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Polysome Profiling Without Gradient Makers or Fractionation Systems.

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Abstract

Polysome fractionation by sucrose density gradient centrifugation is a powerful tool that can be used to create ribosome profiles, identify specific mRNAs being translated by ribosomes, and analyze polysome associated factors. While automated gradient makers and gradient fractionation systems are commonly used with this technique, these systems are generally expensive and can be cost-prohibitive for laboratories which have limited resources or cannot justify the expense due to their infrequent or occasional need to perform this method for their research. Here a protocol is presented to reproducibly generate polysome profiles using standard equipment available in most molecular biology laboratories without the use of specialized fractionation instruments. Moreover, a comparison of polysome profiles generated with and without a gradient fractionation system is provided and strategies to optimize and produce reproducible polysome profiles are discussed. *Saccharomyces cerevisiae* is utilized as a model organism in this protocol, but this protocol can be easily modified and adapted to generate ribosome profiles for many different organisms and cell types.

SUMMARY:

This protocol describes how to generate a polysome profile without using automated gradient makers or gradient fractionation systems.

INTRODUCTION:

Ribosomes are mega-Dalton ribonucleoprotein complexes that perform the fundamental process of translating mRNA into proteins. Ribosomes are responsible for carrying out the synthesis of all proteins within a cell. Eukaryotic ribosomes are comprised of two subunits designated as the small ribosomal subunit (40S) and the large ribosomal subunit (60S) according to their sedimentation coefficients; the fully assembled ribosome is designated as the 80S monosome and polysomes are groups of ribosomes engaged in translating a single mRNA molecule. Polysome fractionation by sucrose density gradient centrifugation is a powerful method that can be used to create ribosome profiles, identify specific mRNAs associated with translating ribosomes, and analyze polysome associated factors[1–13]. This

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DISCLOSURES:
The authors have nothing to disclose.

technique is often used to separate polysomes from single ribosomes, ribosomal subunits, and messenger ribonucleoprotein particles. The profiles obtained from fractionation can provide valuable information regarding the translation activity of polysomes[14] and the assembly status of the ribosomes[15–17].

Ribosome assembly is a very complex process that is facilitated by a group of proteins known as ribosome assembly factors[18–21]. These factors perform a wide range of functions during ribosome biogenesis through interactions with many other proteins including ATPases, endo- and exo-nucleases, GTPases, and RNA helicases, and RNA binding proteins[22]. Polysome fractionation has been a powerful tool used to investigate the role of these factors in ribosome assembly. For example, this method has been utilized to demonstrate how mutations in the polynucleotide kinase Grc3, a pre-rRNA processing factor, can negatively affect the ribosome assembly process[17,23]. Polysome profiling has also highlighted and shown how the conserved motifs within the ATPase Rix7 are essential to ribosome production[16].

The procedure for polysome fractionation begins with making soluble cell lysates from cells of interest. The lysate contains RNA, ribosomal subunits, and polysomes as well as other soluble cellular components. A continuous, linear sucrose gradient is made within an ultra-centrifuge tube. The soluble fraction of cell lysate is gently loaded onto the top of the sucrose gradient tube. The loaded gradient tube is then subjected to centrifugation which separates the cellular components by size within the sucrose gradient by the force of gravity; the larger components travel further into the gradient than the smaller components. The top of the gradient houses the smaller, slower traveling cellular components, whereas the larger, faster traveling cellular components are found in the bottom. After centrifugation, the contents of the tube are collected as fractions. This method effectively separates ribosomal subunits, monosomes, and polysomes. The optical density of each fraction is then determined by measuring the spectral absorbance at a wavelength of 254 nm. Plotting absorbance vs fraction number yields a polysome profile.

Linear sucrose density gradients can be generated utilizing a gradient maker. Following centrifugation, gradients are often fractionated and absorbances measured using an automated density fractionation system[3,7,13,24,25]. While these systems work very well to produce polysomes profiles, they are expensive and can be cost prohibitive to some laboratories. Here a protocol to generate polysome profiles without the use of these instruments is presented. Instead, this protocol utilizes equipment typically available in most molecular biology laboratories.

PROTOCOL:

1. Preparation of 7–47 % Sucrose Gradients

Note: The linear range of the sucrose gradient can be modified to achieve better separation depending on the cell type used. This protocol is optimized for polysome profiles for *S. cerevisiae*.

1.1 Prepare stock solutions of 7 % and 47 % sucrose in sucrose gradient buffer (20 mM Tris-HCl pH 7.4, 60 mM KCl, 10 mM $MgCl_2$, and 1 mM DTT). Filter sterilize the stock sucrose solutions through a 0.22 μm filter and store at 4 °C.

1.2 Prepare 14 mL of 17 %, 27 %, and 37 % sucrose solutions by dispensing and mixing the 7 % and 47 % sucrose stock solutions in the manner described in Table 1.

1.3 Place six polypropylene centrifuge tubes (14 X 89 mm) into a full view test tube rack. Tubes should have enough space between them so that actions with one tube do not disturb the others.

1.4 Attach a long needle to a 3 mL syringe. For this protocol a 9 inch, 22 gauge needle with a blunt tip is recommended (Figure 1), but any needle long enough to reach the bottom of the centrifuge tube will suffice.

Note: It is recommended to perform a test fill and dispensation to ensure that the syringe can hold the sucrose solution without any dripping prior to setting up the gradients.

1.5 Add 2 mL of the 7 % sucrose to the bottom of each centrifuge tube.

1.6 Add 2 mL of the 17 % sucrose beneath the 7 % solution by positioning the needle tip within the immediate vicinity of the tube bottom and dispensing the solution slowly and carefully.

1.7 Repeat with 2 mL each of the 27 %, 37 %, and 47 % sucrose solutions. Each layer should be distinguishable from one another by a line marking the separation of densities (Figure 2).

Note: At this point, prior to their settlement into a continuous, increasing percentage of sucrose, tubes with layered sucrose solutions can be flash frozen in liquid nitrogen and placed into a −80 °C freezer for long term storage.

1.8 Store gradients at 4 °C overnight to allow gradients to settle into a continuous, increasing percentage of sucrose. It takes 8–12 hours for the layered sucrose solutions to settle into a linear sucrose gradient. Linear gradients are stable for up to 48 hours. If using frozen, layered, sucrose solutions overnight storage at 4 °C provides sufficient time for thawing and settlement into a linear gradient. A densitometer can be used to assess gradient quality.

Note: It is critical that the gradients are stored in a stable place where they will not be disturbed as any movements or vibrations will disrupt the gradient.

2. Preparation of Yeast Cell Extracts

2.1 Inoculate yeast strain of interest into 50 mL of YPD media and grow overnight at 30 °C in a shaking incubator to stationary phase.

Note: The temperature may vary depending upon yeast strain requirements.

2.2 Transfer 10 mL of stationary phase culture into 1 L of fresh YPD media. Incubate cells with vigorous shaking at 30 °C (or other suitable temperature) until culture reaches mid-exponential growth phase (OD_{600} = 0.4–0.6).

2.3 At mid-exponential growth phase, add cycloheximide to culture to a final concentration of 0.1 mg/mL. Incubate on ice for 5 minutes.

2.4 Harvest cells by centrifugation at 3,000 x g for 10 minutes at 4 °C.

Note: At this point cells can be frozen and stored at −80 °C.

2.5 Resuspend cells in chilled 700 μL polysome extraction buffer (20 mM Tris-HCl pH 7.4, 60 mM KCl, 10 mM MgCl2, 1 mM DTT, 1 % Triton X-100, 0.1 mg/mL cycloheximide, 0.2 mg/mL heparin). Add 100 units of RNAse Inhibitor and transfer to 1.5 mL centrifuge tube.

2.6 Add ~ 400 μL of pre-chilled glass beads with a size range or 425–600 μm to a centrifuge tube. Disrupt yeast cells by vigorous agitation in a bead-beater for 5 minutes.

2.7 Clarify lysate by centrifugation at 8,000 x g for 5 minutes at 4 °C.

2.8 Determine the concentration of the RNA in the clarified lysate by measuring the absorbance at 260 nm with a spectrophotometer or by using a fluorescence-based RNA detection system.

Note: For very accurate RNA concentration measurements, using fluorescence-based RNA detection kits is recommended.

2.9 RNA concentration should be 0.5–1 μg/μL; if the RNA concentration is too low, reduce the volume of polysome extraction buffer used to resuspend cells in future experiments.

Note: Lysate should be loaded onto gradients immediately after lysis and RNA quantitation. If necessary, lysates can be flash frozen in liquid nitrogen and stored at −80 °C for a few days.

3. Centrifugation of Gradients

3.1 Carefully load lysate onto the top of gradients. Place pipet tip against the inner wall of the polypropylene tube at the top of the tube, gently angle the tube and slowly dispense lysate onto the top of the gradient by dribbling the lysate against the wall. Take great care to not disrupt or disturb the gradient when loading lysate.

Note: The amount of lysate loaded will vary per cell type. The content of the RNA will also vary. It may be necessary to perform a number of experiments in order to determine the amount of lysate needed to generate an optimal polysome profile. For yeast, 300 μG of RNA is a good starting point for optimization.

3.2 Gently place tubes into the pre-chilled buckets of a swinging bucket rotor.

Note: Each polypropylene centrifuge tube should have equal volumes of gradient and the amount of lysate loaded, which can vary from tube to tube, will not be enough to cause an imbalance error during ultracentrifugation.

3.3 Centrifuge gradients at 260,110 x g for 150 minutes at 4 °C.

4. Fraction and Data Collection

4.1 Carefully remove the centrifuge tubes from the swinging bucket rotor and place in a tube holder.

4.2 Label 96 well plates in which fractions will be stored and pre-chill on ice.

Note: It's recommended to use a 96-well plate that is suitable for a spectrophotometer and has an optical window down to 230 nm for nucleic acid determinations at 260 nm/280 nm.

4.3 Collect 100 or 200 ul fractions starting from the top of the gradient by carefully inserting a pipet tip into the top of the gradient. Collect fractions until the entire gradient has been aliquoted; the number of fractions will depend on total gradient volume.

Note: Fractions must be collected in a manner that does not disrupt the rest of the gradient. All fractions should be of equal volume. In addition to manual fractionation, another low-cost method for fractionation is the use of a small peristaltic pump.

4.4 Transfer each fraction to the 96-well plate until the bottom of the centrifuge tube is reached. Keep collected fractions on ice at all times.

4.5 Measure the absorbance at 254 nm of each fraction with a spectrophotometer. The 7 % and 47 % sucrose solutions can be used as blanks.

Note: When measuring the absorbance of fractions, bear in mind that for the majority of spectrometers and colorimeters, the most effective absorbance range is 0.1 to 1. If out-of-range absorbance measurements of 1.0 or above are being taken, the fractions have too much material. Simply dilute the sample and recollect data and then account for the dilution factor when plotting the profile.

4.6 Create the polysome profile by plotting the fraction number versus absorbance.

REPRESENTATIVE RESULTS:

Three representative polysome profiles are shown in Figure 3. All profiles are from the same yeast strain. A typical polysome profile will have well resolved peaks for the 40S, 60S, and 80S ribosomal subunits as well as polysomes. The crest of each ribosomal subunit and polysome peak will be apparent on each profile (Figure 3). A representative profile from an automated density fractionation system is shown in Figure 3A. The sucrose gradients used to generate this profile were prepared by hand as described in this protocol. This profile shown in Figure 3A was produced from a continuous absorbance profile as the sucrose gradient was displaced from the bottom up by a chase solution, through a detector flow cell and collected in fractions. Because these systems continuously measure absorbance, they can record over 1500 data points. It is impractical to manually generate the same number of data points. The fraction volume generally utilized for manual profiles is 100–200 μL. A fraction volume within this range yields a profile with enough detail for most comparative analyses. Representative results from both 100 μL and 200 μL fractionations are shown in Figure 3B and Figure 3C. All three profiles presented utilized sucrose gradients prepared as described in this protocol so that the data yielded can be compared. For an example of a polysome

profile that utilizes a gradient maker to prepare sucrose gradients as opposed to the manual preparation described in this protocol, a recent manuscript by Chikashige et al has several examples of gradient profiles generated using an automated gradient maker and fractionation system[13].

DISCUSSION:

Here a method to create polysome profiles without the use of expensive automated fractionation systems has been described. The advantage of this method is that it makes polysome profiling accessible to labs that do not have automated fractionation systems. The major disadvantages of this protocol are tedious hand fractionation and reduced sensitivity compared to the dedicated density fractionation system.

This protocol entails careful preparation of sucrose gradients with resolution sufficient to separate ribosomal subunits, monosomes, and polysomes. When preparing sucrose gradients, it is critical to not introduce air bubbles while loading gradient layers. Air bubbles rise to the top from the bottom and can disrupt the linearity of the gradient. Additionally, the outside of the needle should be wiped before each use and excess sucrose solution should be wicked off from the lumen to ensure the integrity of each gradient layer. Overnight storage of gradients at 4 °C should be done in a cold room. The vibrations caused by a compressor switching on and off in a refrigerator can disrupt the gradient.

Another critical part of this assay is the volume of the gradient and the concentration of the RNA. The 14 X 89 mm centrifuge tubes can hold a volume of 12 mL, but this is the maximum volume that can be accommodated by these tubes and this volume is typically thought of as overfilling these tubes. A good maximum working volume that does not overfill these tubes is 11.5 mL. The sucrose gradient itself has a volume of 10 mL, therefore the volume of polysome extraction buffer used to resuspend cells should not exceed 1.5 mL. The amount of RNA necessary to generate a good profile will vary by cell type. A number of initial runs should be performed to determine what amount of RNA generates a good polysome profile. Once this amount is determined, it should always be used with that specific cell type to maintain reproducibility and to be able to do comparative analysis. Also, in order to ensure that the same amount of RNA is loaded each time, the method used for RNA quantitation must always be the same. If a spectrophotometer is used to quantitate RNA, then it should be used at all times. If a fluorometer is utilized, then it should be used at all times. Instruments and quantitation techniques vary widely in both accuracy and sensitivity. Utilizing different instruments or techniques to quantitate RNA experiment to experiment will not generate reproducible results.

Finally, this method can be adapted to obtain important information about the status of protein translation in a cell. As mentioned above, the condition of the ribosomal subunits themselves within the assembly process can be determined. Performing experiments in the absence of cycloheximide, which inhibits elongation, enables run off rate analysis, which indicates whether elongation is altered or not[26]. The individual fractions are a valuable source of material for further experiments and analysis. For example, the fractions can be used in Northern or Western blotting protocols to identify a specific RNA or protein that is

associated with ribosomal subpopulations. Finally, RNA can be extracted from the fractions and used to identify mRNAs bound to active ribosomes by microarray analysis[13,27] or by deep sequencing analysis on a DNA library generated from the total mRNA[28].

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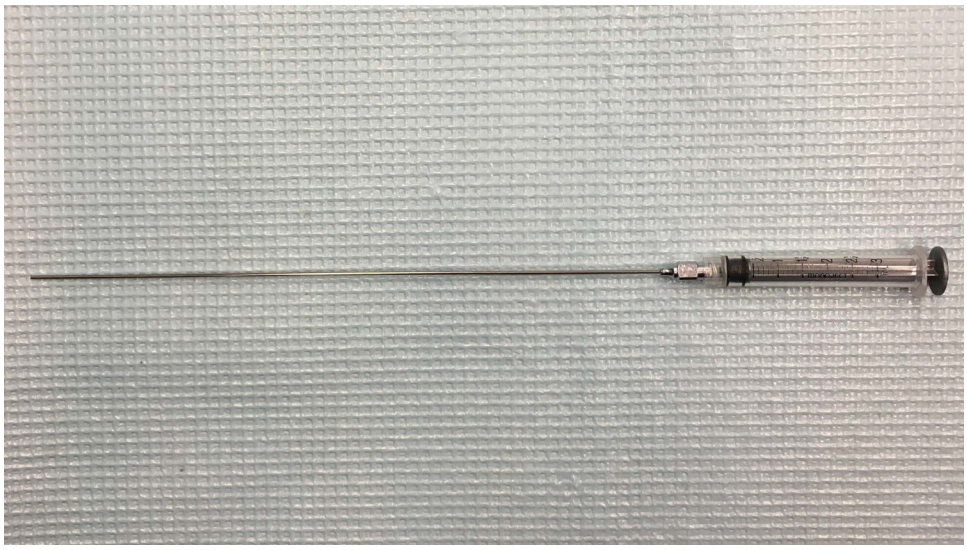

Figure 1:
Assembled needle and syringe. 9 inch, 22 gauge needle with Hamilton Needle Point Style 3 tip attached to 3 mL syringe via a Luer lock mechanism.

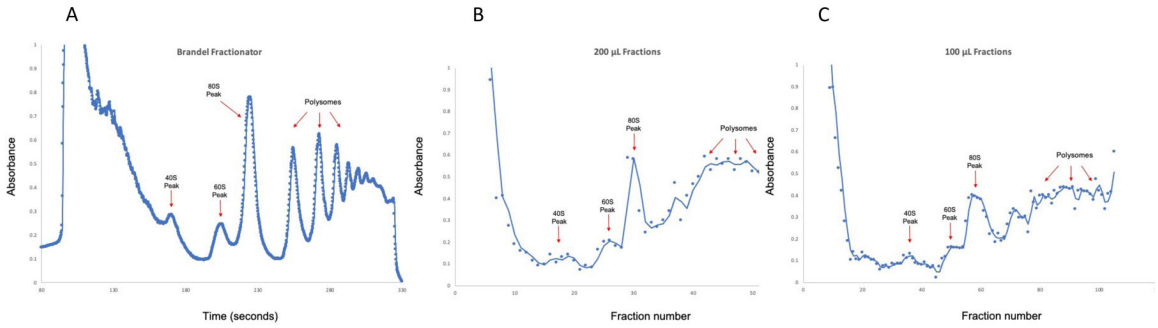

Figure 2:

The final appearance of 7–47 % sucrose gradient layers. 7, 17, 27, 37 and 47 % sucrose solutions layered on top of another as described in the protocol. The 17 and 37 % layers had blue coloring added to help distinguish the layers for a photograph; no coloring should be added when performing an actual experiment.

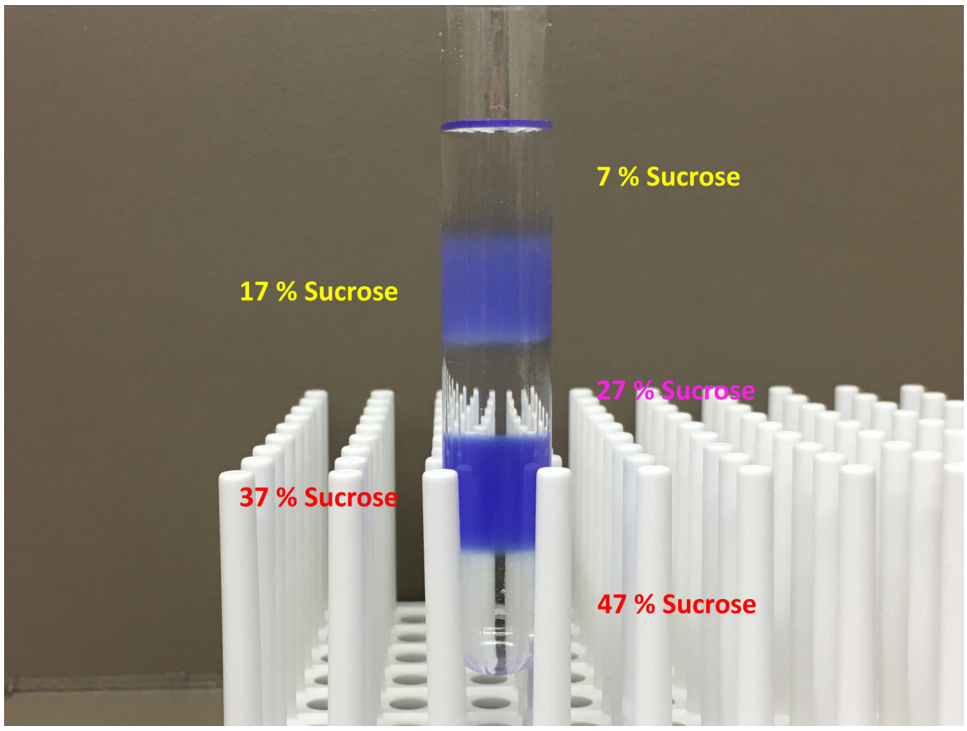

Figure 3:

Polysome profiles; all sucrose gradients used to generate these profiles were prepared using the method described in this protocol. A) The polysome profile generated by an automated fractionation system. B) The polysome profile generated by hand fractionating 200 µL samples described in the current protocol. C) The polysome profile generated by hand fractionating 100 µL samples described in the current protocol. Polysomes, 40S, 60S and 80S peaks are indicated for each profile.

Materials

Name of Material/Equipment	Company	Catalog Number	Comments/Description
Clariostar Multimode Plate Reader	BMG Labtech		
Cycloheximide	Sigma Aldrich	C7698	
Dithiothreitol	Invitrogen	15508–013	
Glass Beads, acid washed	Sigma Aldrich	G8772	425–600 μm
Heparin	Sigma Aldrich	H4784	
Magnesium Chloride, 1 M	KD Medical	CAC-5290	
Needle, 22 Gauge, Metal Hub	Hamilton Company	7748–08	custom length 9 inches, point style 3
Optima XL-100K Ultracentrifuge	Beckman Coulter		
Polypropylene Centrifuge tubes	Beckman Coulter	331372	
Polypropylene Test Tube Peg Rack	Fisher Scientific	14–810-54A	
Potassium Chloride	Sigma Aldrich	P9541	
Qubit 4 Fluorometer	Thermo Fisher Scientific	Q33228	
Qubit RNA HS Assay Kit	Thermo Fisher Scientific	Q32855	
RNAse Inhibitor	Applied Biosystems	N8080119	
Sucrose	Sigma Aldrich	S0389	
SW41 Swinging Bucket Rotor Pkg	Beckman Coulter	331336	
Syringe, 3 mL	Coviden	888151394	
Triton X-100	Sigma Aldrich	X100	
Tris, 1 M, pH 7.4	KD Medical	RGF-3340	
UV-Star Microplate, 96 wells	Greiner Bio-One	655801	

Table 1:

Preparation of sucrose solutions. Prepare 14 mL of 17 %, 27 %, and 37 % sucrose solutions by dispensing and mixing the 7 % and 47 % sucrose stock solutions in the volumes indicated.

Final Concentration	mL of 7% Stock	mL of 47% Stock
7%	14	0
17%	10.5	3.5
27%	7	7
37%	3.5	10.5
47%	0	14