RNA profiling from polysomes

This document 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 (depending entirely on your cell line). 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).

Quick links

1. 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.

1.1 Reagents and materials

- 15mL tubes (VWR, CAT#89093-186)
- 50mL tubes (VWR, CAT#89093-190)
- Centrifuge with 15mL tube buckets (multiple vendors)
- TrypLE express (Thermo Fisher, CAT#12605028)

- DPBS, no calcium or magnesium (Thermo Fisher, CAT#14190144)
- Cycloheximide (Sigma, CAT#C7698)
- Dry ice

1.2 Solution recipes

- Cell culture medium (provided only as an example)
  - DMEM (500mL)
  - FBS (50mL)
- 100mg/mL cycloheximide - 50mg in 500uL of DMSO

1.3 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' and another with 'Media'.
3. To the 'TE' 50mL tube, add 8mL of TrypLE express per sample you plan to harvest.
4. Remove the cell plate from the incubator and pipette 20uL of cycloheximide into the media in the dish (or a volume equivalent to what is present in the dish already, e.g. 15mL of media, add 15uL of cycloheximide stock).
5. Place the plate back in the incubator and leave it for 10-minutes.
6. Remove the cycloheximide treated plate from the incubator and remove the culture medium to the 50mL tube labeled 'Media'.
7. Add 8mL of TrypLE express to the plate and place back into the incubator (see **Note 1**).
8. Once the cells have started to detach, remove the plate from the incubator.
9. With 4mL of media from the 'Media' tube, rinse and collect the detached cells and transfer to the appropriate 15mL tube.
10. Centrifuge the tube with the cells for 3-minutes at 250g.
11. Dump the supernatant to waste (I use an old media bottle for this).
12. Add 5mL of DPBS to the tube with the cell pellet. There is no need to mix here.
13. Centrifuge the tube for 30-seconds at 250g.
14. Dump the supernatant to waste and spirate the remaining liquid to leave as little as possible.
15. Place the cell pellet on dry ice until frozen and keep at -80C until use.

2. Sucrose gradient preparation

Use only RNase-free reagents.

2.1 Reagents and materials

- 50mL tubes (VWR, CAT#89093-190)

- Ultra-clear tubes, 11x60mm, 4mL (Beckman, CAT#344062)
- 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 (Thermo Scientific, CAT#15503022)
- RNA clean water (Thermo Fisher, CAT#10977023)
- BioComp Gradient Station (BioComp)

2.2 Solution recipes

- 4M KCl - 14.9g in 50mL of water
- 0.5M MgCl2 - 5.08g in 50mL of water
- 11% (w/v) sucrose - 55g in 500mL of water (filter and keep at +4C)
- 50% (w/v) sucrose - 250g in 500mL of water (filter and keep at +4C)
- 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
- Light sucrose solution (need 5mL per gradient)
  - 10% sucrose (9mL of 11% stock)
  - 1X polysome base buffer (1mL of 10X stock)
  - water to 10mL (0mL required)
- Heavy sucrose solution (need 5mL per gradient)
  - 45% sucrose (8.2mL of 50% stock)
  - 1X polysome base buffer (1mL of 10X stock)
  - water to 10mL (800uL required)

2.3 Protocol

Make sure you have read and understood the protocol below before proceeding. The gradients are the most important part of this protocol, so take your time here. Practice makes perfect and sucrose is cheap, so practice your technique in a waste tube before hand if you want. I like to make my gradients the day before I plan to run samples.

1. Prepare the light and heavy sucrose solutions according to the recipe's above and allow the solutions to equilibrate to room temperature for 30-minutes or so.
2. Prep your workspace by cleaning it with an RNaseZap wipe.

3. Prepare a 50mL tube containing RNA clean water, and another containing a solution of 10% RNaseZap.
4. Place an ultracentrifuge tube in the appropriate jig and mark the halfway point using a marker (see **Note 2**).
5. Attach a blunt needle to a syringe and draw 3mL of the 10% RNaseZap solution into the syringe and expel back into the same tube. Repeat this process 2-times for a total of 3 rinses.
6. Draw 3mL of RNA clean water into the syringe and expel to waste. Repeat this process 2-times for a total of 3 rinses.
7. Draw a small amount (less than 1mL) of light sucrose solution into the syringe and expel to waste.
8. Draw 3mL of light sucrose solution into the syringe and use it to fill the ultracentrifuge tube to just above the half way point (~1-2mm above line) (see **Note 3**). Repeat this process for all ultracentrifuge tubes.
9. Draw a small amount (less than 1mL) of heavy sucrose solution into the syringe and expel to waste.
10. Draw 3mL of heavy sucrose solution into the syringe and use it to fill, from the bottom and up to the half way point, the ultracentrifuge tubes containing light sucrose solution keeping the needle below the level of the light solution (see **Note 4**). Once the heavy solution is at the half way mark, slowly draw the needle above the heavy layer and then quickly remove it from the tube. Practice makes perfect here.
11. Repeat this process for all ultracentrifuge tubes.
12. Take a short or long tube cap (provided with the BioComp unit) and hold it with the hole in the cap facing your person. Hold the ultracentrifuge tube you plan to cap with your other hand along with a KimWipe. With the cap at a 45 degree angle, insert the cap into the tube such that any liquid that is expelled from the tube enters through the hold in the cap and goes into the top of the cap.
13. Pipette any extra sucrose solution that has expelled into the cap to waste and use the KimWipe in your hand to clean the sides of the tube, if necessary. Repeat this capping process for all tubes.
14. Turn on the BioComp Gradient Station. Press 'GMST' on the unit.
15. Place the bubble level on top of the tube holder and use the 'UP', 'DOWN' commands on the BioComp unit to level the tube holder. Press 'DONE' when finished and return the bubble level to the drawer.
16. Press 'GRAD' and then 'LIST' on the BioComp unit.
17. Choose your rotor, in this case we are using the SW60Ti.
18. Use the 'UP' or 'DN' keys to select the gradient appropriate for your preparation. In this case, we want a Short Sucr 10 - 45% setting. Press 'USE' when finished.
19. Load your gradient tubes in the tube holder and press 'RUN'.
20. Once the gradient cycle is finished, place the tubes in a sample rack in the +4C fridge overnight. Turn off the BioComp unit.

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) (see **Note 5**)
- 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)
- RLT buffer (Qiagen, CAT#79216)
- Qiagen RNeasy Mini Kit (Qiagen, CAT#74104)
- 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
- 50% (w/v) sucrose - 5g in 10mL of water (filter and keep at +4C)
- 10X cOmplete protease inhibitor stock - 1 tablet in 1mL of water
- 100mg/mL cycloheximide - 50mg 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 (200uL of 50% stock)
  - 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)
  - 100U/mL SUPERaseIn (5uL of 20U/uL stock)
  - 200ug/mL cycloheximide (5uL of 100mg/mL stock)

- water to 1mL (470uL of pre-chilled water)

3.3 Protocol

Before starting the lysis protocol, read and understand this process. If you do not know how to properly load the operate the centrifuge, please ask! Turn on the ultracentrifuge and allow it to chill to +4C (see **Note 6**). In addition, turn on a benchtop centrifuge and pre-chill it to +4C. Prepare sufficient 1.5mL tubes for your samples (you need 3 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 thawed, add 250uL of lysis buffer and pipette mix (see **Note 7**).
3. Incubate the tube for 5-minutes on ice with periodic pipetting with a 200uL pipette.
4. Transfer 50uL of the lysis mixture to a fresh 1.5mL tube. This is your total RNA sample. Add 350uL of RLT buffer and extract total RNA using the Qiagen RNeasy Kit following the vendor instructions.
5. Centrifuge the lysate at 5,000g for 5-minutes in the chilled benchtop centrifuge.
6. Recover the supernatant fraction to a fresh tube. Freeze the remaining pellet (nuclei) for later use if desired.
7. Optional: 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 300uL 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.
8. Place the ultracentrifuge buckets in an ice bucket in a vertical position (ideally in the cold room).
9. One at a time, remove the caps from the prepared gradients and place them in the ultracentrifuge buckets (removal is the opposite of insertion, so remove the side with the hole first and work your way backwards) (see **Note 8**).
10. Load 200uL of your recovered lysate on to the top of the gradient (see **Note 9**).
11. Taking care, partially secure the bucket lids to the buckets now containing loaded gradients.
12. Carefully transfer the ice bucket with the gradients to the ultracentrifuge area along with the rotor. One by one and keeping them vertical throughout, pick up the buckets, secure the bucket lids (fingertight, tightening at the base of the lid), and load into the rotor. Repeat for all tubes and ensure

the rotor buckets are loaded in their correct locations and properly.
13. Press 'Open Vent' on the ultracentrifuge. Once the vacuum has released, it will make a chime sound to indicate the lid can be opened.
14. Load the rotor into the unit taking care to keep it vertical (you don't want your gradients to shift).
15. Close the lid and press 'Start Vacuum'.
16. Press 'Start' to begin the spin. Stand and watch to make sure it hits the appropriate speed and that everything is OK.
17. Clean the ultracentrifuge tube caps with water and place back with the BioComp unit.

4. Sucrose gradient fractionation

Use only RNase-free reagents.

4.1 Reagents and materials

- 96-well plate (any 96-well plate with large wells is suitable, not a PCR plate)
- RNA clean water (Thermo Fisher, CAT#10977023)
- Absolute ethanol (multiple vendors)
- Ultra-clear tubes, 11x60mm, 4mL (Beckman, CAT#344062)
- BioComp Gradient Station (BioComp)

4.2 Solution recipes

- 70% ethanol (70mL ethanol + 30mL water)
- 20% ethanol (20mL ethanol + 80mL water)

4.3 Protocol

1. When the ultracentrifuge is approximately 30-minutes from finished, turn on the laptop attached to the BioComp unit and load into Windows. Plug in the USB for the BioComp unit to the laptop.
2. Turn on the BioComp unit and the Gilson fraction collector attached to it.
3. On the BioComp unit, press 'SCAN'.
4. Open the Triax software on the laptop (shortcut is on the desktop). It will give an error about a warmup object, just ignore this and hit OK.
5. Choose your username and the appropriate rotor (SW60Ti in this case) and hit OK.
6. In the page that appears, enter your gradient settings in the boxes at the bottom. Press the 'Goto Scan Setup' button.
7. On this page you can specify how many fractions you would like. Always choose a number beyond what you actually want. For example, for 48 fractions across an entire polysome profile I will choose 64 fractions in the software. The other settings will auto-update from this setting.
8. Press the 'Goto Graph' button.

9. A prompt will appear asking you to name your sample, so do so.
10. A prompt will appear asking you to prepare for calibration. On the BioComp unit, press and hold the 'Rinse' key until the liquid from the syringe reservoir on the right side of the unit is almost entirely drained.
11. Fill the reservoir with 20mL of RNA clean water and hold the 'Rinse' key again until it is almost drained.
12. Fill the reservoir with 50mL of RNA clean water.
13. This time, while pressing and holding the 'Rinse' key, press OK in the software to do calibration and then OK to start the blanking process. Continue to hold the 'Rinse' key throughout the whole process until it says that blanking is complete.
14. While holding the 'Rinse' key, open the gold screw on the top of the BioComp unit until it is completely open. Release the 'Rinse' key.
15. Press the 'Air' key on the BioComp unit for 10-seconds and then release it and close the gold screw.
16. Place a sample collection plate in the fraction collector.
17. Fill a blank ultracentrifuge tube with RNA clean water and place it in the BioComp gradient holder (if you are unsure how to use this, ask).
18. Apply a small amount of grease to the silicon trumpet on the BioComp unit.
19. In the software, press 'Start Run'. Hit 'OK' on the two prompts that follow. The run should start.
20. Once the run is finished, hit 'OK' on the prompt that appears asking about using the air button to expel the remaining material.
21. Hit 'End' on the Gilson Fraction Collector.
22. Remove your fraction plate and replace it with a fresh plate.
23. Press and hold the 'Rinse' key to rinse the tubing and open the gold screw while doing this.
24. Press and hold the 'Air' key for 10-seconds and then release it and close the gold screw.
25. Apply a small amount of grease to the silicon trumpet on the BioComp unit.
26. Press 'New Run' in the software.
27. Press 'Yes' for identical sample, 'No' for doing another blank calibration. Name your sample.
28. When the ultracentrifuge spin has finished, press the 'Open Vent' key and wait for the chime signaling that the door can be opened.
29. Remove the rotor from the unit, close the lid, and power off the machine.
30. Place the ultracentrifuge buckets with your gradients on ice.
31. Using tweezers, remove a sucrose gradient from a bucket and load it into the gradient holder on the BioComp.
32. Hit 'Start Run' in the software and 'OK' on the two prompts that follow.
33. Once the sample is finished, repeat steps 20 - 27 for any remaining samples.
34. When you are finished, repeat steps 20 - 27 with a blank tube of water (the same tube from the previous blank can be re-used).
35. Wipe the silicon trumpet clean with a KimWipe, as well as the BioComp

unit itself if any sucrose has spilled.
36. Clean the gradient holder and the ultracentrifuge buckets with water.
37. On the BioComp unit, press and hold the 'Rinse' button to flush the remaining water from the reservoir while also opening the gold screw.
38. Press and hold the 'Air' key for 10-seconds then release and close the gold screw.
39. Add 20mL of 70% ethanol to the reservoir and use the 'Rinse' key to flush it through the BioComp unit.
40. Add 30mL of 20% ethanol to the reservoir and use the 'Rinse' key to flush 10mL of it through the BioComp unit. There should now be 20mL remaining for storage.
41. Turn the BioComp unit and Gilson fraction collector off. Turn off the laptop and unplug the USB.
42. Your fractions can be frozen for later use or extracted immediately as described in the next section.

5. Polysome RNA extraction

This stage of the protocol is based on extraction of RNA from the fractions. You can use other extraction protocols if you would like, this is just the one I typically use.

5.1 Reagents and materials

- 1.5mL Safe-Lock tubes (Thermo Scientific, CAT#05-402-25)
- Benchtop centrifuge with holder for microcentrifuge tubes (multiple vendors)
- RNaseZAP wipes (Thermo Fisher, CAT#AM9788)
- RNA clean water (Thermo Fisher, CAT#10977023)
- RNA Clean and Concentrator-5 kit, 200 preps (Cedarlane, CAT#R1016) (see **Note 10**)
- Absolute ethanol (multiple vendors)

5.2 Solution recipes

- None

5.3 Protocol

1. Thaw your polysome fractions prepared above and add 2 volumes of RNA Binding Buffer (e.g. 100uL) to each sample and pipette mix.
2. Add 4X the original sample volume of ethanol (e.g. 200uL) to each sample and pipette mix.
3. Transfer up to 750uL of the sample to the Zymo-Spin column in a collection tube and centrifuge for 30-seconds.
4. Repeat the sample loading and spins until all of the sample has been passed through the filter.

5. Add 400uL of RNA Prep Buffer to the column and spin for 30-seconds. Discard the flow-through.
6. Add 700uL of RNA Wash Buffer to the column and spin for 30-seconds. Discard the flow-through.
7. Add 400uL of RNA Wash Buffer to the column and spin for 2-minutes. Discard the flow-through.
8. 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.
9. The extracted RNA samples can be frozen at -80C and used for qPCR or sequencing.

Notes

Note 1 - 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 2 - The jig is provided by Biocomp and should be stored with the machine. There may be multiple jigs available, so use the one appropriate for your tube size (this will be clear as only one of them will actually fit your tube). On the jig there are two levels where you can mark the half way point. If you are using the 'short' lids, mark the tube on the upper level of the jig. If you are using the 'long' lids, mark the tube on the lower level of the jig. The short lids allow you to load around 200uL of sample and the long around 400uL.

Note 3 - Take your time when drawing sucrose liquid into the syringe. The point here is to minimize any potential bubbles or imperfections in the gradient, so draw and expel the liquid slowly. It is OK to go above the half way mark with the light sucrose solution, just be consistent in what you do - what you do for one gradient, do the same thing for all.

Note 4 - As with the light solution, take your time here. The heavy solution is very viscous and can be difficult to draw into the syringe. Once you have drawn in the 3mL for a tube, allow the needle to sit in the heavy liquid for a few seconds just in case there is any remaining vacuum, you don't want air to get drawn into the syringe. Once you are ready, move the syringe into the ultracentrifuge tube with the needle at the bottom of the tube. Move with purpose. Don't fumble around trying to put the lid back on the tube or anything like that, go from one tube immediately to the other. Expel the heavy liquid slowly underneath the light, you want to minimize mixing between the layers. Do not fill beyond the half way marker with the heavy solution. Be consistent with the level you fill to across all of your tubes.

Note 5 - The tubes and rotor you are going to use here is going to depend on what you have access to, and your quantity of material. The two options we have are the SW60Ti rotor along with the 4mL ultraclear tubes, or the SW41Ti rotor with the 13.2mL tubes. If you have a big volume, the SW41Ti rotor is the better choice because it can hold more volume. But, it also results in you having

much larger volumes for your fractions in the end. With the smaller 4mL tubes, you will sacrifice some loading capacity and gradient resolution, but will have your fractions in a much smaller volume.

Note 6 - To turn on the ultracentrifuge, the power switch is on the right side of the unit. Once it has booted up, press the speed box and then select rotor. In this case, we are using the SW60Ti and the 4mL tubes. Set the speed to 36,000rpm. Now select the time box and set it to 2-hours. Now select the temperature box and set it to +4C. In the bottom left, hit the button that says 'Open Vent'. It should now say 'Start Vacuum'. If it does, hit it again to turn on the vacuum and start the chilling process.

Note 7 - Scale the amont of lysis buffer based on the size of your cell pellet. Generally, I will use a volume that I estimate is 2X the size of the pellet (e.g. 200uL of lysis buffer for a 100uL pellet).

Note 8 - The tubes should slide easily into the ultracentrifuge buckets. If they do not, there is likely sucrose that has dried on the side, so try to clean it up. The buckets are balanced across from one another (e.g. bucket 1 is balanced with bucket 4), so pay attention to numbers when loading your samples.

Note 9 - Be careful when loading the samples, you do not want to disturb your gradient. It is best to pipette the sample out slowly onto the side of the gradient tubeso that the sample runs down the side and settles gently on the top of the gradient. Only go to the first stop on the pipette and do not try to expel the last little bit as it will only introduce bubbles.

Note 10 - 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.