

## **Capture and Identification of miRNA Targets by Biotin Pulldown**

### **Reagents:**

- 100 mm dishes
- 3' biotinylated miRNA mimics:
  - o miR-6789-5p
  - o scrambled control (cel-miR-67)
- Transfection Reagent = Lipofectamine 3000 or DharmaFECT 1
- Streptavidin magnetic beads – Preferably the Dynabeads M-280
- Magnetic rack: DynaMag – 2 Magnet
- Wash buffer:
  - o 5 mM Tris-HCl (pH 7)
  - o 500 µM EDTA
  - o 1 M NaCl
- Solution A:
  - o 50 mM NaCl
  - o 100 mM NaOH
- Solution B:
  - o 100 nM NaCl
- TrypLE
- Lysis Buffer:
  - o 20 mM Tris-HCl (pH 7)
  - o 100 mM KCl
  - o 5 mM MgCl<sub>2</sub>
  - o 25 mM EDTA
  - o 0.3 % NP-40
  - o 1x Proteinase Inhibitor Cocktail
- Blocking Buffer:
  - o 1 mg/mL Ultrapure BSA
  - o 200 µg/mL Yeast t-RNA
- Lysis Buffer Plus:
  - o 2.5 mg/mL Ficoll PM400
  - o 7.5 mg/mL Ficoll PM70 (GE Healthcare, USA)
  - o 250 µg/mL Dextran Sulfate 670k (Fluka, USA)
  - o 200 U/mL RNaseOUT
  - o 100 U/mL SUPERase.In in Lysis Buffer (Life Technologies, USA)
- RNase Lysis Buffer:
  - o 2.5 mg/mL Ficoll PM400
  - o 7.5 mg/mL Ficoll PM70

- o 250 µg/mL dextran sulfate 670k
- o 25,000 U/mL RNase T1 in lysis buffer (Fermentas, USA)

### **Buffer Protocols**

- **Wash Buffer:**
  - o 75 µL of 1 M Tris-HCl (pH 7)
  - o 15 µL of 0.5 M EDTA
  - o 3 mL of 5 M NaCl
  - o 12 mL of nuclease-free water
- **Solution A:**
  - o 100 µL of 5 M NaCl
  - o 100 µL of 10 N NaOH
  - o 9.8 mL of nuclease-free water.
- **Solution B:**
  - o Add 100 µL of 5 M NaCl into 4.9 mL of nuclease-free water.
  - o Store at room temperature
- **Lysis Buffer:**
  - o 1 mL of 1 M Tris-HCl
  - o 2.5 mL of 2 M KCl
  - o 250 µL of 1 M MgCl<sub>2</sub>
  - o 2.5 mL of 0.5 M EDTA
  - o 150 µL of NP-40
  - o 43.6 mL of nuclease-free water
  - o 500 µL of Halt Protease Inhibitor Cocktail
  - o Mix it all together by inversion.
- **Blocking Buffer:**
  - o 20 µL of 50 mg/mL Ultrapure BSA
  - o 20 µL of 10 mg/mL Yeast t-RNA (Ambion, USA)
  - o 1 mL of lysis buffer
  - o Mix them all together and scale it up as needed.
- **Lysis Buffer Plus:**
  - o Weigh 2.5 mg of dextran sulfate 670K
  - o Weigh 25 mg of Ficoll PM400
  - o 75 mg of Ficoll PM70
  - o Add these three into a 50 mL conical tube
  - o Add 10 mL of lysis buffer and mix gently by inversion until completely dissolved
  - o Add 50 µL of 40 U/µL RNaseOUT
  - o Add 50 µL of 20 U/µL SUPERase.In

- Mix by inversion. Scale up as needed
- **RNase lysis buffer:**
  - Weigh 500 µg of dextran sulfate 670k
  - Weigh 5 mg of Ficoll PM400
  - Weigh 15 mg of Ficoll PM70
  - Put them in a 50 mL tube
  - Add 2 mL of lysis buffer and mix gently by inversion until completely dissolved.
  - Add 50 µL of 1000 U/µL of RNase T1 and mix by inversion. Scale up as needed

### Protocol

#### Day 0

1. Estimate the total number of cells you will need.
  - a. Each dish should hold 500K cells.
  - b. Usually 3 technical replicates per biological replicate.
2. Warm up EGM, trypsin, and PBS
3. Seed 1 million cells into each well
4. Incubate at 37°C at 5% CO<sub>2</sub> for 48 hours until 80% confluency.

#### Day 2 - Transfection

1. Prepare Lipofectamine 3000 protocol siRNA transfection protocol per the manufacturer's instruction:
  - a. Dilute Lipofectamine 3000 Reagent in EBM media
    - i. 500 µL of EBM + 43 µL of Lipofectamine 3000 Reagent
  - b. Mix the appropriate amount of miRNA needed to Lipofectmaine 3000 to make 50 µM of biotinylated miRNA (1:1 ratio).
  - c. Incubate for 10-15 minutes at room temperature
2. Add complex to wells with 10 mL of growth medium.
3. Incubate for 16 hours.

#### Day 3 – Processing

1. Resuspend magnetic beads (50 µL per 1.5 mL Eppendorf tube per experiment or sample) in 1 mL of wash buffer (maximum of 300 µL of beads per 1 mL of buffer)
2. Mix by inversion and place tubes on magnetic rack for 1 min.
3. Remove wash buffer and repeat twice (total of three times)

4. Resuspend beads in solution A.
5. Mix by inversion and place tubes on magnetic rack for 1 min.
6. Remove solution A and repeat once (for a total of two times).
7. Resuspend beads in solution B.
8. Mix by inversion and place tubes on magnetic rack for 1 min. Remove solution B completely.
9. Resuspend beads in blocking buffer (maximum of an equivalent of 150 µL of beads per 1 mL of buffer)
10. Leave tubes on rotator in 4°C for a minimum of 2 hours.
  - a. Before starting the next step, let the tubes be on the rotator for ~ 1.5 hours
11. Wash all plates with 1 mL of **ice-cold PBS** each and remove PBS completely.
12. Scrape cells off with a cell scraper.
13. Spin cells at 4°C at 500x g for 5 minutes. Discard supernatant and keep cell pellet.
14. Wash cells again with ice-cold PBS for a total of three times.
15. Remove PBS completely and resuspend cell pellet in 500 µL of ice-cold lysis buffer plus.
  - a. Resuspend the cells up and down as much as possible to get them into a single-cell state for optimal lysis.
16. Incubate on ice for 20 minutes, inverting occasionally. While waiting:
  - a. Grab the magnetic bead tubes and place them on magnetic rack for 1 min. Then, remove the blocking buffer.
  - b. Resuspend beads in lysis buffer.
  - c. Mix by inversion and place on magnetic rack for 1 minute. Remove lysis buffer and repeat once (for a total of two times).
  - d. After removing lysis buffer, combine all magnetic beads and resuspend in lysis buffer plus (110 µL of lysis buffer plus for each sample)
  - e. For each sample, aliquot 100 µL of the beads/lysis buffer plus mixture into a fresh 1.5 mL Eppendorf tube and keep on ice.
17. After 20 minutes, spin tubes at 4°C at 5000xg for 5 minutes to pellet nuclei.
18. Remove post-nuclear supernatant, taking care not to touch the nuclear pellet.

- a. For each sample, set aside 50  $\mu$ L of supernatant (cytoplasmic lysate) at 4C for analysis of total cellular RNA.
19. Add 400  $\mu$ L of supernatant (cytoplasmic lysate) to 100  $\mu$ L of prepared beads in lysis buffer plus for each tube.
20. Leave on rotator in 4C for 4 hours.
21. Place bead-containing tubes on magnetic rack for 1 minute.
22. Remove cytoplasmic lysate from all tubes and add 1 ml of lysis buffer.
23. Mix by inversion and place on magnetic rack for 1 minute. Remove lysis buffer and repeat wash steps four more times (for a total of five times).
  - a. For the last wash step, mix by inversion and place on magnetic rack for 2 minutes. Remove lysis buffer completely.
24. Resuspend each sample bead in 100  $\mu$ L of lysis buffer and transfer to a fresh 1.5 mL Eppendorf tube.
25. Add 500  $\mu$ L of TRIzol LS to each tube and mix vigorously.
26. Incubate at room temperature for 5 min, mixing occasionally.
27. Add 100  $\mu$ L of chloroform to each tube and mix vigorously.
28. Spin tubes at 4C > 16000xg for 15 min.
29. For each sample, transfer the organic phase (around 250  $\mu$ L) to a fresh 1.5 mL Eppendorf tube.
30. For each tube, add 5  $\mu$ L of GlycoBlue and subsequently 850  $\mu$ L of 100% ethanol.
31. Mix vigorously and precipitate overnight at -20C

#### Day 4 – RNA Cleanup

1. Spin tubes at 4C at > 16,000xg for 30 min. The blue pellet contains the pulldown RNA. Remove ethanol without touching the pellet.
2. For each sample, add 1 mL of 75% ethanol and mix by inversion.
3. Spin tubes at 4C at > 16,000 xg for 15 min. Remove ethanol without touching the pellet and repeat 75% ethanol wash step once (for a total of two times).
4. Remove ethanol and dry blue RNA pellets in an open fume hood, taking care not to over-dry them as this interferes with resuspension.
5. Resuspend RNA pellets in 200  $\mu$ L of nuclease-free water and place on ice.
6. For each tube, add 200  $\mu$ L of phenol:chloroform:isoamyl (25:24:1)

7. Mix vigorously and spin at > 16,000 xg for 5 min at room temperature.
8. Transfer the aqueous phase (around 200  $\mu$ L) to a fresh 1.5 mL Eppendorf tube, taking care not to touch the interphase.
9. For each tube, add 200  $\mu$ L of chloroform:isoamyl(24:1).
10. Mix vigorously and spin at > 16,000 x g for 5 minutes at room temperature.
11. Transfer the aqueous phase (around 200  $\mu$ L) to a fresh 1.5 mL Eppendorf tube, taking care not to touch the interphase.
12. For each tube, add 20  $\mu$ L of 3M sodium acetate pH 5.5, 4  $\mu$ L of GlycoBlue, and subsequently 1 mL of 100% ethanol.
13. Mix vigorously and precipitate overnight at -80C.

#### Day 5 – Wrapping Up

1. Spin tubes at 4C at > 16,000 x g for 30 min. The blue pellet contains RNA. Remove ethanol without touching the pellet.
2. For each sample, add 1 mL of 75% ethanol and mix by inversion.
3. Spin tubes at 4C at > 16,000 xg for 15 min. Remove ethanol without touching the pellet and repeat 75% ethanol wash step once (for a total of two times).
4. Remove ethanol and dry blue RNA pellets in an open fume hood.
5. Resuspend RNA pellets in 50  $\mu$ L of nuclease-free water and place on ice.
6. Measure RNA concentration in NanoSight