POLYSOME PROFILE PROTOCOL

(Revised 9.29.04)

NB: Make sure everything is DEPC treated as you will be isolating RNA.

DAY BEFORE EXPERIMENT

A. **DEPC Treat & Autoclave:** Cell scrapers
Polyallomer Tubes 9/16 x 3 1/2 in (Beckman cat # 331372)

B. **Check for:** Recombinant RNAse Inhibitor (Invitrogen cat # 15518-012) at –20ºC , 70% Sucrose at 4ºC

C. Solutions to make:

1. Polysome Lysis Buffer (500ml)

	Amount	Final Concentration
KCL	3.728g	100mm
$MgCl_2$	0.508g	5mm
Hepes pH 7.4	1.19g	10mm
NP-40	2.5ml	0.5%

-QS to 500ml with RNAse/ DNAse free water.
-Add 100μg/ml of CHX to PLB:
For 10ml of PLB, add 0.001g of CHX.
-After CHX addition, sterile filter into 50ml conical tubes.
-Store at 4ºC.

2. 1X Phosphate Buffered Saline with CHX (100μg/ml)
-Start with 500ml of room temperature sterile filtered 1X PBS, add 0.05g of CHX (CHX does not dissolve quickly at cold temperatures).
-Store at 4ºC.

3. *Polysome Gradient Buffer (500ml)*

	Amount	Final Concentration
KCl	3.728g	100mm
$MgCl_2$	0.508g	5mm
Hepes pH 7.4	1.19g	10mm

-QS to 500ml with RNAse/ DNAse free water and sterile filter into 50ml conical tubes.
-Store at 4ºC
NB: The 1M $MgCl_2$ that we have is in liquid form, so use 2.5ml for a final concentration of 5mm.

4. 15% Sucrose (use DEPC treated 100ml bottle)
-100ml Polysome Gradient Buffer
-15g Ultra Pure Sucrose
-Store at 4ºC

5. 45% Sucrose (use DEPC treated 100ml bottle)
-100ml Polysome Gradient Buffer
-45g Ultra Pure Sucrose
-Store at 4ºC

6. 70% Sucrose (use DEPC treated 125ml bottle)
-100ml Polysome Gradient Buffer
-70g Ultra Pure Sucrose
-Store at 4ºC

**You will need ≅ 6ml of each sucrose reagent/ gradient. You can do a maximum of 6 gradients at a time.*

D. **Prepare the Gradients (Do this the night before and store at 4˚C for ~18 hours)**
   1. Take out 2 polyallomer tubes (plastic, autoclaved) and label. Place each tube inside of the 5ml silver holder and mark the tube along the rim of the barrel to indicate the 5ml mark.
   2. Stand both tubes upright in the magnetic tube holder on top of the Seton Gradient maker.
   3. Aspirate ~ 6ml of **15%** sucrose with a 10cc syringe and metal barrel needle. Put the needle at the bottom of the tube and begin dispensing the 15% sucrose along the side. As the level rises, bring up the needle along the side of the tube keeping the needle submerged just below surface level. Once at the 5ml line, add an additional ml.
   4. Using the same syringe and metal needle, aspirate ~ 6ml of **45%** sucrose. Insert the needle to the BOTTOM of the tube and SLOWLY dispense 45% sucrose along the side of the tube. As the 15% / 45% interface rises, bring the needle up along the side of the tube keeping the needle submerged just below surface level.
   5. Fill the tube until the top almost overflows and pull out the needle very quickly.
   6. Put the LONG BLACK CAP in the tube, inserting the side with the hole first to prevent air bubbles in the gradient.

E. **Processing the Seton Gradient Maker**
   1. Turn on (button on bottom of machine)
   2. You want to turn it 90º, so hit "level" and use the "scroll up/ down" buttons until the bubble on the leveler is centered between the lines.
   3. Hit "end of run", "now", "level".
   4. Now hit "mode" and it will display 3 settings:
      - Time = 4.06 min and hit "mode"
      - Angle = 80º and hit "mode"
      - Speed = 8 and hit "mode"
   5. Hit "start". The machine will beep once it is complete. Store the gradients at 4˚C overnight, being careful not to disturb them in transit.

DAY OF EXPERIMENT

Starting Material: 6 plates of 3T3-L1 preadipocytes OR
3 plates of Day 8/Day 10 3T3-L1 adipocytes

A. Set-Up
   1. You will need one large ice bucket for the 10cm 3T3-L1's and one small ice bucket for reagents. For each gradient, place 3 RNAse/ DNAse free 1.5ml microfuge tubes on ice to chill.
   2. Turn on the Beckman ultracentrifuge, make sure the vacuum is on and the temperature is set at 4°C.
   3. Turn on the Eppendorf centrifuge and fast cool to 4ºC.
   4. Get the SW-41 rotor and buckets from the cold room and place in the 4ºC fridge till ready for use.

B. Preparing the Lysate
   5. Place the 10cm 3T3-L1's on ice, aspirate the media and wash twice with 3ml of ice cold 1X PBS + CHX (refer to C2 under Day Before).
   6. For preadipocytes, add 100μl Polysome Lysis Buffer + CHX per plate.

Ex: If you have 12 plates of Preads, you will scrape in 2 sets of 6 plates. For each set add:
-600µl of PLB + CHX (refer to C1 under Day Before)
-6µl Recombinant RNAse Inhibitor
-Add the 606ul of PLB and RNAse Inhibitor to one plate, scrape the cells and then transfer to the next plate and scrape. Do this until all 6 plates are scraped, then transfer the lysate to an RNAse/ DNAse free 1.5ml microfuge tube.

7. For adipocytes, add 200ul Polysome Lysis Buffer + CHX per plate.
   Ex: If you have 6 plates of Ads, you will scrape in 2 sets of 3 plates. At this point, follow the same procedure for the preadipocytes.

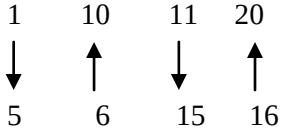
8. Passage the lysate 5 to 7x with a 1ml syringe and 26 gauge needle to achieve complete lysis of the cells.
9. Spin at 13,200rpm in the Eppendorf centrifuge at 4ºC for 6 minutes to pellet the nuclei.
10. Transfer the supernatant from the nuclei pellet into another RNAse/ DNAse free 1.5ml microfuge tube. Repeat steps 9 and 10 one more time.

C. Preparing for Centrifugation

11. Remove the gradients from the 4˚C fridge and place on the bench in their holder. Extract about 1ml of the top portion of the gradient with a 1ml insulin syringe to accommodate the lysate.
12. **SLOWLY** layer the lysates onto each gradient using a 1mL filter tip and pipet, being especially careful to prevent mixing of the lysate with the gradient. **THIS STEP IS CRITICAL IN GENERATING A GOOD PROFILE!!**
13. **GENTLY** place the gradients inside of the SW-41 holders for the centrifuge rotor. Use the balance to make sure they are equivalent weights so they will balance in the centrifuge. If necessary, add Polysome Lysis Buffer to the top of the gradient to balance them.
14. Screw the metal caps onto the holders and place on the rotor. Make sure they are paired correctly when putting on the rotor (1& 4, 2 & 5, 3 & 6).
15. Spin at 35K for 2 hours at 4ºC. Make sure the BRAKE IS OFF!!! Make sure the vacuum is pulling as well. It will take ~ 45 minutes for the centrifuge to slow down.

D. Fraction Collecting

16. Turn the ISCO UA-6 UV-VIS Detector to "operate" and let it warm up for 15 minutes.
17. Samples can remain in the ultracentrifuge until you are ready to begin. Alternatively, they can also be stored and are stable at 4ºC for up to 6 hours.
18. Place 20 0.5ml conical microfuge tubes (Continental Lab Products, Cat #3434.S) in the white microfuge rack as follows:



NB: You may not use all of these tubes for your gradient.

E. Brandel Pump

19. Assemble the 50ml glass syringe and the barrel. Attach the rubber tubing to the syringe and the metal needle to the tubing. Fill the syringe with 50ml of **70% sucrose** (you will not use this much 70% sucrose, but it is a good idea to have a full syringe).
20. Turn on the Brandel and place the glass syringe in the holder. The motor will need to reverse to accommodate the syringe.
    "Rev" switch down

"Rapid" switch up
Turn both off when the syringe fits snuggly.

21. Now you will need to get the air bubbles out of the tubing.
    "Forward" up
    1.5ml/min
22. Assemble the Brandel optical unit by placing the black circular knob into the screw assembly with the needle facing up.
23. Attach the tubing from the 50ml syringe to the underside of the black circular knob.
24. Now, turn "Forward" up and flow rate to 1.5ml/min until you see sucrose coming through the needle on the knob. Allow it to flow to the top of the needle and then stop.

F. UV-VIS Detector

25. Refer to the ISCO UA-6 "Baseline Adjustment for Absorbance" above the unit. Perform steps 1-10 to set your baseline.
26. Set the following: Noise Filter = 0.5
    Sensitivity = 1.0 (Preads), 2.0 (Ads)
    Peak Separator = off
    Chart speed = 30cm/hr
    Place the red pen into the black holder above the chart paper

G. Optical Unit Assembly

27. Remove one polyallomer gradient tube from the rotor holder and CAREFULLY insert it into the collar on the optical unit. Be sure not to create any waves in the gradient!! Adjust the stage and screw assembly so that the needle on the black knob is directly centered beneath the SW-41 gradient tube.
28. CAREFULLY push upward on the stage while keeping the needle centered under the tube until it pierces the tube. *Be sure to apply the appropriate amount of controlled force so as not to bend the needle.*
29. Tighten the screws so the stage is secure.
30. Secure white microfuge tube rack on the fractionator along with 20 0.5ml conical microfuge tubes with screw caps off.
31. Place the rubber tube from the top of the optical unit into the top of the fractionator.
32. Fractionator Settings: "Power" up, "Main" up, "Drop" down and set on 31 drops per tube.

H. Begin Collecting

33. Brandel Pump Settings: "Rapid" = down
    "Forward" = up
    ml/min = 0.750
    NB: You will notice the 70% / Gradient interface rise about 2.5-3ml before the first drop falls into the first 0.5ml conical tube in the rack.
34. Once all fractions for that sample have been collected, reverse the pump so that the 70% sucrose goes back into the syringe. *Be careful not to draw any of the sample back into the tubing attached to the glass syringe.*
35. You will need to flush the optical unit and tubing with RNAse/ DNAse free water and isopropanol using a 10ml syringe and needle. Repeat this flush again, and end with one RNAse/ DNAse free water flush.
36. Make sure all water is out of the tube before the next sample is processed.
37. Label the gradient fractions and store at -20˚C.

I. Clean-up

38. Empty 70% sucrose back into its bottle. Wash and soak in dH_2O along with tubing and metal needle.