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Polysome Profiling Without Gradient Makers or Fractionation Systems.

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TITLE:

Polysome Profiling Without Gradient Makers or Fractionation Systems

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SUMMARY:

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

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 that 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 specialized fractionation instruments. Moreover, a comparison of polysome profiles generated with and without a gradient fractionation system is provided. Strategies to optimize and produce reproducible polysome profiles are discussed. *Saccharomyces cerevisiae* is utilized as a model organism in this protocol. However, this protocol can be easily modified and adapted to generate ribosome profiles for many different organisms and cell types.

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 comprise 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. Polysomes are groups of ribosomes engaged in translating a single mRNA molecule. Polysome fractionation by sucrose density gradient centrifugation is a powerful method used to create ribosome profiles, identify specific mRNAs associated with translating ribosomes and analyze polysome associated factors[1–13]. This 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 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, 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 ultracentrifuge 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 at 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 polysome 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 sucrose stock 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. Ensure enough space between the tubes 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, use a 9 in, 22 G needle with a blunt tip (**Figure 1**), but any needle long enough to reach the bottom of the centrifuge tube will suffice.

NOTE: 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. Ensure that each layer is 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, flash freeze tubes with layered sucrose solutions in liquid nitrogen and store in 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 h for the layered sucrose solutions to settle into a linear sucrose gradient. Linear gradients are stable for up to 48 h. Overnight storage at 4 °C provides sufficient time for thawing and settlement into a linear gradient for using frozen, layered sucrose solutions. Use a densitometer to assess the gradient quality.

NOTE: It is critical to store the gradients 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 the yeast strain of interest into 50 mL of yeast extract peptone dextrose (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 the cells with vigorous shaking at 30 °C (or other suitable temperature) until the culture reaches the mid-exponential growth phase (OD_{600} = 0.4–0.6).

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

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

NOTE: At this point, cells can be frozen and stored at -80 °C.

2.5. Resuspend the cells in chilled 700 µL of 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 of cycloheximide, 0.2 mg/mL of heparin). Add 100 units of RNase Inhibitor and transfer it to a 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 the yeast cells by vigorous agitation in a bead-beater for 5 min.

2.7. Clarify the lysate by centrifugation at 8,000 x *g* for 5 min 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 using a fluorescence-based RNA detection system.

NOTE: For very accurate RNA concentration measurements, use fluorescence-based RNA detection kits.

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

NOTE: Load the lysate onto the gradients immediately after lysis and RNA quantitation. If necessary, flash freeze the lysates in liquid nitrogen and store at -80 °C for a few days.

3. Centrifugation of gradients

3.1. Carefully load the lysate onto the top of the gradients. Place the pipette tip against the inner wall, at the top of the polypropylene tube. Gently angle the tube and slowly dispense the lysate onto the top of the gradient by dribbling against the wall. Take great care not to disrupt or disturb the gradient when loading the 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 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 the 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 the lysate loaded. This can vary from tube to tube. Ensure that the variation does not cause an imbalance error during ultracentrifugation.

3.3. Centrifuge the gradients at 260,110 x *g* for 150 min at 4 °C.

4. Fraction and data collection

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

4.2. Label the 96-well plates to store the fractions and pre-chill on ice.

NOTE: Use a 96-well plate suitable for a spectrophotometer that has an optical window down to 230 nm for nucleic acid determinations at 260 nm/280 nm.

4.3. Collect 100 µL or 200 µL 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 is aliquoted. The number of fractions will depend on total gradient volume.

NOTE: Collect the fractions in a manner that does not disrupt the rest of the gradient. Ensure that all the fractions have equal volume. In addition to manual fractionation, another low-cost method for fractionation is to use a small peristaltic pump.

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

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

NOTE: When measuring the absorbance of fractions, bear in mind that for most spectrometers and colorimeters, the most effective absorbance range is 0.1–1. If the absorbance measurements are out of range (≥1.0), the fractions have too much material. Dilute the sample, recollect the 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 1,500 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. The representative results obtained 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 to compare the data yielded. 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].

FIGURE AND TABLE LEGENDS:

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.

Figure 1: Assembled needle and syringe. A 9 in, 22 G needle with Hamilton needle point style 3 tip attached to a 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 (Brandel Fractionator). (**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.

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 not to 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 must 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 mm 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, 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 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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DISCLOSURES:

The authors have nothing to disclose.

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Figure 1

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Figure 2

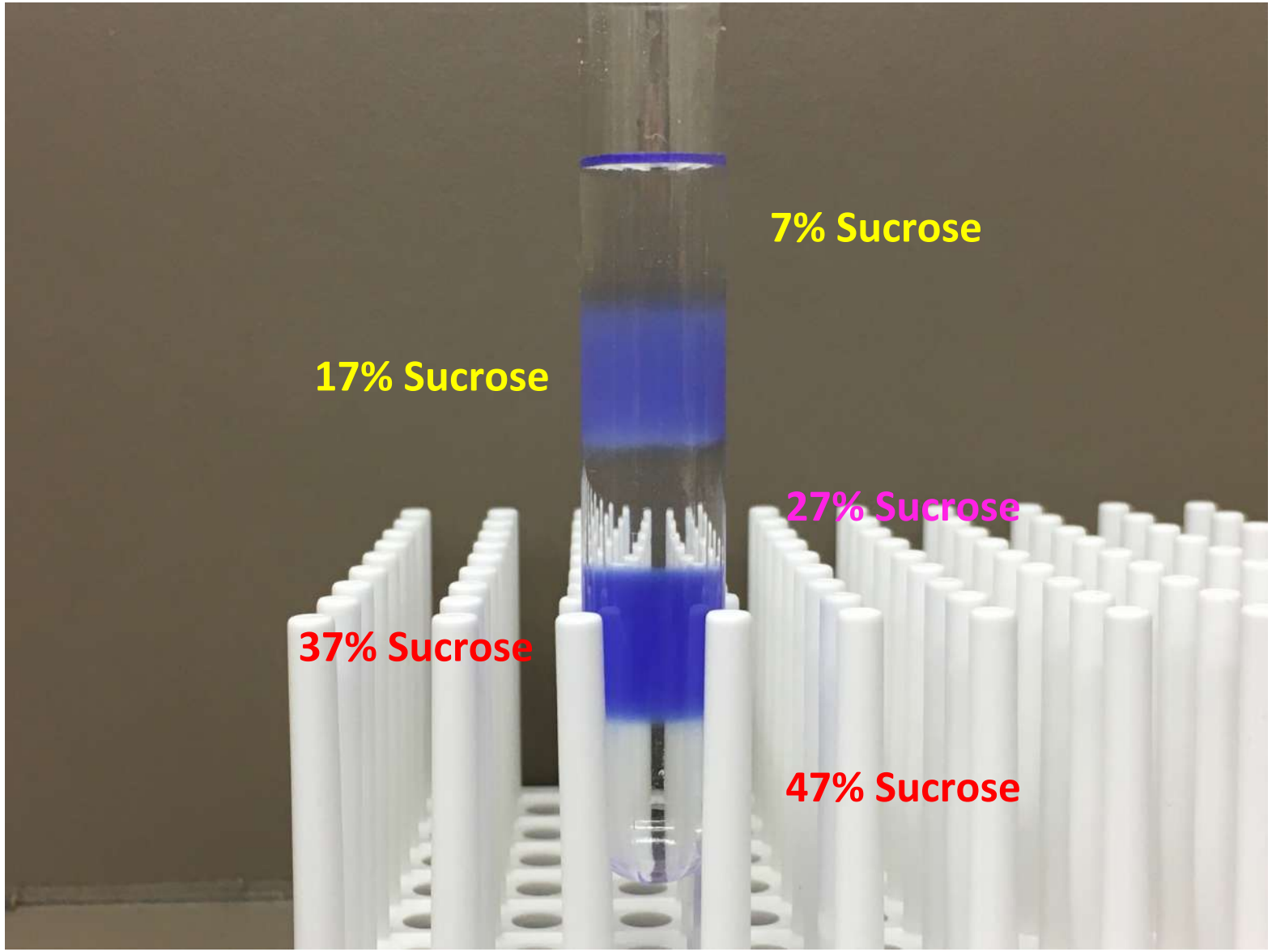

Figure 3

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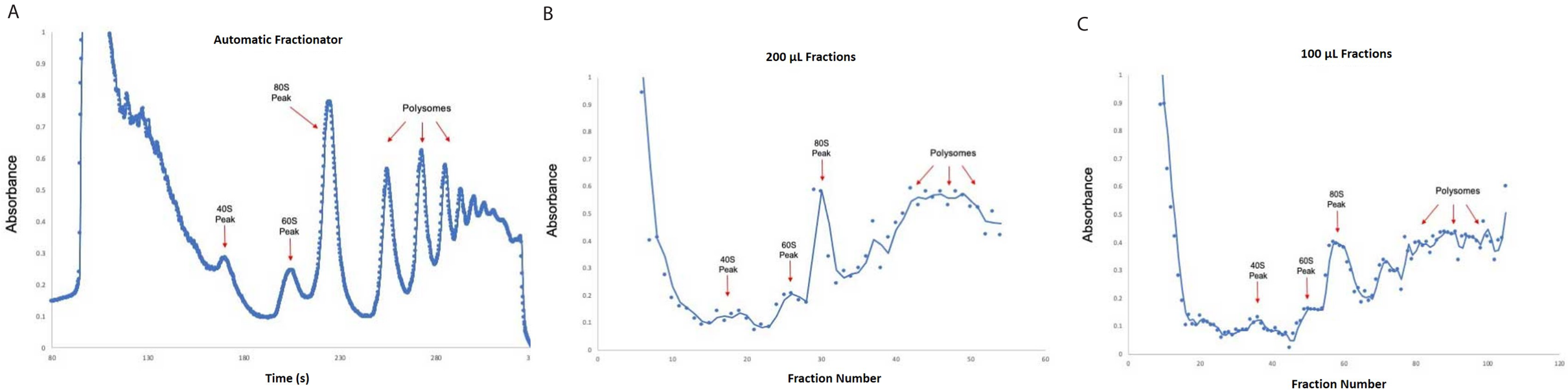

Table 1

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

Name of Material/Equipment	Company	Catalog Number
Automatic Fractionator	Brandel	
Clariostar Multimode Plate Reader	BMG Labtech	
Cycloheximide	Sigma Aldrich	C7698
Dithiothreitol	Invitrogen	15508-013
Glass Beads, acid washed	Sigma Aldrich	G8772
Heparin	Sigma Aldrich	H4784
Magnesium Chloride, 1 M	KD Medical	CAC-5290
Needle, 22 G, Metal Hub	Hamilton Company	7748-08
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
Tris, 1 M, pH 7.4	KD Medical	RGF-3340
Triton X-100	Sigma Aldrich	X100
UV-Star Microplate, 96 wells	Greiner Bio-One	655801

Comments/Description
425–600 µm
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Editorial comments:

Changes to be made by the Author(s):

1. Please take this opportunity to thoroughly proofread the manuscript to ensure that there are no spelling or grammar issues. Please define all abbreviations at first use.

We have thoroughly proofread the manuscript to ensure that there are no spelling or grammar issues and have defined all abbreviations.

2. Increase the word count of the abstract to be 150-300 words.

The word count of the abstract has been increased to 163.

3. Please provide an email address for each author.

Email addresses haven now been provided for both authors.

4. Please revise the following lines to avoid overlap with previously published work: 31, 100-101, 186-188, 256-257, 259-260, 261-262, 263-264

These lines have been revised/rewritten to avoid overlap.

5. Please add only one space after punctuation.

There is now only one space after punctuation.

6. Please remove the embedded Table from the manuscript. All tables should be uploaded separately to your Editorial Manager account in the form of an .xls or .xlsx file. The table legend or caption (title and description) should appear in the Figure and Table Legends section after the Representative Results in the manuscript text.

The embedded Table has been removed. A new table has been generated using Excel and a Table Legend has been added to the revised manuscript.

7. Please revise the text, especially in the protocol, to avoid the use of any personal pronouns (e.g., "we", "you", "our" etc.).

All personal pronouns have been removed.

8. Please ensure that all text in the protocol section is written in the imperative tense as if telling someone how to do the technique (e.g., "Do this," "Ensure that," etc.). The actions should be described in the imperative tense in complete sentences wherever possible. Avoid usage of

phrases such as “could be,” “should be,” and “would be” throughout the Protocol. Any text that cannot be written in the imperative tense may be added as a “Note.” However, notes should be concise and used sparingly. Please include all safety procedures and use of hoods, etc.

All instances of “could be,” “should be,” and “would be” have been removed unless mentioned in a “Note”. The protocol has been edited to conform to the imperative tense.

9. Please note that your protocol will be used to generate the script for the video and must contain everything that you would like shown in the video. Please ensure you answer the “how” question, i.e., how is the step performed? Alternatively, add references to published material specifying how to perform the protocol action. There should be enough detail in each step to supplement the actions seen in the video so that viewers can easily replicate the protocol.

The details of each step in the described in this manuscript has been checked to ensure viewers can replicate the protocol.

10. After including a one line space between each protocol step, highlight up to 3 pages of protocol text for inclusion in the protocol section of the video. This will clarify what needs to be filmed.

One line space is between each protocol step.

11. In figures, e.g., Fig 3, please ensure there is a space between the number and units, e.g., 200 mL not 200mL.

The figures have been edited to ensure that there is a space between numbers and units.

12. Please ensure that the references appear as the following: [Lastname, F.I., LastName, F.I., LastName, F.I. Article Title. Source (ital.). Volume (bold) (Issue), FirstPage–LastPage (YEAR).] For more than 6 authors, list only the first author then et al. Please include volume and issue numbers for all references, and do not abbreviate journal names.

References have been corrected. All references were generated using EndNote and the 2020 JoVE style.

13. Please sort the Materials Table alphabetically by the name of the material.

The Materials Table is now in alphabetical order.

Reviewers' comments:

Reviewer #1:

Manuscript Summary:

Polysome profiling by sucrose density gradients is a key method to analyze cellular ribosomes and their association with mRNAs and translation factors. Most commonly, to achieve high

quality and reproducibility, polysome profiling requires expensive automated gradient makers and fractionation systems. The manuscript of Sobhany and Stanley describes a simplified protocol aimed to obtain polysome profiles using sucrose density gradients without the need of the costly instrumentation. The approach is rather simple: the gradient is formed by layering five different stocks of sucrose solutions and, following ultracentrifugation, the gradients are manually fractionated by pipetting small aliquots from top to bottom to then analyze the obtained fractions. I agree with the authors that the approach could be easily implemented in any lab. In addition, the protocol is well-written and, as far as I can tell, it would be easily followed by any lab member with basic lab skills. However, I find critical issues that may discourage the readers to use the described approach.

Major Concerns:
1. Fractionation seems to be very time consuming. I'd estimate that withdrawing 200 µL aliquots of a ~10 mL gradient would take approximately 12-14 min (~15 sec to pipette the aliquot from the tube to the 96-well plate). This time would be for a single gradient and would be doubled (30 min!) if to take 100 µL aliquots. Readers need to be made aware of this point, moreover taking into account that labor is very expensive.

The reviewer is correct that manual fractionation can be time consuming, however with our experience manual fractionation takes roughly the same amount of time (or can actually be faster) than using a commercial fractionation system. We have considerable experience using the Brandel BR-188 Density Gradient Fractionation System. It takes five minutes and thirty seconds for the chase solution to entirely push a ~10mL gradient through the detector flow cell and into the fraction collector. After reversing the syringe pump, it takes another five minutes to remove the chase solution from the polypropylene tube and reset for the next tube. When you factor in placement and removal of the polypropylene tube into the tube piercing system, and replacing centrifuge tubes in the fraction collector, it takes roughly 15 minutes per gradient to generate a profile. For an experienced user it takes less than ten minutes to manually load 100 µL aliquots into 96-well plates.

2. Resolution is very low, moreover that of the polysome fractions. Besides a peak corresponding to disomes, no distinct peaks for groups of more than two ribosomes can be distinguished. The low resolution issue will make it really difficult to reach any solid conclusions about mRNAs or proteins associated with a particular group of translating ribosomes.

The reviewer is correct that the resolution of the peak is lower for manual versus automated systems, however the relative peak height is distinguishable between samples and can indicate differences in ribosomal profiles. This protocol is not meant to be a replacement for automated fractionation but rather an option for those who do not have access to automated instruments.

3. Reproducibility of the results seems low. It is worrisome that the representative profiles shown in the manuscript reveal inconsistencies. For example, even though more aliquots are collected in the profile shown in Figure 3C, the 60S peak is not defined. Also, the number of data points in the 80S peak is as few as three in panel B of the same figure and something like 10-12 in panel C. These inconsistencies reflect irreproducibility of the data.

While there is a significance difference in resolution of the chromatogram with manual versus automated fractionation in our experience, we have not had issues with data reproducibility with manual fractionation. There are seven data points for the 80S peak in panel B and fourteen in panel C. This is consistent with the aliquot volumes. Even in commercial fractionation systems, the 60S peak can be ill-defined.

An additional point is that authors could mention that a well-known alternative technique to fractionate sucrose gradients without the use of an expensive automated instrument is to simply carefully insert a needle/capillary tube to the bottom of the gradient tube, attach it to a peristaltic pump, properly adjust the flow and collect aliquots with either a fraction collector or manually. This approach is less labor intense and yields reproducible data with very acceptable resolution.

We thank the reviewer for this suggestion and have added this as a Note in the protocol.

Reviewer #2:

Manuscript Summary:

This manuscript introduces a new and easy method to profile polysome without commercial gradient makers. The method is simple and detailed in the manuscript. Therefore, for labs with limited resources, this manuscript will be helpful.

Major Concerns:

None.

We thank the reviewer for their support of our manuscript and valuable suggestions for refining the manuscript which are detailed below.

Minor Concerns:

(1) in the manuscript it states that overnight incubation is used to create linear gradient of sucrose. However, the authors didn't detail why overnight is sufficient, or how to judge whether a satisfied gradient has been built.

The reviewer is correct with this assessment. Additional details about length of incubation have been included. Also, we now suggest the use of a densitometer to assess the quality of a gradient.

(2) Just to confirm, Fig 3A is the results from a commercial fractionator, not the gradient maker.

The reviewer is correct. However, we realize that the Representative Results section of our manuscript lacked clarity on this issue, and therefore added several lines to eliminate any potential confusion.

(3) How the profiling results are compared with that from a commercial gradient maker? It will be better if the authors can provide or refer to some profiling results obtained from commercial gradient makers.

The reviewer is correct; comparison with a commercial gradient maker would make the manuscript better. We now refer to a paper that utilizes the Biocomp Gradient Maker.

Reviewer #3:

Manuscript Summary:

The manuscript by Sobhany and Stanley describe a method to generate polysome gradients without the need of expensive devices using only commonly available lab equipment. Although not novel, the beauty of this manuscript is the direct comparison between these 2 techniques (shown in figure 3) showing that for most of the common purposes (differentiate between monosomes and polysomes) the resolution obtained with this technique is enough. This also can be used as a way to perform preliminary experiments before deciding on the purchase of an expensive machine or the collaboration with other groups.

This report could also motivate the use of polysome profiles in laboratories that are not necessarily experts in the field and therefore expand the range of techniques and results produced by those labs.

I still have some criticisms/suggestions.

Major Concerns:

Please indicate in each possible point when the sample can be flash frozen and stored (during cell harvesting and sample preparation). This is a crucial piece of information in an experimental manuscript.

The reviewer is correct; this is crucial information and the text has been revised to include when samples and gradients can be flash frozen and stored.

Representative results: It is not clear if the profiles shown in figure 3 were generated using a gradient maker or the hand pouring/overnight incubation described in the manuscript. If not only the gradients harvesting but also their generation are different the authors should show the effect of each of these 2 steps play in the final gradient quality (for example by generating the gradients manually but harvest them with a automated fractionation system.

The reviewer is correct; we had not made this point clear. The manuscript has been revised to clearly state that all profiles in Figure 3 were created using gradients that had been prepared as described in the protocol.

Minor Concerns:

Line 36: a single mRNA molecule

This has been changed.

Labe 145: RNA, lysates or both could be frozen down?

This has been revised to “lysates”.

Lane 152-154: Not RNA but lysates are pipetted on top of the gradient

This has been corrected.

Lane 160: using which buffer?

This step of the protocol has been revised accordingly.

182: the word reach is missing: until you reach the bottom of the tube

"Reach" has been added.

Point 2.6: In our experience cooling the sample between shacking is better (by doing for example 5 rounds of 1min with 1min incubation on ice in between).

Thank you for this suggestion. We have not tested this variation of the protocol so we did not note this in the manuscript, but this is something to keep in mind for the future.

Reviewer #4:
Here the authors describe a layman's method of polysome fractionation. This reviewer appreciates the clarity of their presentation, along with an impressive figure showing the non-linear sucrose gradient using blue dyes. There is no concern except that this reviewer recommends to cite the latest work of high quality related to polysome profiling below.
Recommendation
This reviewer wants to highlight the recent work of polysome profiling (Chikashige et al., 2020) that is worth being cited here in the lines 38, 69 and 264.

Reference to be added
Chikashige, Y., Kato, H., Thornton, M., Pepper, W., Hilgers, M., Cecil, A., Asano, I., Yamada, H., Mori, C., Brunkow, C., et al. (2020). Gcn2 eIF2α kinase mediates combinatorial translational regulation through nucleotide motifs and uORFs in target mRNAs. Nucleic Acids Research 48, 8977-8992.

We thank the reviewer for their support of our manuscript. The recommended reference is now specifically described within the body of text in the Representative Results section and cited in the introduction and discussion as suggested.