

Polysome Analysis

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[Abstract] Polysome analysis is a method to separate mRNAs from a cell into actively translating and non-translating fractions depending on their association with polysomes. By this protocol, cell lysates are fractionated by sucrose density gradient ultracentrifugation. Free mRNA fraction and various ribosomal fractions, such as 40S, 60S, monosomes and polysomes are collected by fractionation. Association of particular mRNAs with these fractions is detected by reverse transcription – PCR to investigate the translational state of the mRNA.

Keywords: Translation, Ribosome fractionation, Polysome, mRNA, Sucrose density gradient, Ultracentrifugation

[Background] The cellular mRNAs are distributed into an actively translating and a non-translating pool at any point of time and can dynamically redistribute between these pools in response to various stimuli. The actively translating mRNAs have a higher number of ribosomes associated with them and the number of ribosomes associated with an mRNA is a measure of the translation state of the mRNA. Therefore on fractionating the ribosomes from a cell, actively translating mRNAs will be found in the polysomal fraction whereas non-translating/poorly-translating mRNAs will be either found in the free mRNA fraction or associated with 40S ribosomal subunits. Polysome analysis is therefore a method to separate mRNAs from a cell into actively translating and non-translating fractions depending on their association with polysomes (Ray *et al.*, 2009; Poria *et al.*, 2016). Association of individual mRNAs with translating/non-translating fractions can be detected by RT-PCR, whereas the entire translation or non-translating pool of mRNAs can be identified by RNA sequencing or microarray analysis.

Materials and Reagents

1. Ultracentrifuge tube for SW41Ti (Seton Scientific, catalog number: 7030)
2. Permanent ink marker
3. 10 ml syringe with wide gauge luer lock cannula (Vita Needles, gauge 14)
4. 10 cm dish (Eppendorf, catalog number: 0030702115)
5. 1.5 ml tube (RNase and DNase free) (Corning, Axygen®, catalog number: MCT-150-C or equivalent)
6. MCF7 human breast carcinoma cell line (ATCC, catalog number: HTB22)
7. Cycloheximide (CHX) (AMRESCO, catalog number: 94271)
8. Phosphate buffered saline (PBS) (Thermo Fisher Scientific, Gibco™, catalog number:

- 10010023)
9. DEPC treated water (AMRESCO, catalog number: E174)
 10. Citrate saturated phenol (pH 4.5) (Sigma-Aldrich, catalog number: P4682)
 11. Chloroform (Sigma-Aldrich, catalog number: C2432)
 12. 75% ethanol
 13. Nuclease free water
 14. Oligo dT primer
 15. dNTP mix
 16. 5x RT buffer
 17. Dithiothreitol (DTT) (Thermo Fisher Scientific, Thermo Scientific™, catalog number: R0861)
 18. RNase inhibitor (Thermo Fisher Scientific, catalog number: EO0381)
 19. cDNA synthesis reagents (Thermo Fisher Scientific, Invitrogen™, catalog number: 28025013)
 20. 10x PCR buffer (with 15 mM MgCl₂)
 21. 10 μM forward primer for gene of interest/control gene
 22. 10 μM reverse primer for gene of interest/control gene
 23. PCR reagents (New England Biolabs, catalog number: M0273L)
 24. Sucrose (RNase and DNase free) (AMRESCO, catalog number: 0335)
 25. Potassium chloride (KCl) (AMRESCO, catalog number: 0395)
 26. Magnesium chloride (MgCl₂) (AMRESCO, catalog number: 0288)
 27. HEPES (pH 7.4) (Thermo Fisher Scientific, Affymetrix, catalog number: 16926)
 28. IGEPAL CA-360 detergent (Sigma-Aldrich, catalog number: I3021)
 29. Protease inhibitor cocktail (AMRESCO, catalog number: M250)
 30. 10% sucrose solution (for 8 ml) (see Recipes)
 31. 50% sucrose solution (for 8 ml) (see Recipes)
 32. Polysome lysis buffer (see Recipes)

Equipment

1. Gradient Station or any equivalent gradient fractionators (Biocomp, catalog number: 153-002)
2. Tube holder
3. Ultra-centrifuge (Beckman Coulter, model: Optima L-70 or equivalent)
4. Rotor SW41Ti (Beckman Coulter, catalog number: 331362)
5. 200 μl pipette
6. Spectrophotometer (Thermo Fisher Scientific, Thermo Scientific™, model: NanoDrop™ 2000)
7. Fraction collector (Gilson, model: FC203B)
8. UV monitor (Bio-Rad Laboratories, model: EM1-Econo™)
9. Balance
10. Vortex

Software

1. Gradient Master software (included with the Gradient Station)

Procedure

A. Gradient preparation

1. Prepare 8 ml of 10% and 50% sucrose solution each in 1x sucrose gradient buffer for a single gradient (see Recipes, Table 1).
2. Degas the solution under vacuum for 5 min.
3. Mark ultracentrifuge tubes at 50% of tube height with permanent ink marker using the marker block provided with the Gradient Station.
4. Carefully add 10% sucrose solution upto the 50% mark of the tube with a wide gauge cannula.
5. Aspirate the 50% sucrose solution into a syringe and insert the cannula directly to the bottom of the tube through the 10% sucrose solution then slowly layer the heavier solution to the bottom of the tube without disturbing the 10% sucrose solution. The heavier sucrose solution will slowly push the 10% solution up, and the 50% sucrose solution should reach up to the 50% marking of the ultracentrifuge tube.
6. Cover the tube with rubber cap and place the tube in the tube holder on the rotor of the gradient station.
7. Run the following pre-set program in the Gradient Master software
Program name: Short Sucrose 10-50% (w/v) Fast
Program specification: 10-50%; Step: S1/1; Time: 1 min 45 sec; Angle: 81.5; rpm: 25 (Video 1).
8. Carefully take out the tubes and store vertically at 4 °C for 2 h without disturbance.

Video 1. Gradient preparation



B. Cell lysate preparation for polysome profiling

1. Treat 70-80% confluent cells in a 10 cm dish with 100 µg/ml cycloheximide (CHX) by directly

- adding it to the culture media at 15 to 30 min prior to harvesting.
2. Wash the cells twice with PBS containing 100 µg/ml CHX, and harvest them by scraping into 10 ml ice cold PBS containing 100 µg/ml CHX. Centrifuge at 600 x g for 5 min at 4 °C and discard the supernatant carefully.
 3. Lyse the cell pellet by adding 200 µl of polysome lysis buffer (see Recipes). Mix gently with 200 µl pipette for a number of times and incubate on ice for 15 min.
 4. Centrifuge at 10,000 x g for 20 min at 4 °C.
 5. Transfer the supernatant to a pre-chilled 1.5 ml tube.
 6. Measure OD at 254 nm using a spectrophotometer.
 7. Overlay 25-50 OD equivalent of lysate gently on top of the gradient in the ultracentrifuge tube without disturbing the gradient. Leave around 2 mm of space in the gradient tube on top of the lysate layer (Figure 1).
 8. Weigh each gradient tube in a balance and adjust the weight of each tube containing the gradient to be exactly equal by adding polysome lysis buffer to the overlaid layer without disturbing the gradient.

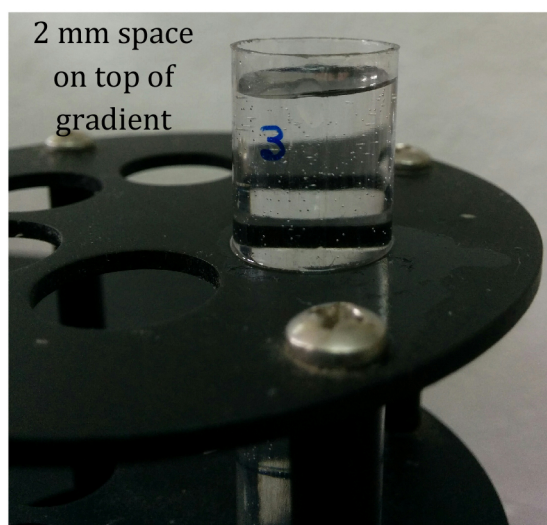


Figure 1. 10%-50% sucrose gradient showing 2 mm space on top of gradient

C. Ultracentrifugation and fraction collection

1. Pre-cool the rotor assembly and the tube buckets at 4 °C.
2. Load the tubes carefully into the buckets without disturbing the gradients. Load the buckets on the rotor assembly ensuring equal weights in opposing positions. Perform the ultracentrifugation at 111,000 x g (Avg RCF) for 4 h at 4 °C at maximum vacuum.
3. During the last hour of the ultracentrifugation switch on the gradient profiler, UV detector, gradient profiling software and wash the fractionator tubing with running DEPC treated water for several times and dry by running air.
4. Carefully take out the tubes, one at a time, from the buckets and load into the tube holder of the

- gradient fractionator, and place at the proper position under the collector piston.
5. Run water through the tubing and press auto zero on the UV monitor.
6. Set up the tube length, gradient length (75 cm for SETON open top polyclear tube), fraction number (12 to 16) in the set up window.
7. Run the fractionation and collect fractions (Figure 2) in 1.5 ml tubes and transfer to ice immediately.

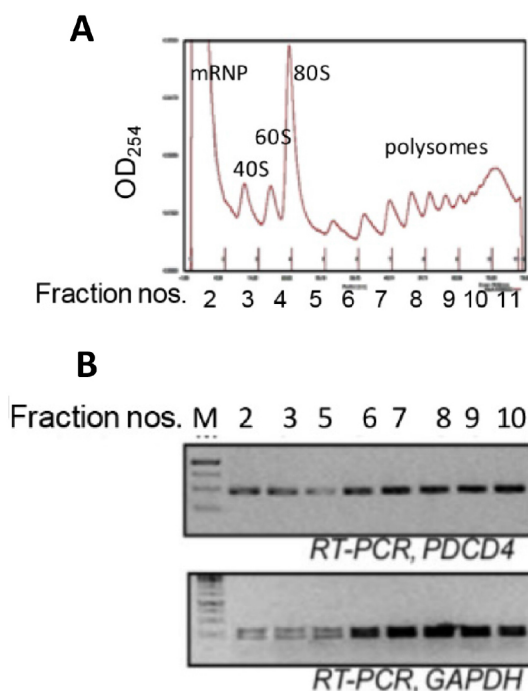


Figure 2. Ribosomal fractions from MCF7 cells were analysed by 10-50% sucrose density gradient fractionation. A. Ribosomal RNA content, measured at 254 nm, is plotted against fraction numbers (top panel). B. RNA isolated from selected fractions was analyzed by semiquantitative RT-PCR using *PDCD4* and *GAPDH* primers (bottom panel).

D. RNA isolation and RT-PCR

1. Add 300 μ l phenol (citrate saturated, pH 4.5) and 300 μ l chloroform to the tubes containing the gradient fractions.
2. Vortex for 30-60 sec.
3. Centrifuge at 16,000 $\times g$ for 15 min at 4 $^{\circ}$ C.
4. Carefully transfer the supernatants to new tubes.
5. Add 10 μ g glycogen (RNase free) and equal volume of isopropanol and mix by inverting the tubes several times. Incubate at room temperature for 30 min.
6. Centrifuge at 16,000 $\times g$ for 30 min at 4 $^{\circ}$ C.
7. Wash the RNA pellet with 1 ml ice cold 75% ethanol.
8. Air dry the RNA pellet and dissolve into 10-20 μ l of nuclease free water.

9. Measure the absorbance at 260 nm in a spectrophotometer to determine RNA concentration.
10. cDNA synthesis: Setup the following reaction for cDNA synthesis for each RNA.

RNA	8 µl
Oligo dT primer	1 µl
10 mM dNTP mix	1 µl

65 °C for 5 min. Keep on ice for 1 min.

Add,

5x RT buffer	4 µl
100 mM DTT	2 µl
RNase inhibitor	0.5 µl
M-MLV RT (200 U/µl)	1 µl
Nuclease free water	2.5 µl

Incubate at 37 °C for 1 h. Then at 70 °C for 15 min.

Store at 4 °C.

11. PCR

10x PCR buffer (with 15 mM MgCl ₂)	2 µl
10 µM forward primer for gene of interest/control gene	1 µl
10 µM reverse primer for gene of interest/control gene	1 µl
<i>Taq</i> DNA polymerase (1 U/µl)	1 µl
cDNA	2 µl
Nuclease free water	13 µl

Run thermal cycling for 30-35 cycles (Denaturation at 95 °C for 30 sec, annealing at 55 °C for 30 sec, elongation at 72 °C for 30 sec) and run the products on agarose gel (Figure 2).

Recipes

1. 10% sucrose and 50% sucrose solution (for 8 ml) (Table 1)

Table 1. Recipe for sucrose density gradient preparation

	10% sucrose solution (for 8 ml)	50% sucrose solution (for 8 ml)
Sucrose	0.8 g	4.0 g
2 M KCl	400 µl	400 µl
1 M MgCl ₂	40 µl	40 µl
100 mM DTT	160 µl	160 µl
1 M HEPES pH 7.4	160 µl	160 µl
100 mg/ml Cycloheximide	8 µl	8 µl
DEPC water	Upto 8 ml	Upto 8 ml

2. Polysome lysis buffer
100 mM KCl

5 mM MgCl₂
20 mM HEPES (pH 7.4)
0.5% NP-40
100 µg/ml CHX
2 mM DTT
40 U/ml RNase inhibitor
1x protease inhibitor cocktail

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