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Analysis of mRNA Translation Rate in Mouse Embryonic Stem Cells

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Abstract

Regulation of gene expression is essential to enable embryonic stem cells (ESCs) to either self-renew or to differentiate. Translational regulation of mRNA plays a major role in regulating gene expression and has been shown to be important for ESC differentiation. Sucrose gradients can be used to separate mRNAs based on the number of associated ribosomes and this can be used as a readout of the rate of translation. Following centrifugation through a sucrose gradient, mRNAs can be recovered, purified, and analyzed by quantitative real-time polymerase chain reaction (qRT-PCR) to determine their ribosomal load in different cell states. Here, we describe how to differentiate mouse ESCs to Neural Precursor Cells (NPCs) and analyze the rate of translation of individual mRNAs by qRT-PCR following polysome fractionation.

Keywords: Stem cells, Translation, Polysome, Differentiation, Quantitative real-time PCR

1 Introduction

Embryonic stem cells (ESCs) have the unique ability to self-renew or to differentiate into multiple different cell lineages. This is driven by changes in gene expression that are coordinated at many levels. Regulation of gene expression is essential for directed differentiation and it includes regulation of transcription, RNA stability, translation, and protein stability. In ESCs, global profiling of the translation state of mRNAs has demonstrated widespread regulation at the level of translation highlighting the limitation of analyzing mRNA levels as a readout of gene expression (1–3). Translational control of an mRNA is regulated largely by the binding of RNA binding proteins or miRNAs (4–6). These trans-acting factors generally target translation initiation and thus decrease the ribosomal load on an mRNA. The number of ribosomes associated with an mRNA can be used as a readout of its rate of translation. mRNAs heavily loaded with ribosomes are considered to be highly translated while those with fewer ribosomes are considered to be nontranslated or inefficiently translated. While current advances in sequencing technologies allow the analysis of ribosome footprints on a genome wide level (7), analysis of the translation rate of

individual mRNAs can be done using sucrose gradients to separate the mRNAs based on size. When cytoplasmic mRNAs are spun through a linear sucrose gradient, mRNAs heavily loaded with ribosomes sediment into the heavy polysome fractions while the mRNAs with fewer ribosomes fractionate with the lighter polysome fractions. The mRNAs can then be isolated and quantitative real-time PCR (qRT-PCR) can be used to determine the relative ribosomal load of individual mRNA species. The use of splice variant specific primers enables the analysis of differences in translation rate between different splice variants within the same cell or between different cell states.

Mouse ESCs can be efficiently differentiated to Neural Precursor Cells (NPCs) using established protocols (8, 9). These NPCs can then be further differentiated into the three neural sub-types neurons, astrocytes and oligodendrocytes. The protocol detailed here describes how to differentiate mouse ESCs into NPCs using published protocols (8, 9) and then to analyze the ribosomal load of individual mRNA species as a readout of translation rate in the two cell types. This method can be used on a wide range of cell types including both adherent and suspension cell cultures.

2 Materials

All glassware and plasticware should be RNAse free. Autoclave glassware at 120 °C for 30 min prior to use. Prepare all solutions using nuclease, protease, and pyrogen free biotechnology grade water and analytical grade reagents. To protect against RNA degradation, decontaminate workbench with RNase Away followed by DEPC water before the start of experiment. Wear gloves and use barrier tips for all steps of this protocol. Ensure proper waste disposal in accordance with waste disposal regulations.

2.1 Cell Culture Reagents

1. DMEM (Gibco® BRL, Bethesda, MD, USA).
2. ES Fetal Bovine Serum (ES FBS) (Gibco® BRL, Bethesda, MD, USA).
3. β-mercaptoethanol (Gibco® BRL, Bethesda, MD, USA).
4. L-glutamine (100×) (Gibco® BRL, Bethesda, MD, USA).
5. MEM nonessential amino acids (100×) (Gibco® BRL, Bethesda, MD, USA).
6. ESGRO® Leukemia inhibitory factor (LIF), 10^7 U/mL (Merck Millipore ESG1107).
7. Neurobasal® Medium (Gibco® BRL, Bethesda, MD, USA).
8. DMEM/F-12 Medium (Gibco® BRL, Bethesda, MD, USA).
9. N-2 Supplement (100×) (Gibco® BRL, Bethesda, MD, USA).

10. B-27® Supplement (50×), serum free (Gibco® BRL, Bethesda, MD, USA).
11. 0.1 % Gelatin in water (STEMCELL Technologies Inc., Vancouver, British Columbia, Canada).
12. 0.25 % Trypsin–EDTA (Gibco® BRL, Bethesda, MD, USA).
13. Corning® 100 × 10 mm petri dish with cover.

2.2 Preparation of Cell Culture Medium

1. DMEM for ESCs: Prepare 500 mL of ESC culture medium by supplementing ES DMEM with 15 % FBS, 0.2 mM β-mercaptoethanol, 2 mM L-glutamine, 1× MEM nonessential amino acids, and 1,000 U/mL of LIF.
2. N2B27 medium for Neural Precursor (NP) cells: Prepare 400 mL of NP cell culture medium by supplementing with 730 μL of β-mercaptoethanol, 2 mL of N-2 supplement, 1 mL of L-glutamine, 4 mL of B-27 supplement in a (1:1) mix of Neurobasal®–DMEM/F-12 medium.

2.3 Polysome Extraction Components

1. Diethylpyrocarbonate (DEPC) (Sigma Chemical Company, St. Louis, MO, USA).
2. DEPC (0.1 %) treated deionized water.
3. Dulbecco's Phosphate-buffered saline (DPBS), without magnesium and calcium (Gibco® BRL, Bethesda, MD, USA).
4. 10 % Triton X-100: 1 mL Triton X-100 top up to 10 mL with water.
5. 10 % Tween 20: 1 mL Tween 20 top up to 10 mL with water.
6. 10 % sodium deoxycholate stock: Dissolve 10 g of sodium deoxycholate in 100 mL water using a magnetic stirrer (*see* **Note 1**) (Sigma Chemical Company, St. Louis, MO, USA).
7. 1 M Tris–HCl, pH 7.4: Add 2 mL of Tris–HCl (pH 8) (Ambion®, Austin, Texas) to 8 mL of Tris–HCl (pH 7) (Ambion®, Austin, Texas).
8. Sucrose (Sigma Chemical Company, St. Louis, MO, USA).
9. Cycloheximide (100 mg/mL), Ready-made solution (Sigma Chemical Company, St. Louis, MO, USA).
10. 5 M NaCl (Ambion®, Austin, Texas).
11. 1 M $MgCl_2$ (Ambion®, Austin, Texas).
12. 2 M KCl (Ambion®, Austin, Texas).
13. Heparin (10 mg/ml) stock: Add 0.01g of heparin sodium salt to 1mL RNase free water (Sigma Chemical Company, St. Louis, MO, USA).
14. 20U/μL SuperaseIn (Ambion®, Austin, Texas).
15. 10 % SDS solution (Gibco® BRL, Bethesda, MD, USA).

16. RNAse free Proteinase K (20 mg/mL) (Gibco® BRL, Bethesda, MD, USA).
17. TRIzol® Reagent (Ambion®, Austin, Texas).
18. 50 mL syringes.
19. 10 mL syringes.
20. 0.2 μm syringe filters.
21. Long blunt-end needle.
22. RNase Away (Molecular BioProducts Inc., San Diego, CA).

2.4 Preparation of 10 % and 50 % W/V Sucrose

1. 10 % sucrose solution: Weigh 5 g of sucrose in a 50 mL Falcon tube. Add RNase free water to 35 mL. Add 1.875 mL of 2 M KCl, 75 μL of 1 M $MgCl_2$, and 500 μL of 1 M Tris–HCl, pH7.4 to the sucrose solution. Place tubes in boiling distilled water for 15 min, gently agitating the sucrose mix until the sucrose is completely dissolved. Cool on ice. Make volume up to 50 mL with RNase free water and filter through a 0.2 μm syringe filter into a fresh 50 mL Falcon tube.
2. 50 % sucrose solution: Weigh 25 g of sucrose in a 50 mL Falcon tube and prepare a 50 mL sucrose solution as described above. Sucrose solutions can be stored at −20 °C and thawed to room temperature before use.

2.5 Preparation of Buffers and Reagents

1. 2× Resuspension buffer (RSB): Add 200 μL of 1 M Tris–HCl, pH 7.4, 40 μL of 5 M NaCl, 30 μL of 1 M $MgCl_2$ and top up to 10 mL with water. Store at 4°C (*see* **Note 2**).
2. 1× RSB: Add 150 μL of 2× RSB, 16 μL of SuperaseIn (320 U), 0.6 μL of cycloheximide (200 μg/mL), 3 μL of 10 mg/mL heparin and top up to 300 μL with water (*see* **Note 3**).
3. Lysis buffer (LB): Add 500 μL of 2× RSB, 100 μL of 10 % Triton-X, 200 μL of 10 % Tween-20, 100 μL of 10% sodium deoxycholate, 20 μL of heparin (10 mg/mL) and top up to 1 mL with water (*see* **Note 4**).
4. Add 100 μg/mL cycloheximide (1:1,000 dilution) to warm PBS, 0.25 % Trypsin–EDTA, neutralizing medium & ice cold PBS before start of experiment.

2.6 Polysomal RNA Extraction and cDNA Synthesis Components

1. Phenol, pH 6.6–chloroform–isoamyl alcohol (25:24:1) (Ambion®, Austin, Texas).
2. Chloroform–isoamyl alcohol (24:1) (Sigma Chemical Company, St. Louis, MO, USA).
3. 3 M sodium acetate, pH 5.2 (Ambion®, Austin, Texas).
4. Glycogen, 5 mg/mL (Invitrogen®, Carlsbad, CA, USA).
5. 7.5 M Lithium chloride (LiCl) (USB Corporation, Cleveland, OH, USA).
6. Isopropanol (Applichem Biochemica, Germany).

7. 75 % Ethanol in water (Sigma Chemical Company, St. Louis, MO, USA).
8. GeneChip Poly-A RNA Control Kit (Affymetrix, Cleveland, USA).
9. SuperScript® III First Strand Synthesis System for qRT-PCR (Invitrogen®, Carlsbad, CA, USA).

2.7 qRT-PCR Components

1. Power SYBR® Green Master Mix (Applied Biosystems®, Carlsbad, CA, USA).
2. MicroAmp® Optical 384-Well Reaction Plate with Barcode (Applied Biosystems®, Carlsbad, CA, USA).

2.8 Bacterial Spike-in mRNA Primer Sequences

Primers targeting the bacterial RNAs from the polyA RNA control kit.

DAP Forward 5′-CCC ACA GTG ATG ATG TCG AG-3′.

DAP Reverse 5′-GCT GCT TCA GCT GCT TCT TC-3′.

THR Forward 5′-CTC GCT CAA GCT GTC ATG TAC-3′.

THR Reverse 5′-CGG TGA TTT CTC ACA GAT GG-3′.

2.9 Equipment

1. BioComp gradient master model 107ip (BioComp Instruments, Inc. New Brunswick, Canada).
2. Ultracentrifuge model Optima L-Series (Beckman Coulter, Fullerton, CA).
3. SW41Ti rotor and swinging buckets (Beckman Coulter, Fullerton, CA).
4. Seton open-top Polyclear™ Centrifuge Tubes, 14 × 89 mm (BioComp Instruments, Inc. New Brunswick, Canada).
5. Piston gradient fractionator (BioComp Instruments, Inc. New Brunswick, Canada).
6. FC 204 Fraction collector (Gilson, Inc., Germany).
7. Monitor UV-M II (Bio-Rad, Hercules, CA USA).
8. Gradient Profiler Software Version 1.58 (BioComp Instruments, Inc. New Brunswick, Canada).
9. Ultrospec™ 2100 pro UV/Visible Spectrophotometer (GE Healthcare).
10. Thermal cycler (Bio-Rad, Hercules, CA USA).
11. ABI PRISM 7900 Sequence Detection Systems (Applied Biosystems® Carlsbad, CA, USA).
12. Vacuum dryer.
13. Spectrophotometer NanoDrop ND-1000 (Biofrontier Technology).

2.10 Preparation of Centrifuge Buckets and Tubes

1. Chill centrifuge buckets on ice for 30 min before spin. Insert Kimwipes into the bucket to collect condensation. Rinse centrifuge tubes and caps with RNase Away followed by DEPC water and leave it to dry before use. Prechill Eppendorf tubes and Falcon tubes before the start of the experiment (*see* **Note 5**).

3 Methods

All procedures (from Section 3.3) are to be carried out on ice unless otherwise stated. Centrifuges should be prechilled to 4 °C before use.

3.1 Growing ESC and Differentiating Them to NPC

1. Pre-coat 10 cm tissue culture dishes with 0.1 % gelatin 24 h prior to the start of experiment.
2. Seed mouse ESCs at a density of 5×10^6 cells in 2×10 cm dishes in ES DMEM medium 2 days prior to polysome extraction. Incubate cells in a 37 °C, 5 % CO_2 incubator. Change medium 16–20 h before experiment. Harvest cells at no more than 70–80 % confluency (*see* **Note 6**).
3. To differentiate ESCs to NPCs, seed mouse ESCs at a density of 5×10^6 cells in 4×10 cm dishes in N2B27 medium 6 days prior to polysome extraction. Incubate cells in a 37 °C, 5 % CO_2 incubator. Change medium every day.
4. Efficiency of differentiation can be monitored by staining for the neural marker SOX1 in the cells. Efficient differentiations should yield over 80 % SOX1 positive cells.

3.2 Preparation of Sucrose Gradients

Prepare gradients before harvesting cells.

1. Fit 14×89 mm Polyclear Seton tubes into the SW41Ti gradient master tube holder.
2. Add 6 mL of 10 % filtered sucrose to each tube.
3. Use a long blunt needle to gently underlay 5.5 mL of 50 % filtered sucrose at the bottom of the tube being careful to keep the two layers from mixing. Fit in the Biocomp long caps gently to avoid air bubbles and remove any displaced liquid from the cap.
4. Use the Biocomp gradient master as per manufacturer's instructions to make the gradient. Level the machine and choose the program, SW41-List-Long caps-Sucrose-10–50 % w/v.
5. After gradient formation, remove the caps gently (*see* **Note 7**).
6. Weigh the tubes and adjust the weight of the tubes by adding 10 % sucrose to the top of the gradient drop wise.
7. Place the gradients in the chilled centrifuge buckets before use. Gradients must be of equal weight before loading into buckets.

3.3 Polysome Cell Extraction

1. Treat cells with 100 μg/mL cycloheximide for 10 min in 37 °C, 5 % CO_2 incubator (1:1,000 dilution).
2. Wash cells with 5 mL of warm (37 °C) PBS containing 100 μg/mL cycloheximide. Remove PBS and harvest cells with 1 mL of trypsin containing 100 μg/mL cycloheximide. Incubate at 37 °C for 1–2 min. Neutralize trypsin with 4 mL of ice cold medium (with 100 μg/mL cycloheximide). Transfer cells to a prechilled Falcon tube.
3. Spin cells at 380 rcf for 5 min at 4 °C using prechilled centrifuge.
4. Discard supernatant, and resuspend cell pellet in 1 mL of ice-cold PBS (with 100 μg/mL cycloheximide). Transfer cells to a new prechilled 1.5 mL Eppendorf tube.
5. Take out 100 μL of cells and add 1 mL of TRIzol® for total RNA extraction if required.
6. Spin and wash cells twice with 1 mL of ice-cold PBS (with 100 μg/mL cycloheximide) 400 rcf, 4 °C for 2 min.
7. Discard supernatant and resuspend cell pellet in 140 μL of fresh 1× RSB (*see* **Note 8**).
8. Transfer cells (i.e., 160 μL, depends on cell size) to a fresh cold Eppendorf tube. Add an equal volume (i.e., 160 μL) of fresh 1× lysis buffer. Incubate on ice and gently shake the tube every 2 min for 10 min.
9. Spin samples for 3 min at 4 °C, full speed (16,000 rcf) using a microcentrifuge to pellet nuclei (*see* **Note 9**).
10. Transfer supernatant to a new cold tube and spin for 10 min at 4 °C, full speed (16,000 rcf). Transfer supernatant into a fresh tube carefully avoiding the pellet (*see* **Note 10**).
11. Take out 50 μL of extract as unfractionated lysate and add 1 mL of TRIzol® for RNA extraction if required.
12. Take out 10 μL of extract and perform a 1/50 dilution in water to measure the OD units at 254 nm. Prepare 1X RSB at 1/50 dilution in water as blank.
13. Add 400 μL of 10 % sucrose to the top of the gradient gently to avoid mixing of sucrose with the lower layers. Load equal OD units of cell extract onto the sucrose gradients. Top up with 1× RSB if necessary. If sample volume exceeds 250 μL add less sucrose to the top of the gradients.
14. Label the side of each tube with sample name and note which bucket is used.
15. Seal bucket tightly with the lid and spin samples at 8 °C, 36,000 rpm (220,000 rcf) for 1.5 hs in a Beckman ultracentrifuge. Carefully remove the tubes from the rotor and place them in 4 °C fridge.

3.4 Collection of Polysomal Fractions

1. Prepare and label 12 RNase free 1.5 mL Eppendorf tubes per sample on ice. Program the fractionator as follows:

   Speed: 0.3 mm/s, 6.83 mm/fraction, 11 fractions, Total distance: 75.13 mm.
2. Adjust absorbance unit (AU) on software and UV spectrometer monitor according to the sample OD readings (samples with a higher OD reading require a higher absorbance unit) (*see* **Note 11**).
3. Align 1.5 mL Eppendorf tubes on the fraction collector. Collect 11, 1 mL fractions using a gradient piston fractionator simultaneously detecting the absorbance at 254 nm. Manually collect fraction 12 from the bottom of the gradient.
4. Save the UV trace in .csv format (*see* **Note 12**) (Fig. 1).
5. Rinse fractionator between samples using DEPC treated water.
6. After fractionation, add 110 μL of 10 % SDS and 12 μL of 20 mg/mL proteinase K to each fraction.
7. Incubate and shake at 42 °C for 30 min.
8. Samples can be stored at −80 °C or used immediately for RNA extraction.

3.5 RNA Extraction from Polysomal Fractions

1. Bacterial spike-in poly(A) RNAs (containing, DAP and THR mRNAs) are prepared in 1/20 dilution aliquots and stored in −20 °C.
2. 1/20 diluted aliquots are further diluted to 1/100 prior to use. Add 10 μL of diluted spike-in to fresh cold RNase free 2 mL Eppendorf tubes. Transfer 900 μL of polysomal fraction to the tube containing spike-in. Add 1 Volume of 25:24:1 phenol–chloroform–isoamyl alcohol to sample (i.e., 900 μL). Mix well by inverting or vortexing (*see* **Note 13**).
3. Spin at 12,000 rcf at room temperature for 10 min.
4. Transfer 850 μL of aqueous phase into a new RNAse free tube, carefully avoiding the middle layer.
5. Add 1 Volume of 25:24:1 phenol–chloroform–isoamyl alcohol to sample (i.e., 850 μL). Mix well by inverting or vortexing.
6. Spin at 12,000 rcf at room temperature for 10 min.
7. Remove 800 μL of aqueous phase into a new RNAse free tube, carefully avoiding the middle layer.
8. Add 1 volume (800 μL) of chloroform–isoamyl alcohol (24:1). Mix vigorously by vortexing.
9. Spin at 12,000 × *g* for 5 min at room temperature.
10. Remove 700 μL of aqueous phase into a new RNAse free tube on ice.

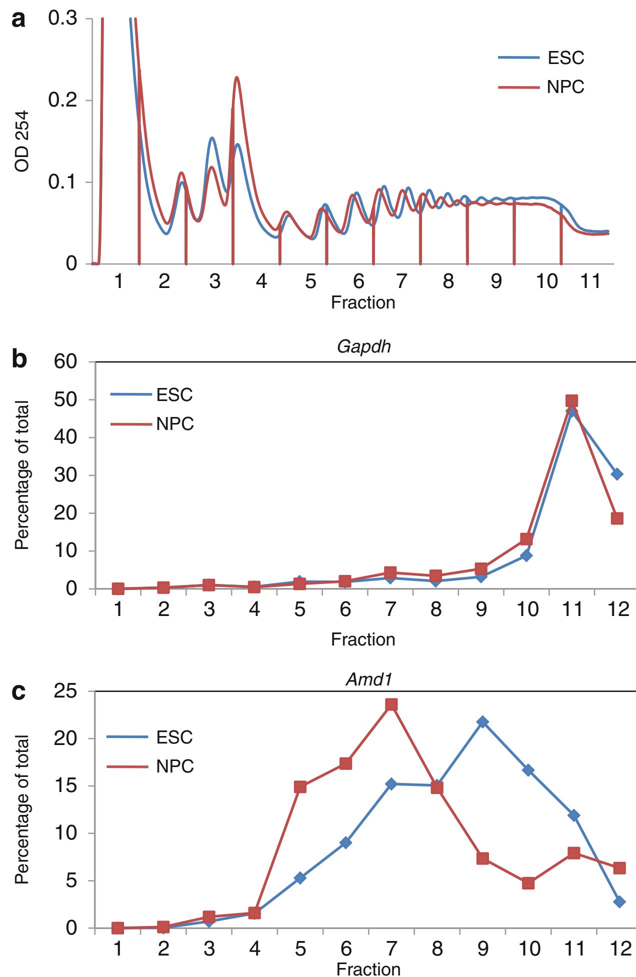

Fig. 1 (**a**) Representative polysome profiles from ESCs and NPCs showing 11 fractions captured on the Biocomp fractionator. Fraction 12 is recovered from the bottom of the gradient. (**b**) qRT-PCR analysis of *Gapdh* and *Amd1* mRNAs in polysomal fractions from ESCs and NPCs. *Gapdh* is similarly loaded with ribosomes in both cell types suggesting an equal rate of translation. *Amd1*, a regulator of the polyamine pathway, is in the heavy fractions (peak in fraction 9) in ESCs and moves to the lighter fractions (peak in fraction 7) on differentiation to NPCs suggesting it is translationally repressed in NPCs (3)

11. Add 1/10th volume of 3 M sodium acetate, pH 5.2 (70 μL) and 3 μL of 2 mg/mL glycogen and mix (*see* **Note 14**).
12. Add 1 volume of cold isopropanol (*see* **Note 15**).
13. Incubate at −80 °C for at least 1 h to overnight (*see* **Note 16**).
14. Spin samples at 4 °C for 30 min at full speed (16,000 rcf) using a microcentrifuge. Look for pellet and carefully discard supernatant.

15. Wash pellet in 700 μL of 75 % ethanol.
16. Spin at 4 °C for 15 min at full speed (16,000 rcf) a microcentrifuge.
17. Resuspend pellet in 50 μL of RNAse free water and add 100 μL of 7.5 M LiCl (*see* **Note 17**). Add 2.5 volumes of 100 % ethanol. Incubate overnight at −20 °C.
18. Spin samples at 4 °C for 30 min at full speed (16,000 rcf) using a microcentrifuge. Carefully discard supernatant. Pellet may not be visible.
19. Wash pellet in 100 μL of 75% ethanol.
20. Spin at 4 °C for 15 min at full speed (16,000 rcf) using a microcentrifuge. Carefully discard supernatant.
21. Resuspend pellet in 100 μL of RNase free water. Add 1/10th volume of 3 M sodium acetate, pH 5.2 (10 μL) and 1 μL of 2 mg/mL glycogen and mix (*see* **Note 18**).
22. Add 2.5 volumes of cold ethanol and mix.
23. Incubate at −20 °C for at least 1 h to overnight.
24. Spin samples at 4 °C for 30 min at full speed (16,000 rcf) using a microcentrifuge. Look for pellet and carefully discard supernatant.
25. Wash pellet in 100 μL of 75% ethanol.
26. Spin at 4 °C for 15 min at full speed (16,000 rcf) using a microcentrifuge. Discard supernatant and dry pellet using a vacuum dryer for 1–5 min (*see* **Note 19**). Resuspend pellet in 30 μL of RNase free water or as desired.
27. Measure RNA concentration and purity using a nanodrop (*see* **Note 20**). RNA can be stored at −80 °C. Avoid repeated freeze thawing of RNA.

3.6 Preparation of cDNA Using Polysomal RNA

1. Calculate the volume for 1 μg of RNA based on the highest concentration among the RNA fractions.
2. Add the same volume of RNA for all the fractions in fresh 0.2 mL PCR tubes. The ng amount will be different for each fraction.
3. Add 1 μL of 1:1 mix of 50 μM oligo$(dT)_{20}$ and 50 ng/μL random hexamers primer and 1 μL of 10 mM dNTP mix, top up to 10 μL with nuclease and RNase free water (*see* **Note 19**).
4. Incubate mixture at 65 °C for 5 min, and cool on ice for at least 1 min.

   Prepare the following cDNA synthesis mix, by adding the reagents in the order as indicated below:

Reagents	1× reaction volume
10× RT buffer	2 μL
25 mM $MgCl_2$	4 μL
0.1 M DTT	2 μL
RNaseOUT™ (40 U/μL)	1 μL
SuperScript® III RT (200 U/μL)	1 μL

5. Add 10 μL of cDNA synthesis mix to the RNA–primer mixture, flick tubes gently and collect by brief centrifugation.
6. Incubate mixture in a thermal cycler as follows:

   25 °C for 10 min, followed by 50 °C for 50 min, and terminate the reaction at 85 °C for 5 min.
7. Store cDNA at −20 °C or use for qRT-PCR immediately.

3.7 qRT-PCR Reaction

1. Dilute 6 μL of cDNA in 194 μL of RNase free water, to make up to 200 μL of diluted cDNA.
2. Prepare primer pair stocks containing 2.5 μM of each gene specific primer in RNase free water.
3. Dilute gene specific primers in Power SYBR® Green master mix at a ratio of 1:5.
4. Load 4 μL of diluted cDNA into each well of a 384-well PCR plate.
5. Load 6 μL of primer/SYBR® Green mix to each well. Centrifuge at 380rcf for 2 min.
6. Load plate into ABI PRISM 7900 Sequence Detection Systems.
7. Perform qRT-PCR reaction as follows:

   Stage 1: 90 °C for 2 min,

   Stage 2: 95 °C for 10 min,

   Stage 3: 95 °C for 30s, 60 °C for 30 s followed by 72 °C for 1 min, for a total of 40 cycles,

   Stage 4: 95 °C for 15 s, 60 °C for 15 s, and 95 °C for 15 s.
8. Normalize raw candidate CT values to spike-in controls DAP and THR. As these were present at equal concentrations in each fraction any differences in the efficiency of RNA extraction between fractions will be reflected in the CT values of these controls. The CT values for the spike-in RNAs should be very similar across all fractions if RNA extractions were equally efficient.
9. Convert resultant CT values to arbitrary units and present relative RNA levels as a percentage of the total RNA where 100 % RNA is calculated as the sum of fractions 1–12 (Fig. 1).

4 Notes

1. Sodium deoxycholate takes a long time to dissolve. It can be stored at room temperature but if precipitates form redissolve before use.
2. 2× RSB can be stored at 4 °C for up to a month.
3. Prepare 1× RSB fresh before the start of the every experiment. The recipe given above is for two sample preps and should be adjusted accordingly.
4. Prepare 1× lysis buffer fresh before the start of every experiment.
5. It is crucial to prechill all Eppendorf, Falcon, and centrifuge tubes as there is increased likelihood of ribosome run-off or RNA degradation if tubes are not kept cold. If polysome peaks do not look as expected on first run, it is likely that the process needs to be done faster and colder.
6. Number of cells seeded should be adjusted as required. Cells should be actively growing, as once they reach stationary phase of growth, translation may be repressed.
7. Avoid tilting the tubes which will disrupt the gradient.
8. Adjust volume of 1× RSB added depending on the size of the cell pellet. A bigger cell pellet may require more than 140 μL of 1× RSB to resuspend.
9. Nuclear pellet will be smaller and whiter than the cell pellet.
10. Take out maximum volume of cell lysate possible. Cell lystate can be snap-frozen and stored at −80 °C for later fractionation.
11. 1.0 AU works best for seven or more OD units of sample loaded. For samples above three OD units, set to 0.5 AU; for samples between one and three OD units, set to 0.2 AU to enhance sensitivity of detection.
12. Open file using an Excel spreadsheet format. Replace the last absorbance value of each fraction with "0" to indicate the end of each fraction. Plot a smooth line of absorbance profile at 254 nm (*y*-axis) against fraction number (*x*-axis).
13. TRIzol® is not recommended for polysome RNA purification from sucrose gradients, and the RNeasy columns can give unequal RNA yields. Phenol–chloroform extraction is recommended with this protocol to enable equal extraction efficiency across all fractions.
14. Glycogen acts as a carrier and helps in the precipitation of RNA.
15. When using isopropanol, the RNA pellet tends to be loose and will be dislodged easily; care should be taken not to disrupt the RNA pellet.

16. Longer precipitation increases RNA yield, and thus, overnight incubation is encouraged. In cases where RNA purity is compromised, an additional ethanol precipitation step is necessary.
17. Heparin inhibits reverse transcriptase activity and has to be removed from RNA samples. Lithium chloride precipitation is performed to remove heparin.
18. Sodium acetate precipitation is performed to remove lithium chloride from RNA.
19. Dry the RNA pellet for about 1–5 min, checking on the pellet every 1 min. Pellet should appear glassy before resuspension. Overdried RNA pellet appears white and will not dissolve easily.
20. For RNA purity, the 260/280 ratio should be over 2 and the 260/230 ratio should be over 1.8.
21. Prepare a master mix of oligoDT, random hexamer primers, and dNTPs according to the number of reactions. Avoid pipetting volumes below 1 μL which may lead to high inaccuracies.

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