RNA profiling from polysomes

This protocol was last updated - May 10, 2020 by Chris Hughes.

This protocol describes protocols for preparing polysomes in order to isolate RNA for downstream analysis. This protocol is designed to work with a cell pellet derived from a single 15-cm dish at approximately 75% confluency that would yield approximately 20 million cells. Scale based on the yield of your own cell line. This protocol can also be modified to be used for tissues (modify the lysis conditions to add some level of mechanical disruption of the tissue). I prefer to use a sucrose step gradient for this, but you could use a cushion if you prefer.

Quick links

1. Sucrose gradient preparation

Try to maintain RNase-free conditions for all aspects of this protocol.

1.1 Reagents and materials

- Ultra-clear tubes, 11x60mm, 4mL (Beckman, CAT#344062)
- 15mL tubes (VWR, CAT#89093-186)
- BD Luer-lock syringes, 5mL (VWR, CAT#B309646)
- Blunt ended needles, 20-gauge, 15cm (VWR, CAT#20068-676)
- Nalgene Rapid-Flow filter units, 500mL, 0.2um filter (VWR, CAT#73520-984)
- RNaseZAP wipes (Thermo Fisher, CAT#AM9788)
- 1M HEPES pH 7.3 (Thermo Fisher, CAT#BP299-1)
- MgCl2 (Sigma, CAT#M8266)

- KCl (Sigma, CAT#P9333)
- Sucrose (Sigma, CAT#84097-1KG)
- RNA clean water (Thermo Fisher, CAT#10977023)
- Accessories from a Biocomp Gradient Station (BioComp Instruments)

1.2 Solution recipes

- Polysome base buffer (10X)
  - 200mM HEPES pH 7.3 (100mL of 1M stock)
  - 1.5M KCl (56.0g)
  - 150mM MgCl2 (7.1g)
  - water to 500mL
  - filter and store at +4C
- Sucrose solution (20%)
  - sucrose (10g)
  - 1X polysome base buffer (5mL of 10X stock)
  - water to 50mL
  - filter and store at +4C
- Sucrose solution (50%)
  - sucrose (25g)
  - 1X polysome base buffer (5mL of 10X stock)
  - water to 50mL
  - filter and store at +4C
- RNaseZAP cleaning solution
  - 10% RNaseZAP (10mL of stock)
  - water to 50mL

1.3 Protocol

Before proceeding, allow the sucrose solutions to equilibrate to room temperature. I recommend making the sucrose step gradients a couple of hours before you plan to use them, or the day before. Clean your working area with RNaseZAP.

1. Place a tube in the jig provided with the BioComp Gradient Maker instrument.
2. Using a black marker, make a mark at lower level of the notched jig. Repeat for the same number of tubes as you have samples to process.
3. Attach a blunt ended needle to a 10mL plastic syringe and draw RNaseZAP cleaning solution into it, and then expel the liquid to waste.
4. Repeat **Step 3** one further time for a total of 2 rinses.
5. Repeat **Step 3** two times but instead rinsing with clean water.
6. Draw 4mL of 20% sucrose into the syringe and add it to the bottom of an ultra-clear sucrose gradient tube until you reach the level of the mark made on the tube previously. Discard any leftover the waste.
7. Draw 4mL of 50% sucrose into the syringe and add it to the bottom of the same ultra-clear tube until you reach the level of the mark (see **Note 1**). Discard any leftover to waste.

8. Rinse the syringe twice with water, followed by two times with RNaseZAP cleaning solution. Expel all liquid from the needle and store for autoclaving at a later date.
9. Place a short tube cap on top of the prepared gradient tube and place in an upright position at +4C until use (see **Note 2**).

2. Cell culture and harvest

Use whatever cell culture methods you are compfortable with here. This stage of the protocol is only given as an example of a process that is validated to work and is by no means the only way to achieve a successful result in this section.

2.1 Reagents and materials

- 15mL tubes (VWR, CAT#89093-186) - need 1 per sample
- 50mL tubes (VWR, CAT#89093-190) - need 3 in total
- Centrifuge with 15mL tube buckets (multiple vendors)
- TrypLE express (Thermo Fisher, CAT#12605028)
- DPBS, no calcium or magnesium (Thermo Fisher, CAT#14190144)
- Dry ice

2.2 Solution recipes

- Cell culture medium (provided only as an example)
  - DMEM (500mL)
  - FBS (50mL)

2.3 Protocol

This protocol assumes you have your cells growing in 15cm dishes. For other dish sizes, simply scale the reagents for your purpose.

1. Label 1x15mL tube for each sample you plan to harvest.
2. Label a 50mL tube with 'TE', another with 'Media', and a last with 'PBS'.
3. To the 'TE' 50mL tube, add 8mL of TrypLE express per sample you plan to harvest.
4. To the 'PBS' 50mL tube, add 5mL of DPBS per sample you plan to harvest.
5. Remove the cell plate from the incubator and ipette the media the cells are growing in into the 50mL tube labeled 'Media'.
6. Add 8mL of TrypLE express to the plate and place back into the incubator (see **Note 3**).
7. Once the cells have started to detach, remove the plate from the incubator.
8. With 4mL of media from the 'Media' tube, rinse and collect the detached cells and transfer to the appropriate 15mL tube.
9. Centrifuge the tube with the cells for 3-minutes at 250g.
10. Dump the supernatant to waste (I use an old media bottle for this).

11. Add 5mL of DPBS from the 'PBS' tube to the tube with the cell pellet. There is no need to mix here.
12. Centrifuge the tube for 30-seconds at 250g.
13. Dump the supernatant to waste and spirate the remaining liquid to leave as little as possible.
14. Place the cell pellet on dry ice until frozen and keep at -80C until use.

3. Cell lysis and ultracentrifugation

Use only RNase-free reagents.

3.1 Reagents and materials

- 1.5mL Safe-Lock tubes (Thermo Fisher, CAT#05-402-7)
- Benchtop centrifuge with holder for microcentrifuge tubes (multiple vendors)
- SW60Ti rotor (Beckman) - other rotors can be substituted here.
- Ultracentrifuge compatible with the SW60Ti rotor (Beckman)
- Qubit RNA HS Assay Kit, 100 assays (Thermo Fisher, CAT#Q32852)
- Qubit Assay Tubes, 500 tubes (Thermo Fisher, CAT#Q32856)
- RNaseZAP wipes (Thermo Fisher, CAT#AM9788)
- TURBO DNase, 2U/uL (Thermo Fisher, CAT#AM2238)
- SUPERaseIN RNase inhibitor, 20U/uL (Thermo Fisher, CAT#AM2694)
- Cycloheximide (Sigma, CAT#C7698)
- 1M HEPES pH 7.3 (Thermo Fisher, CAT#BP299-1)
- MgCl2 (Sigma, CAT#M8266)
- KCl (Sigma, CAT#P9333)
- NP-40 (Sigma, CAT#NP40)
- Deoxycholate (DOC) (Sigma, CAT#D6750)
- cOmplete protease inhibitor tablets, EDTA free (Sigma, CAT#11836170001)
- Dimethylsulfoxide (Sigma, CAT#D8418)
- RNA clean water (Thermo Fisher, CAT#10977023)

3.2 Solution recipes

- 4M KCl - 14.9g in 50mL of water
- 0.5M MgCl2 - 5.08g in 50mL of water
- 10% (v/v) NP-40 - 1mL in 9mL of water
- 10% (w/v) DOC - 1g in 10mL of water
- 10X cOmplete protease inhibitor stock - 1 tablet in 1mL of water
- 20mg/mL cycloheximide - 10mg in 500uL of DMSO
- Lysis buffer (need 1mL per sample)
  - 20mM HEPES pH 7.3 (20uL of 1M stock)
  - 150mM KCl (37uL of 4M stock)
  - 5mM MgCl2 (10uL of 0.5M stock)
  - 10% (w/v) sucrose (1g powder)

- 0.5% (v/v) NP-40 (50uL of 10% stock)
- 0.5% (v/v) DOC (50uL of 10% stock)
- 0.5X cOmplete protease inhibitor (50uL of 10X stock)
- 20U/mL TURBO DNase (10uL of 2U/uL stock)
- 50U/mL SUPERaseIn (2.5uL of 20U/uL stock)
- 100ug/mL cycloheximide (5uL of 20mg/mL stock)
- water to 1mL (766uL of pre-chilled water)

3.3 Protocol

Before starting the lysis protocol, turn on the ultracentrifuge and allow it to chill to +4C. In addition, turn on a benchtop centrifuge and pre-chill it to +4C. Prepare sufficient 1.5mL tubes for your samples (you need 1 per sample) and chill them on ice. Prepare sufficient Lysis buffer for your samples and allow it to cool to +4C.

1. Remove the frozen cell pellet from the freezer and allow to partially thaw at room temperature.
2. Once the pellet is partially, but not completely thawed, add 700uL of lysis buffer and pipette mix.
3. Incubate the tube for 5-minutes on ice with periodic pipetting with a 200uL pipette.
4. Centrifuge the lysate at 5,000g for 5-minutes in the chilled benchtop centrifuge.
5. Recover the supernatant fraction to a fresh tube. Freeze the remaining pellet (nuclei) for later use if desired.
6. Measure RNA concentration using a Qubit RNA HS assay.
    1. Dilute 2uL of the supernatant fraction with 98uL of water in a fresh 1.5mL tube.
    2. Add 20uL of this dilution to a Qubit assay tube.
    3. Add 1.5uL of Qubit dye to 200uL of the Qubit dilution buffer.
    4. Add 180uL of the prepared dye/buffer mix to the Qubit tube with the 20uL of diluted material.
    5. Measure on the Qubit instrument and record the concentration.
7. Place the empty buckets for the centrifuge rotor on ice and one by one, remove the gradient caps and load the gradients into the buckets (see **Note 4**).
8. Remove 400uL of the sucrose from the gradient tube and add 600uL of sample to the top of the gradient (see **Note 5**).
9. Secure the bucket lids on the buckets.
10. Transfer the loaded buckets to the SW60 rotor and load into the centrifuge (if you don't know how to do this properly, ask someone!).
11. Spin the gradients at 35,000rpm for 2-hours (+4C temperature).

4. Polysome fractionation and RNA isolation

This stage of the protocol is based on the use of a BioComp Fractionation Station, but you can use whatever device you have available. Use only RNase-free reagents.

4.1 Reagents and materials

- 1.5mL Safe-Lock tubes (Thermo Fisher, CAT#05-402-7)
- RNaseZAP wipes (Thermo Fisher, CAT#AM9788)
- RNA clean water (Thermo Fisher, CAT#10977023)
- BioComp Fractionation Station (BioComp Instruments)
- RNA Clean and Concentrator-5 kit, 200 preps (Cedarlane, CAT#R1016)

4.2 Solution recipes

- No solutions are required for this stage of the protocol.

4.3 Protocol

Prepare sufficient 1.5mL tubes for your samples (you need 2 per sample) and chill them on ice.

1. Turn on the BioComp instrument (switch on the back left when facing the instrument).
2. Press the button corresponding to the 'SCAN' option on the instrument.
3. Turn on the fraction collector (switch on left side when facing the instrument). Disconnect the green connector on the back of the fraction collector that is used to trigger the unit, we will do this manually.
4. Turn on the laptop (password is 'Science').
5. Open the 'Triax' software on the computer. Ignore the warmup error.
6. Choose your username and the appropriate rotor.
7. Click the 'Scan Setup' button.
8. On the GST instrument, use the 'Rinse' button to flush the liquid in the syringe reservoir on the right side of the instrument (do not let it go dry).
9. Fill the reservoir with 30mL of clean water and flush using the 'Rinse' button.
10. Fill with a further 50mL of water.
11. Back on the laptop, hit the 'Graph' button.
12. Name your sample in the prompt that appears.
13. Prompts will appear asking you about blanking the instrument. Hold the 'Rinse' button on the GST instrument and while holding 'Rinse', hit 'Ok' on the laptop to start the blanking procedure. It will notify you when it is complete.
14. Apply some gasket grease to the plunger on the GST instrument.
15. Insert your first gradient tube (or a tube with just water to run as a blank) into the gradient tube holder on the GST instrument.

16. Unscrew the gold screw on top of the plunger unit and hold the 'Air' button to flush liquid from the system (hold for about 10 seconds). Re-screw the gold screw until it is finger tight. Do not overtighten.
17. Ensure you have collection tubes in place. I like to place 4 tubes in the sample rack: two that will be waste, one for my sample, and an extra just in case.
18. On the laptop, click 'Start scan'. Prompts will appear reminding you of everything you should do prior to running a sample, so it is a good idea to double check you have done these things.
19. Hit 'Ok' to run your sample. The plunger should move down until it reaches your gradient and pause for a second before proceeding. The UV trace should appear on the laptop as the plunger proceeds through the gradient.
20. Hit 'Start' on the fraction collector. It should move to the first tube.
21. Monitor the elution. The polysome peak is going to be around the interface of the 20% and 50% sucrose, so about halfway in.
22. When you start to observe the polysome peak is eluting (green line on the screen), hit ADV again to move the fraction collector to your sample tube.
23. Once the polysome peak has eluted, hit ADV again to move off of your sample tube. You can now hit END on the fraction collector to move it back to waste.
24. Remove your sample tube and place it on ice.
25. After the gradient has completed, the plunger will move back to the top position. Remove and discard the dispensed gradient tube.
26. Press 'Ok' on the laptop when it asks about using air to push out the last part of the gradient.
27. Press 'End' on the fraction collector unit.
28. Press 'Rinse' on the GST instrument to flush out the lines (10 seconds or so).
29. Unscrew the gold screw and flush with air, as before. Re-screw the gold screw when finished.
30. You are now ready to run your next sample, or clean up.
31. To run your next sample:
    1. Hit 'New sample' on the laptop. It will ask if you want another identical sample, say yes. It will ask if you want to blank again, say no. Name your sample.
    2. Insert your gradient tube into the holder, apply grease to the plunger, refresh your collection tubes, and proceed just as above.
32. For clean-up:
    1. Flush the system using the 'Rinse' button for at least 30mL of water.
    2. After the water, flush with air.
    3. After the air, flush with 20mL of 70% ethanol.
    4. Add 30mL of 20% ethanol to the syringe reservoir and flush 10mL through the GST instrument.
    5. Leave the instrument sitting in 20% ethanol for long-term storage.
33. You can now freeze your sample at -80C until you are ready to process it

further, or proceed to RNA extraction (see **Note 6**):

1. The polysome sample volume is generally around 350uL. Add 2 volumes of RNA Binding Buffer (e.g. 700uL) to the polysome sample and pipette mix.
2. Add an equal volume of ethanol (e.g. 1050uL) and pipette mix.
3. Transfer 750uL of the sample to the Zymo-Spin column in a collection tube and centrifuge for 30 seconds.
4. Discard the flow-through and reload the column with another 750uL of sample and spin.
5. Repeat the sample loading and spins until all of the sample has been passed through the filter.
6. Add 400uL of RNA Prep Buffer to the column and spin for 30 seconds. Discard the flow-through.
7. Add 700uL of RNA Wash Buffer to the column and spin for 30 seconds. Discard the flow-through.
8. Add 400uL of RNA Wash Buffer to the column and spin for 2 minutes. Discard the flow-through.
9. Transfer the filter to a fresh 1.5mL elution tube and add 15uL water to the filter, incubate for 1 minute, then spin for 30 seconds.

Notes

Note 1 - When you draw the heavy solution into the syringe, be careful not to introduce bubbles by trying to go too quickly, take your time. After you have the liquid in the syringe, allow it to sit in the solution for a couple of seconds to release any negative pressure that may introduce air bubbles. When you transfer the syringe to the gradient tube, be deliberate. Transfer it directly to the bottom of the gradient tube and start expelling the liquid slowly. Again, take your time.

Note 2 - When you put the lids on, take note of the hole in the lid. Place the cap into the gradient tube on an angle with the hole side pointed up, and gradually seal the tube with the cap. Liquid that gets expelled should go through that hole in the cap and end up inside the cap itself. If liquid gets on the side of the tube, wipe it off immediately to avoid the sucrose drying out and make the side of the tube sticky.

Note 3 - Depending on your cell line, the time you leave it in here will vary. For example, for HEK293 cells I will only leave it for 1 - 2 minutes, but for U2OS I would leave it for longer.

Note 4 - To remove the caps, simply do the reverse of what you did to place them originally. Peel them back on an angle, removing the side with the hole first and working away from this. Take your time to avoid disturbing the gradient.

Note 5 - Pipette the sample onto the side of the gradient tube to avoid disrupting

the gradient. Take your time.

Note 6 - You can use whichever RNA extraction method here that you would like. Other methods, like Trizol, phenol-chloroform, or another kit should work equally well. We use the Zymogen kit because it is fast and offers great flexibility in elution volume.