

Polyribosome Profiling

Wei Zheng*

Keck Biotech Services, Yale University, New Haven, USA

*For correspondence: wei.zheng.madison@gmail.com

Materials and Reagents

1. Acetone
2. Sucrose
3. Cyclohexamide
4. Bromophenol blue (BPB)
5. Urea
6. Tris-HCl
7. Tris-Acetate
8. MgCl₂
9. DTT
10. NH₄Cl
11. NaCl
12. DEPC-treated water
13. Heparin
14. Stocks (see Recipes)
15. Lysis buffer (see Recipes)
16. 60% sucrose (see Recipes)
17. 7% sucrose solution (see Recipes)
18. 47% sucrose solution (see Recipes)
19. Diluted sucrose (see Recipes)
20. Cyclohexamide to add to cells (see Recipes)

Equipment

1. Autoclaved, RNase free 500 ml centrifuge bottles (1 per sample)
2. Autoclaved, RNase free 40 ml centrifuge bottles (1 per sample)
3. Centrifuges
4. Colored Epi tubes. Need 30/sample
5. 15 ml, 50 ml falcon tubes (BD Biosciences, Falcon®)

6. Ultracentrifuge tubes (Don't autoclave) (Beckman Coulter, catalog number: 344059)
7. Beckman JA-10, JA-20, and Epi rotors to 4 °C (Beckman Coulter)
8. Ultra SW-51 buckets to 4 °C
9. RNase-free glass beads and bead scoop
10. Foxy fraction collector
11. BioRad Econo UV monitor (Bio-Rad)
12. Peristaltic pump

Procedure

Day 1

1. Make sure all stock solutions are previously prepared.
2. Inoculate an overnight yeast cell culture from fresh plates.

Day 2

1. Get together all the necessary equipment prior to starting as this is a time-sensitive experiment.
2. Aliquot acetone - if you are harvesting protein pellets.
 - a. 7 parts acetone to 2 parts water.
 - b. Aliquot 0.9 ml into colored Eppendorf tubes. Label 1-24, P & C. Store at -20 °C.
3. Pour gradients
 - a. Make sucrose solutions and dilutions.
 - b. Into 15 ml Beckman tubes, add 2.4 ml (3 x 800 µl) of 47% sucrose solution to tube.
 - c. Freeze at -80 °C for 30 min.
 - d. Carefully add 37% same way. Do this carefully. Don't want dimples.
 - e. Repeat with 27% and 17%.
 - f. Add 800 µl 7% gradient 2 times. Add 600 µl 7% solution to very top of tube (add as much as possible).
 - g. Freeze 2-3 h. Transfer to fridge that will not be disturbed and thaw O/N.
4. Inoculate 100 ml of media with O/N.
5. Put JA-10 rotor in Beckman. Leave at 4 °C. Move JA-20 and Epi rotors to fridge.
6. Put ultracentrifuge baskets in fridge.

Day 3

A. Cell extraction

1. Take OD of culture. Want between 0.4-0.6.
2. Get 2 buckets of ice, turn on Marathon to cool down.

3. Make lysis buffer and weigh out cyclohexamide.
4. Pour culture into 500 ml centrifuge bottle containing cyclohexamide. Swirl for 30 sec. and fill 3/4 full with ice.
5. Balance tubes and centrifuge in JA-10 7 k for 5 min (5 min from beginning of spin-not to when it gets up to speed). Dump ice and cells out aggressively.
6. Resuspend cells in 20 ml cold lysis buffer (do NOT throw away remaining lysis buffer -store on ice). Transfer to 40 ml centrifuge tube and spin at 7 K for 3 min. This step is very time sensitive. Balance as fast as possible.
7. Resuspend pellet in remaining liquid. Measure and bring volume up to 600 μ l with lysis buffer in colored Epi tube. A vigorous dumping will leave about 600 μ l.
8. Add glass beads.
9. Vortex in cold room for 3 min.
10. Puncture hole in bottom. Stuff on another tube. Put whole thing in 50 ml Falcon, spin in Marathon 2 min at 5 K.
11. Transfer supernatant to new tube. Centrifuge at 7 K in the JA-18 Eppendorf tube rotor in Beckman for 10 min.
12. Transfer supernatant to Eppendorf tube.
13. Start precooling ultracent - turn on, set to 4 $^{\circ}$ C, pull vacuum. Get rotor out.

B. Quantitate RNA

1. Spec 5 μ l of RNA in 995 μ l water at UV 260 nm.
2. Calculate volume of extract to load onto gradients: μ l loaded = 10 OD units * 1,000 μ l ml⁻¹ / (OD₂₆₀ * dilution factor).
3. Set aside 1/24th of volume that will be loaded onto gradients for control.

C. Ultracentrifuging

1. Load 10 OD units of sup on top of gradient by dropping gently onto top.
2. Grease all baskets with SPINKOTE for rotor SW-41. Line up in pairs: 1&4, 2&5, 3&6.
3. Load tubes into baskets. Write down who went where.
4. Hang baskets onto rotor. Give a little wiggle to make sure they are on securely.
5. Spin at 39 K for 2.5 h at 4 $^{\circ}$ C. Decel = 7. This breaks until RPM = 2,000. Once that hits, it stays on hold until you stop it (by hitting stop). Takes approximately 4 min to come down.

D. Collecting fractions

1. While samples are centrifuging, make 7% sucrose & 60% sucrose + BPB.
 - a. 7% sucrose solution for baselining ~ 50 ml.
 - b. 60% sucrose + 100 μ l 5 mg/ml bromophenol blue + 100 μ l DEPC ~ 50 ml.

2. Turn on reader about 20 min before the end of the spin.
3. Once the reader is warmed up, baseline reader:
 - a. Turn top of reader to Max Open, sensitivity = 1, noise filter = 1.5. Filter = 5 mm path length, 2 filters. Both need to be same setting (one that reads, one that is a reference).
 - b. Turn on pump (not foxy). Fill spill tube with 7% solution. Run 7% through so drips out the fraction collector.
 - c. Click "autobaseline" and move pen to position 50 on paper.
 - d. Once 7% is moving through reader, move pen manually down to position 20 on chart. Want consistent, straight line.
 - e. Turn off paper, move pen up off of paper.
 - f. Take out 7% and run dry for a while to get rid of droplets in thin tubing going to fraction collector. Very important to get this dry.
 - g. Wipe down seals with D.D. water.
 - h. Get blue sucrose moving through large tubing through to metal tube. Once starts dripping, stop pump and wipe off blue drop.
 - i. Pump set at 80 x 10. Turn on fraction collector first, then turn on pump – now foxy controls the pump.
 - j. Foxy program: E=edit, simple rack, last= 24, collect by drops = 10.
 - k. Program #2.
4. Load acetone tubes onto collector.
5. When spin is done, take out baskets. If doing more than 2 gradients, store holder in fridge. If only doing two, move holder to bench top. Wipe down rotor and put away.
6. Take tube out of basket with forceps. Assemble white seal on top of tube and attach to pump.
7. Start chart at 60 or 150 (write down speed). Move pen on paper!
8. On Foxy, hit run - fraction collector will move.
9. Time between time that measurement is taken (the pen will mark all the way up) and when you see first drop into tube. Write down.
10. When done, turn off foxy, reverse flow on pump until get the Beckman tube about 1/2 full of blue dye.
11. Dump off blue sucrose and invert tube. Pellet is at the bottom. Add 500 μ l lysis buffers to pellet and resuspend by vortexing hard. Transfer to Epi tube with acetone marked "P". Add 500 μ l lysis buffer to 1/24th sample saved on ice before spinning. Transfer to Epi tube with acetone marked "C".
12. Put all samples in -20 °C O/N.
13. Clean tube holder and run thin tubing to Foxy dry. Must have no droplet. If there is any problem run 7% through and repeat process with blue.

14. To clean system run 250 ml D.D. water + 2 drops Ivory Soap through to fraction collector and rinse with 2 x 250 ml of D.D. water.

Day 4

A. Precipitating samples

1. Centrifuge acetone samples 5 min at full speed 4 °C. Aspirate sup - pellets may not be visible.
2. Dry covered to prevent dust from going into tube ~ 10 min or until there is no more acetone fumes. Can save dry pellets in -20 °C until use.

B. Preparing for westerns

1. Resuspend pellets in 10 µl loading buffer by flicking tube.
2. Boil 2-3 min, vortex, quick spin.
3. Load onto gel (do not load insoluble pellet).
4. Loading buffer - for protein detection. 3 parts 6 M urea to 1 part 4 x Laemmli buffer.

Recipes

1. Stocks

- 1 M Tris-HCl (pH 7.5 at 4 °C)
- 1 M Tris-Acetate (pH 7.0 at 4 °C)
- 1 M MgCl₂
- 1 M DTT (store at -20 °C)
- 1 M NH₄Cl - do not autoclave.

2. Lysis buffer

- 10 mM Tris-HCl (pH 7.5 at 4 °C)
- 0.1 M NaCl
- 30 mM MgCl₂
- Filter sterilize, store at RT.

3. 60% sucrose

- Make 60% sucrose in DEPC-treated water. Use baked spatulas and baked glassware.
- Filter sterilize and store at RT.

4. 7% sucrose solution (200 ml) (enough for 4 samples)

- | | |
|---------|-------------------------------|
| 23.3 ml | 60% sucrose, 7% |
| 10 ml | 1 M Tris-Acetate, 50 mM |
| 10 ml | 1 M NH ₄ Cl, 50 mM |
| 2.4 ml | 1 M MgCl ₂ , 12 mM |
| 0.2 ml | 1 M DTT, 1 mM |

- 0.2 ml DEPC, 0.1 %
 Up to volume with DEPC-treated water
5. 47% sucrose solution (200 ml)
- 156.7 ml 60% sucrose, 7%
 10 ml 1 M Tris-Acetate, 50 mM
 10 ml 1 M NH₄Cl, 50 mM
 2.4 ml 1 M MgCl₂, 12 mM
 0.2 ml 1 M DTT, 1 mM
 0.2 ml DEPC, 0.1 %
 Up to volume with DEPC-treated water
6. Diluted sucrose (12 ml)
- 37% = 9 ml 47% + 3 ml 7%
 27% = 6 ml 47% + 6 ml 7%
 17% = 3 ml 47% + 9 ml 7%
7. Cyclohexamide to add to cells (assuming cell volume =100 ml)
- 5 mg cyclohexamide resuspended in water, 50 µg/ml
8. Lysis buffer (make 25 ml per sample)
- 1.25 mg cyclohexamide, resuspend in water, 50 µg/ml
 5 mg heparin, resuspend in water, 200 µg/ml
 50 µl DEPC, 0.2%

References

1. Baim, S. B., Pietras, D. F., Eustice, D. C. and Sherman, F. (1985). [A mutation allowing an mRNA secondary structure diminishes translation of *Saccharomyces cerevisiae* iso-1-cytochrome c.](#) *Mol Cell Biol* 5(8): 1839-1846.