

# Polysome profiling (and mRNA isolation) protocol

This protocol is partially based on [Aeschimann \*et al.\*, Methods, 2015.](#)

## Material and reagents

The following solutions should be prepared in advance and be filter-sterilized. They can be stored at 4°C for months. *All solutions should be prepared initially without CHX and DTT, added only fresh before each experiment.*

Lysis buffer:

- 20mM HEPES pH 7.4
- 150mM KCl
- 15mM MgCl<sub>2</sub>
- 100µg/ml CHX
- 1mM DTT
- 2% NP40
- Complete EDTA-free protease inhibitor
- 10U/ml RNA inhibitor

15% Sucrose solution:

- 20mM HEPES pH 7.4
- 150mM KCl
- 15mM MgCl<sub>2</sub>
- 15% (w/v) sucrose
- 100µg/ml CHX
- 1mM DTT

60% Sucrose solution:

- 20mM HEPES pH 7.4
- 150mM KCl
- 15mM MgCl<sub>2</sub>
- 60% (w/v) sucrose
- 100µg/ml CHX
- 1mM DTT

For washing:

- 1xPBS
- 100µg/ml CHX

Generating sucrose gradients:

- Biocomp Gradient Master

- Gradient Forming Accessories (Biocomp; 105–914B): tube holder, tube caps, and marker block to indicate heavy-light sucrose interface on centrifugation tubes
- Polypropylene tubes (14 × 95 mm; Beckman Coulter; 331374)
- Sterile 30ml syringe with metal cannula

#### Ultracentrifugation:

- Optima™L-80 XP Ultracentrifuge (Beckman Coulter)
- SW 40 rotor with metal adaptors for Beckman Coulter 14 × 95 mm polypropylene tubes

#### Fraction collection:

- 70% (w/v) sucrose solution (to push out gradient)
- Tris peristaltic Pump (Teledyn ISCO)
- Gradient Fractionator (Brandel; BR-184-X)
- fraction collector (Gilson; FC-203B)
- Econo UV monitor EM-1 (Biorad) (measures absorbance at 254nm)
- LabJack U6 data acquisition device using DAQFactory-Express software

#### mRNA isolation:

- TRIzol reagent (LifeTechnologies)
- RNeasy Micro Kit with DNase (Qiagen)

## **Procedure**

### **Day 1**

1. Plate 2.5 million HeLa cells in a 15cm dish. The cells should be 80-90% confluent on day 3 in order to prevent excessive contact and translation inhibition.

### **Day 3**

#### **Preparation of sucrose gradients**

2. Add 100µg/ml CHX and 1mM DTT to each of the two sucrose solutions (about 15ml of each required for two gradients).
3. Mark centrifugation tubes at half their height using the lower edge of the marker block and fill them with approximately 6ml of 15% sucrose solution to just slightly above the mark. To avoid air bubbles, pipette the solution to the wall of the tubes.
4. The 60% sucrose solution is layered underneath the 15% solution. Insert the cannula quickly to the bottom of the tube and begin layering approximately 6ml of 60% sucrose solution until the heavy-light interface rises precisely to the mark. This guarantees tubes of identical weight for ultracentrifugation.
5. Slowly add the cap of the tube while making sure all the air and the excess sucrose can escape through the cap's overflow valve. Remove the excess 15% sucrose solution from the central reservoir of the cap.
6. Create gradients at room temperature with the Gradient Master by selecting the appropriate program (Long Sucr 15–60% wv) and indicating the rotor type (here: SW 40). The program will run for ca. 4min.
7. Move the gradient-containing tubes carefully and store at 4°C until cell lysates are added (stable for at least 6 hours).
8. Start pre-cooling the ultracentrifuge.

#### **Cell treatments and lysis**

9. Perform required induction of reporter or desired experiment. For polysome run-off experiments: 60min 100uM Sodium arsenite.
10. Immobilize mRNAs on ribosomes by replacing media with warm DMEM + 100µg/ml CHX (stock is 100mg/ml, 1/1000 dilution).
11. Return cells to incubator for 10min.
12. Wash cells twice with cold PBS+100µg/ml CHX. Aspirate completely.
13. Add 1ml cold PBS+100µg/ml CHX and harvest cells by scraping.
14. Pellet cells by centrifuging at 500g for 5min at 4°C. Aspirate supernatant.
15. Resuspend cells in 600µl cold lysis buffer and pipet up-and-down 5 times.
16. Incubate cells on ice for 5min.
17. Centrifuge at 13,000rpm for 10min at 4°C.
18. Transfer supernatant to a new tube.

19. Carefully remove caps from previously prepared gradient-containing ultracentrifuge tubes.
20. Carefully layer 500uL of the cell lysate (by pipetting against the tube wall) onto the sucrose gradient.

### **Ultracentrifugation**

21. Carefully hang gradient-containing ultracentrifuge tubes into the SW40 rotor.
22. Ultracentrifuge at 39k rpm for 3h at 4°C.

### **Fractionation of centrifuged sucrose gradients**

23. Set the baseline of the UV monitor by running water through the system. Remove the water again from all tubings by pushing air through the system. Pump the 70% sucrose solution through the tubing until the first drops come out of the needle (preventing air bubbles from disturbing the gradient).
24. Attach an ultracentrifuged sucrose gradient tube to the gradient fractionator system, fixing it in an upright position (grease the centrifugation tube with vaseline to prevent leaking).
25. With the needle, pierce the tube from the bottom and raise the needle until its opening is entirely within the tube.
26. Start the data acquisition and unload the gradient by pumping 70% sucrose underneath the gradient, pushing it towards the fraction collector. Start the fraction collector as soon as the first drop reaches it.
27. Store the collected fractions on ice. If desired, the fractions can also be snap-frozen in liquid nitrogen and stored at -80 °C overnight. Fraction collection requires ca. 15–20min per gradient.

## **Day 4**

### **mRNA isolation**

28. Analyze the acquired data. Small subunit, large subunit, monosome and polysome peaks should be visible.
29. As a backup, split each fraction into two tubes (0.5 ml each) and add 1 ml of Trizol Reagent to each tube. Flash-freeze the samples in liquid nitrogen and store them at -80°C or proceed with the RNA isolation.
30. Use the TRIzol reagent according to the manufacturer's protocol.

## **Day 5**

31. Due to the high sucrose concentrations and a resulting potential phenol contamination after the TRIzol extraction all samples should be purified with an RNeasy Micro Kit

according to the manufacturer's protocol. DNase treatment for mRNA seq can be performed as well.