C hybridisation oven for 30min.
5. To remove aspecifically-bound RNA species, clear the lysate from beads twice using the magnetic stand and transfer it to a 5ml round-bottom tube.
6. Mix the lysate with 2ml of supplemented Hybridisation Buffer.
7. Add a total amount of 100pmol probes (ODD/EVEN capture probes or scrambled control probes, 1µl from a 100µM stock solution).
8. Rotate in the 37°C hybridisation oven for 4h.
9. Wash 2x 100µl of beads three times with 1ml of unsupplemented Lysis Buffer and, at the end of the washes, resuspend them in 2x 100µl of supplemented Lysis buffer.
10. Add 100µl of beads to the sample and rotate in the hybridisation oven for 30min at 37°C.
11. Pellet the beads with the the EasySep magnet and resuspend in 1ml of Wash Buffer pre-warmed to 37°C. Once separated with the magnet, resuspend and collect the beads in 1ml of Wash buffer, then wash again the tube with additional 300ul of Wash buffer and add to the sample.
12. Transfer beads/buffer to a 1.5ml tube and rotate in a 37°C hybridisation oven for 5min.
13. Wash 3 more times in a similar manner with 1ml of pre-warmed Wash Buffer for a total of 5 washes.
14. Resuspend beads with 1ml pre-warmed Wash Buffer.
15. Spin down beads after last wash and remove all remaining Wash Buffer.

RNA elution protocol

1. Resuspend the beads in 185µl of proteinase K buffer and add 15µl of 20mg/ml proteinase K. Treat also the Lysate with proteinase K, as input control.
2. Incubate the samples at 45°C for 1h under constant and vigorous agitation.
3. Incubate the samples at 95°C for 10min.
4. Add 1ml Trizol directly to the beds, vortex and incubate for 10min at room temperature.
5. Proceed with RNA isolation or store at -80°C.