Video Article

Eukaryotic Polyribosome Profile Analysis

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Abstract

Protein synthesis is a complex cellular process that is regulated at many levels. For example, global translation can be inhibited at the initiation phase or the elongation phase by a variety of cellular stresses such as amino acid starvation or growth factor withdrawal. Alternatively, translation of individual mRNAs can be regulated by mRNA localization or the presence of cognate microRNAs. Studies of protein synthesis frequently utilize polyribosome analysis to shed light on the mechanisms of translation regulation or defects in protein synthesis. In this assay, mRNA/ribosome complexes are isolated from eukaryotic cells. A sucrose density gradient separates mRNAs bound to multiple ribosomes known as polyribosomes from mRNAs bound to a single ribosome or monosome. Fractionation of the gradients allows isolation and quantification of the different ribosomal populations and their associated mRNAs or proteins. Differences in the ratio of polyribosomes to monosomes under defined conditions can be indicative of defects in either translation initiation or elongation/termination. Examination of the mRNAs present in the polyribosome fractions can reveal whether the cohort of individual mRNAs being translated changes with experimental conditions. In addition, ribosome assembly can be monitored by analysis of the small and large ribosomal subunit peaks which are also separated by the gradient. In this video, we present a method for the preparation of crude ribosomal extracts from yeast cells, separation of the extract by sucrose gradient and interpretation of the results. This procedure is readily adaptable to mammalian cells.

Video Link

The video component of this article can be found at http://www.jove.com/video/1948/

Protocol

1. Preparation of 7-47% sucrose gradients

1. Prepare sucrose gradients one day before use to allow gradients to become continuous. Make sterile 7% and 47% sucrose solutions containing 50mM NH_4Cl, 50mM Tris-Acetate pH 7.0 and 12mM $MgCl_2$ and store at 4°C [1].
2. For 6 gradients mix the 7% and 47% sucrose stock solutions to make 48 mL of each of the following sucrose concentrations. Add DTT (1M stock) to each sucrose solution to a final concentration of 1mM.

Final Concentration	7% sucrose	47% sucrose
7%	48 mL	0
17%	36 mL	12 mL
27%	24 mL	24 mL
37%	12 mL	36 mL
47%	0	48 mL

3. In order to pour the gradients, attach a Pasteur pipette to a 20 mL syringe by securing and sealing with Parafilm or use a long needle. Add 7 mL of 7% sucrose to the bottom of a 1 x 3.5" polyallomer ultracentrifuge tube. Next add 7 mL of 17% sucrose solution under the 7% solution by placing the Pasteur pipette tip near bottom of tube and pipetting no faster than 0.3 mL/sec. Repeat with 7 mL each of 27%, 37% and 47% sucrose solutions. You should observe clear lines between the layers, indicating that minimal mixing has occurred. Store gradients at 4°C overnight along with the rotors, bottles and tubes that will be used to harvest samples.

2. Preparation of the yeast extract

1. Grow 125 mL of yeast culture to an OD_{600} of 0.8-1.0. Add cycloheximide to the culture to a final concentration of 100 µg/mL and continue shaking at 30°C for 15 min.
2. Meanwhile, prepare lysis buffer containing 80 µg/mL cycloheximide, 200 µg/mL heparin, 0.2% DEPC, and 10 mM Tris-HCl pH 7.5, 0.1M NaCl, 30mM $MgCl_2$. Keep everything on ice from this point on.

3. Pour the culture into a 250 mL centrifuge bottle and fill with ice. Centrifuge at 8,000 x g (7,250 rpm in a SLA-1500 rotor) for 5 min at 4°C. Wash the pellet once with 10 mL of ice cold lysis buffer and transfer to 15 mL polypropylene tubes. Centrifuge at 5,900 x g (~6,000rpm in a SLA-600TC rotor) for 3 min at 4°C. Resuspend the pellet in 0.5 mL of lysis buffer, transfer to 1.5mL tube and add 0.5 mL of chilled, baked acid-washed glass beads. Vortex the cells for four 30 sec intervals, cooling the tube on ice for 30 sec between each interval. Add another 0.5 mL of lysis buffer into the 1.5 mL tube. Centrifuge at maximum speed in a microcentrifuge (14,000 rpm in an Eppendorf 5417R microcentrifuge) for 10 min at 4°C. Transfer supernatant to a new 1.5 mL microcentrifuge tube. Remove 10 µl to a separate tube to determine the OD_{260} / OD_{280} ratio (see step 3.1 below). Store extracts at -80°C.

3. Preparation of extracts from mammalian cells

1. For adherent cells, grow in 100 mm dish to ~70% confluence. You will need one 100 mm dish per 11 ml gradient (typical yield is ~20 OD_{260} units). Scale the amount linearly for larger or smaller gradient sizes. For non-adherent cells, approximately 1 x 10^7 cells are needed per 11 ml gradient.
2. Prior to lysis, add cycloheximide to 100 µg/mL. Incubate at 37°C for 15 min. Transfer plates to ice and rinse cells twice in ice cold PBS. All future steps MUST be carried out at 0-4°C.
3. Remove all traces of PBS by aspiration (let plates drain on an angled bed of ice) and add 1 mL lysis buffer, scrape and transfer to pre-chilled 1.5 mL tube. Lysis buffer components will need to be optimized to the cell type and growth conditions. A good starting buffer would be 20 mM HEPES-KOH, pH 7.4, 15 mM $MgCl_2$, 200 mM KCl, 1% Triton X-100 (v/v), 100 µg/mL cycloheximide, 2 mM DTT, and 1 mg/mL heparin. The key variables are the concentrations of magnesium and potassium chloride as well as the inclusion or exclusion of non-ionic detergents to improve extraction of cytoskeletal or membrane bound polysomes. Lyse cells by passing through 27 gauge syringe needle twice or by 5-10 strokes of dounce homogenization using Type B pestle. The syringe or homogenizer should be pre-chilled prior to use.
4. Spin at 14,000 x g for 5 min in refrigerated microcentrifuge. Transfer fresh lysate to the sucrose gradient (for mammalian cells this is made up in lysis buffer lacking Triton X-100 and heparin but otherwise as described in 1.2 and 1.3) or flash freeze in liquid nitrogen for later use.

4. Centrifugation of gradients

1. Thaw polyribosome lysates on ice. Determine the concentration of nucleic acid in the lysate by adding the reserved 10 µL sample to 1 mL of water and reading the OD_{260}/OD_{280} ratio with a spectrophotometer. Typical yield from a yeast culture grown as described is 50 OD units.
2. Using a pipette with the tip placed against the wall of the tube near the surface of the gradient, gently layer 25 OD_{260} units of the lysate on top of the gradient. For a 35 mL gradient 500 µl of lysate is typically loaded. Lysis buffer can be used to assure all gradients are loaded with an equal volume. Lower amounts of material yield better resolution. Using forceps, carefully lower the gradients into buckets of a swinging bucket rotor.
3. Centrifuge the gradients at 100,000 x g (23,000 rpm in a Surespin 630 rotor) for 4 hr at 4°C. This protocol can be adapted for smaller volume gradients.

5. Collection of data and fractions

1. Approximately 30 min prior to the completion of the 4 hr spin attach the needle to the Model 184 tube piercer. Attach the pen to the online Isco Model UA-5 Absorbance/Fluorescence Monitor. Power on the absorbance monitor to allow a stable baseline to be reached prior to analyzing samples. Electronic acquisition of the polysome profile can easily be obtained by attaching a DI-148U data acquisition unit (DATAQ Instruments) to the chart recorder. Profiles can then be collected in real time and saved for later annotation using the accompanying WinDaq software.
2. Fill the syringe pump with 50mL of Fluorinert using the reverse flow, rapid setting. Make sure that there are no air bubbles present. Connect the hose from the syringe to the tube piercer and briefly turn on the flow of Fluorinert at 3 mL/min in the forward direction to clear the hose of any air bubbles.
3. Place a waste beaker or series of tubes if collecting fractions under the hose at the end of the flow cell to collect sample run off.
4. Carefully remove the polyallomer centrifuge tubes containing the sucrose gradient from centrifuge rotor and place them on ice (pre-form the wells in the ice with an empty tube to avoid disturbing gradients).
5. To analyze cell extracts, place the gradient tube on the tube piercer. Connect the top of the tube to the outlet and secure. Raise the bottom stage to the centrifuge tube so that the needle pierces the bottom of the tube. Once the needle has protruded through the bottom of the centrifuge tube, begin the flow of Fluorinert in the forward direction at 6mL/min.
6. Once the Fluorinert has begun flowing into the tube, monitor the movement of the needle on the online absorbance monitor. Once the needle begins to move, turn on the chart movement to a speed setting of 60. The monitor will record the OD_{254} starting with the free material followed by 40S ribosomal subunit detection and continuing through to polyribosome complexes. If you are collecting fractions for RNA or protein analysis, typically 0.2 min fractions are taken (1 mL). For RNA analysis, fractions should be collected directly into tubes containing Trizol (Invitrogen), Phenol or SDS/proteinase K, depending on the preferred method of extraction.
7. When the end of the polyribosome profile has been reached, turn off the flow of Fluorinert to the centrifuge tube and stop the chart movement of the absorbance/fluorescence monitor.
8. Retract the Fluorinert from the centrifuge tube into the syringe by beginning the flow in the reverse direction at a rapid rate making sure not to draw sucrose from the tube into the syringe. Unscrew the centrifuge tube from the tube piercer, lower the needle, and remove the tube.
9. Repeat steps 5.5 through 5.8 for each sucrose gradient.
10. When all sucrose gradients have been analyzed, retract the Fluorinert from the hose back into the syringe. Remove each part of fractionator (tube piercer and flow cell) including the waste hose located at the top and rinse well with water.

6. Representative Results

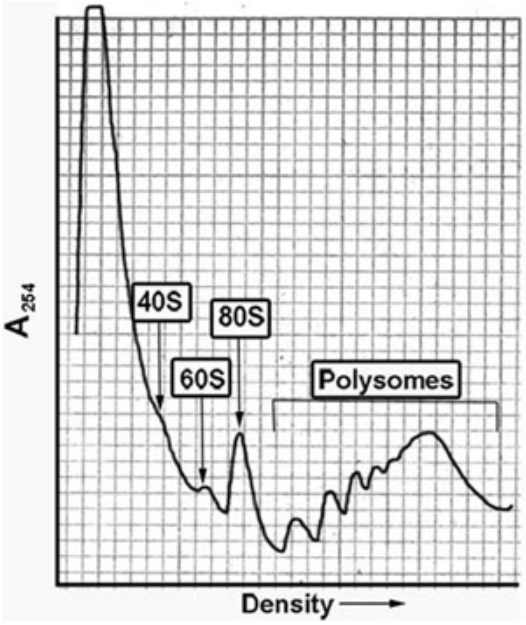

Figure 1. Representative trace of ribosome extract prepared from yeast strain BY4741 (*mat***a** *his3Δ1 leu2Δ0 met15Δ0 ura3Δ0*) in the presence of cycloheximide. The ribosomal extract was fractionated utilizing a 7 47% sucrose gradient and analyzed using a pump syringe apparatus attached to a UV detector. Peaks containing polyribosomes and 80S, 60S and 40S ribosomes are indicated.

Discussion

The information obtained from the polyribosome profile can provide valuable insight into the translational status of the cell. In addition, the status of the assembly of ribosomal subunits themselves can be determined[2]. For example the presence of halfmers or 80S and larger peaks with a slight shoulder to the right on the profile indicates a bound 40S subunit awaiting 60S subunit joining. Performing the experiment in the absence of any added cycloheximide or other inhibitor of elongation allows for analysis of the rate of run off, which indicates whether or not elongation is altered[3]. The fractions themselves are a rich source of reagents for the subsequent determination of the association of a specific mRNA or protein with ribosomal subpopulations by Northern or Western blotting of the fractions. The total pool of mRNAs associated with active ribosomes can be determined via an associated microarray[4] or deep sequencing analysis[5]. The protein associations can also be stabilized by the appropriate addition of a cross linking reagent [6].

Polyribosome analysis from mammalian cells is also worth mentioning as a distinct protocol in terms of the apparent difficulties in establishing the conditions required to obtain stable polysomes. The buffer systems reported in the literature are widely variable [7-10], thus there may be a requirement for cell-type specific optimization of the lysis buffer, or it is equally plausible that the buffer system is not as important as keen attention to working with fresh lysates kept at low temperatures. To our knowledge a systematic evaluation of the parameters affecting mammalian polysome formation has not been reported.

Disclosures

No conflicts of interest declared.

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