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) - 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

1.2 Solution recipes

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

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', 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 pipette 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 1**).
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.

2. Cell lysis and ultracentrifugation

Use only RNase-free reagents.

2.1 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 2**)
- Ultra-clear tubes, 11x60mm, 4mL (Beckman, CAT#344062)
- TLA100 rotor (Beckman) (see **Note 2**)
- Open-Top Thickwall Polypropylene Tube, 7 x 20mm (Beckman, CAT#343621)
- Ultracentrifuge compatible with your chosen rotor (Beckman)
- 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)
- 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)
- Sucrose (Thermo Scientific, CAT#15503022)
- RNA clean water (Thermo Fisher, CAT#10977023)

2.2 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
- 20mg/mL cycloheximide - 10mg in 500uL of DMSO
- 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 (30%)
  - sucrose (15g)
  - 1X polysome base buffer (5mL of 10X stock)
  - water to 50mL
  - filter and store at +4C
- 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)
  - 50U/mL SUPERaseIn (2.5uL of 20U/uL stock)
  - 100ug/mL cycloheximide (5uL of 20mg/mL stock)

- water to 1mL (466uL of pre-chilled water)

2.3 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 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 partially, but not completely thawed, add 250uL of lysis buffer and pipette mix (see **Note 3**).
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. Freeze at -80C until RNA extraction is performed in the next section.
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 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.
8. Place your ultracentrifuge tubes in a steady rack in preparation for loading (ideally in the cold room).
9. Using the luer lock syringe and blunt needle (you could also use a pipette for accuracy), add 30% sucrose solution to the tubes for each sample (see **Note 4**).
10. Load the sample on top of the 30% cushion (see **Note 5**).
11. Transfer the loaded buckets to the rotor and load into the centrifuge and spin (+4C temperature) (see **Note 6**). If you do not know how to properly load the operate the centrifuge, please ask!

3. Polysome RNA and protein isolation

This stage of the protocol is based on extraction of both RNA and proteins from the samples. You can just extract one or the other if desired. Use only RNase-free reagents.

3.1 Reagents and materials

- 1.5mL or 2.0mL Safe-Lock tubes (Thermo Scientific, CAT#05-402-25 or CAT#05-402-7)
- Benchtop centrifuge with holder for microcentrifuge tubes (multiple vendors)
- ThermoMixer with a 2mL block (Eppendorf)
- RNaseZAP wipes (Thermo Fisher, CAT#AM9788)
- 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)
- Dithiothreitol (Bio-Rad, CAT#1610611)
- 20% SDS (Thermo Scientific, CAT#BP1311)
- RLT buffer (Qiagen, CAT#79216)
- RNA clean water (Thermo Fisher, CAT#10977023)
- RNA Clean and Concentrator-5 kit, 200 preps (Cedarlane, CAT#R1016) (see **Note 7**)

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
- Pellet solubilization solution (need 100uL per tube)
  - 100mM HEPES pH 7.3 (100uL of 1M stock)
  - 0.5% (v/v) NP-40 (50uL of 10% stock)
  - 0.5% (v/v) DOC (50uL of 10% stock)
  - water to 1mL (800uL)
- 0.2M of dithiothreitol (DTT) - 15mg in 500uL of water, prepare fresh and keep on ice
- 2X Protein solubilization solution (need 50uL per tube)
  - 100mM HEPES pH 7.3 (200uL of 1M stock)
  - 2% SDS (100uL of 20% stock)
  - 20mM DTT (100uL of 0.2M stock)
  - water to 1mL (600uL)

3.3 Protocol

Prepare sufficient 1.5mL tubes for your samples (you need 2 per sample) and chill them on ice. Preheat a Thermomixer with a 2mL block to +60C.

1. Remove the centrifuged tubes from the rotor and carefully extract the supernatant to waste using a pipette or blunt ended needle.
2. Reconstitute the remaining pellet in 120uL of Pellet solubilization solution being careful to not lose sample or generate bubbles.
3. Transfer 50uL to a fresh 1.5mL tube on ice. This is your polysome RNA sample.
4. Transfer the remaining 50uL to a fresh 1.5mL tube and add 130uL of 2X Protein solubilization solution. Transfer the tube to the pre-heated Thermomixer and incubate for 30-minutes at +60C with mixing at 1,000rpm.
5. You can now freeze the polysome RNA sample at -80C until you are ready to process it further, or proceed to RNA extraction:
    1. Thaw your total RNA sample you 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.
6. By now, the protein sample should be finished incubating. Allow to cool to room temperature and proceed with SP3 for trypsin digestion and proteomics analysis as described elsewhere on this web page. The extracted protein samples can be stored indefinitely at -80C at this point and used for Western blotting or MS-based proteomics.

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 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 TLA100 rotor with the listed tubes. If you have a big volume, the SW60Ti rotor is the better choice because it can hold more volume. But, it also requires longer spin

times and can only spin 6 tubes at a time. The TLA100 rotor can spin 20 tubes in a single run and only requires 2-hours. However, the tube volume is limited to ~230uL.

Note 3 - 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 4 - If you are using the SW60 rotor, load ~3.75mL to each tube. If you are using the TLA100, load 150uL.

Note 5 - If you are using the SW60 rotor, load 200uL of sample to each tube. If you are using the TLA100, load 100uL across two individual tubes.

Note 6 - If you are using the SW60 rotor, spin at 45,000rpm for 12-hours. If you are using the TLA100, spin at 70,000rpm for 2-hours.

Note 7 - 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.