

Polysome Profiling Analysis

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[Abstract] Polysome profiling is a method that allows monitoring of translation activity of mRNAs in cells and tissues. Once each polysome fractions are collected, the translation activity of each mRNA is analyzed using various molecular biology techniques such as Northern blotting, RT-PCR, microarray or deep-sequencing.

Materials and Reagents

1. Sucrose
2. 1 M HEPES-KOH (pH 7.6)
3. 2 M KCl
4. 1 M MgCl₂
5. PBS
6. 1 M Tris-HCl (pH 7.5)
7. 10 mg/ml cycloheximide (Sigma-Aldrich, catalog number: C7698) in MilliQ water
Note: Cycloheximide inhibits protein synthesis by blocking translation elongation. This molecule interferes with the translocation of tRNAs with the mRNA and the ribosome, resulting in fixed ribosomes on mRNAs.
8. Protease inhibitor cocktail (EDTA-free) (F. Hoffmann-La Roche, catalog number: 04 693 159 001)
9. RNase inhibitor (RNasin 40 units/μl) (Promega Corporation, catalog number: N2511)
10. 10% Triton X-100
11. 10% Sodium deoxycholate (Sigma-Aldrich, catalog number: D6750)
12. RNaseZap (Life Technologies, Ambion[®], catalog number: AM9780)
13. Trizol (Life Technologies, Invitrogen[™])
14. Oligo dT primer and Super Script III (Life Technologies, Invitrogen[™], catalog number: 18418012 and 18080093)
15. 10x sucrose gradient buffer (see Recipes)
16. 10-50% sucrose solutions (see Recipes)

 17. Hypotonic buffer (see Recipes)

Equipment

1. Ultra centrifuge and rotor (Beckman, model: Optima L-80; SW40Ti rotor)
2. UV detector and fraction collector (Teledyne ISCO)
3. NanoDrop (Thermo Fisher Scientific)
4. 0.22 μm filter

Software

1. TracerDAQ software (MicroDAQ)

Procedure

A. Preparation of sucrose gradients

1. Prepare 50 ml of 60% (w/v) sucrose solution in MilliQ water and 10 ml of 10x sucrose gradient buffer. Filter solutions with 0.22 μm filter.
2. Prepare 10-50% sucrose solutions using 60% sucrose. Prepare gradients at least one day before cell lysis to allow gradient to diffuse overnight at 4 °C.
3. Carefully add 1.2 ml of each sucrose solution (starting with the 50% sucrose solution) to Beckman tubes (12 ml total volume). To improve efficacy at this step, the gradients can be flash frozen in liquid nitrogen following each addition of sucrose solution. This helps in generating equally poured gradients and prevents too much diffusion.
4. Cover tube with parafilm and incubate at 4 °C overnight. Alternatively, sucrose gradients can be stored at -80 °C indefinitely.

B. Isolation of polysomes

1. Prepare cells at least one day before lysing. Confluency is very important. About 80-90% confluency gets the maximum polysomes. 1-5 15-cm dishes are needed to see polysome profile.
2. Prior to lysing the cells, treat cells with 100 $\mu\text{g/ml}$ cycloheximide at a final concentration for 5 min.
3. Wash cells twice with 10 ml of ice-cold 1x PBS containing 100 $\mu\text{g/ml}$ cycloheximide.
4. Scrape cells with 5 ml of ice-cold 1x PBS containing 100 $\mu\text{g/ml}$ cycloheximide, collect them in 15 ml tube and rinse with 5 ml of ice-cold 1x PBS containing 100 $\mu\text{g/ml}$ cycloheximide.

5. Centrifuge cells at 1,200 rpm (300 x g) for 5 min at 4 °C.
6. Aspirate supernatant, resuspend cells in 425 µl of hypotonic buffer and transfer all to a new pre-chilled 1.5 ml tube.
7. Then add:
 - 5 µl of 10 mg/ml cycloheximide
 - 1 µl of 1 M DTT
 - 100 units of RNasin
8. Vortex for 5 sec.
9. Then add:
 - 25 µl of 10% Triton X-100
 - 25 µl of 10% Sodium Deoxycholate
10. Vortex for 5 sec.
11. Centrifuge at 15,000 rpm (21,000 x g, a maximal speed on a bench top centrifuge in 1.5 ml eppendorf tube) for 5 min at 4 °C.
12. Transfer supernatant (about 500 µl) to a new pre-chilled 1.5 ml tube. Measure OD_{260nm} for each sample using NanoDrop.

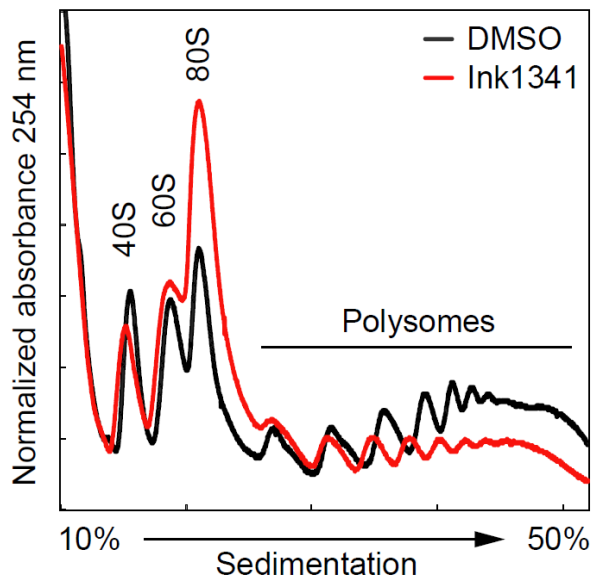


Figure 1. Polysome profiling from MEFs treated with DMSO or Ink1341 (250 nM) for 8 h from Dowling *et al.* (2010). 40 S, 60 S and 80 S denote the corresponding ribosomal subunits and monosome, respectively.

13. Load the same OD amount of lysate onto each gradient (10-30 OD_{260nm} is loaded). Keep 10% of lysates as an input.
14. Weight and balance each gradient.

15. Centrifuge at 35,000 rpm for 2 h at 4 °C using SW40Ti rotor in a Beckman Coulter (Optima L-80 ultra centrifuge). No brake of brake at 1 (maximum) for deceleration. Acceleration does not matter much.
16. While the samples are centrifuging, clean fraction collector with warm MilliQ water containing a bit of RNase ZAP (a few sprays).
17. Carefully remove tubes from the rotor and place them at 4 °C until they are ready for running.
18. Switch on computer, pump, UV detector and fraction collector. Set pump at 1.5 ml/min and the fraction collector by time. Place 2 ml tubes on fraction collector.
19. Open TracerDAQ program.
20. Run chasing solution (60% (w/v) sucrose with bromophenol blue) through the system until it reaches the needle. Make sure to see at least one drop coming out of the needle such that no bubbles are introduced into the gradient.
21. Screw each tube onto the ISCO UV detector and then pierce the tube with the needle.
22. Begin running the chasing solution through the gradient. Run solution with the pump at 1.5 ml/min. Click on acquire data button and press run on the fraction collector.
23. Place fractions on dry ice or liquid nitrogen.
24. Add 750 µl of Trizol for RNA isolation.

C. RNA isolation and RT-qPCR

1. Add 150 µl of chloroform to each fraction.
2. Isolate RNA according to manufacturer's Trizol protocol.
3. Measure the RNA concentration of each fraction or input.
4. Prepare the following solutions in 500 µl tubes or PCR tubes.

0.5 µg of RNA	x µl
Oligo (dT) primer	1 µl
dNTP (10 mM each)	1 µl
Water	x µl/total 13 µl
5. 65 °C for 5 min.
6. On ice for at least 1 min.
7. Add the following solutions (7 µl premix) on ice. Make premix before adding.

5x buffer	4 µl
1 M DTT	1 µl
RNasin 40 units/µl	1 µl
RTase	1 µl
8. Mix gently and spindown on ice.
9. 50 °C for 60 min.

10. 70 °C for 15 min.
11. Keep at 4 °C until next step.
12. Add 180 µl of MQ.
13. qPCR according to manufacturer's protocol.

2x SYBR	10 µl
5' Primer (10 µM)	0.4 µl
3' Primer (10 µM)	0.4 µl
RT sample	5 µl
MQ	4.2 µl/Total 20 µl

Recipes

1. 10x sucrose gradient buffer (50 ml)
 - 200 mM HEPES (pH 7.6)
 - 1 M KCl
 - 50 mM MgCl₂
 - 100 µg/ml cycloheximide
 - 1x protease inhibitor cocktail (EDTA-free)
 - 100 units/ml RNase inhibitor
2. 10-50% sucrose solutions

Final (%)	60% stock (ml)	water (ml)	10x sucrose gradient buffer (ml)
50	8.3	0.7	1
45	7.5	1.5	1
40	6.7	2.3	1
35	5.8	3.2	1
30	5.0	4.0	1
25	4.2	4.8	1
20	3.3	5.7	1
15	2.5	6.5	1
10	1.7	7.3	1
3. Hypotonic buffer
 - 5 mM Tris-HCl (pH 7.5)
 - 2.5 mM MgCl₂
 - 1.5 mM KCl
 - 1x protease inhibitor cocktail (EDTA-free)

References

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