Protocol: Power's Tight-Couple Ribosome Preparation

IMPORTANT: Everything should be done at 4 ºC! Use ice-cold Buffers, French Press, Rotors, etc. Use RNase free lab wares and reagents.

1. Inoculate FOUR 5 ml LB with MRE600 glycerol stock. Grow overnight at 37 ºC in shaker.

2. Inoculate SIX 500 ml LB with 2 ml of saturated, overnight MRE600 culture. Grow at 37 ºC in a shaker until A650 is 0.5. (Takes approximately 4 hours)

3. Place cultures on ice for 30 minutes. Transfer to 500 ml centrifuge bottles.

4. Spin at 8000 RPM for 15 min at 4 ºC in BECKMAN 10.500 rotor.

5. Resuspend each cell pellet in 15 ml of buffer A.

6. Pool all the resuspended cells in one centrifuge bottle. Spin at 8000 RPM for 15 min at 4 ºC in BECKMAN 10.500 rotor. <u>You can store the cell pellets at this stage at –80 ºC</u>.

7. Resuspend the cell pellet in 90 ml of buffer A on ice.

8. Lyse the cells using a French Press at 18000 psi. Two passes through the press is usually sufficient.

9. Add 50 µl of RQI RNAse Free, DNase I (Promega).

10. Spin at 15,000 RPM (=30,000 g) at 4 ºC for 30 min in BECKMAN JA 17 rotor.

11. Transfer supernatant (S30) to new tubes and repeat spin (step 10).

12. Adjust the ammonium chloride conc in the S30 to 0.5 M.

13. Transfer the S30 supernatant to FOUR clean "Red-capped" 25 ml Ultra Centrifuge bottles (BECKMAN Cat# 355618).

14. Spin at 40,000 RPM for 4 hours at 4 ºC in BECKMAN Ti-70 rotor.

15. Prepare SIX 10% to 40% sucrose gradients in buffer B. Each gradients is made using 18 mls of 10% sucrose + 18 mls of 40% sucrose in BECKMAN Ultra-Clear centrifuge tubes (Cat# 344058). **USE PINK NEEDLE AT THE END OF THE TUBE ATTACHED TO THE GRADIENT MAKER.** Store the gradients at 4 ºC for atleat 2 hours before use.

16. Wash the crude ribosome pellet with 5 ml of buffer B. This can be accomplished by adding the buffer to the centrifuge bottles and gently twrilling the bottles. Remove the buffer by decanting carefully.

17. Resuspend the crude ribosome pellets in 1.5 ml buffer B on ice using a small magnetic stir-bar. Do not resuspend the brown "junk" at the bottom of the pellet.

18. Load 1 ml of resuspended ribosomes per gradient.

19. Spin gradients at 20,000 for 13 hours at 4 ºC in BECKMAN SW-28 rotor.

20. Set-up the ISCO/BRANDEL gradient fractionator. Warm it up for 30 min before use. Use ice-cold 50% sucrose to pump the gradients.

21. Monitor the gradient at A254 using the UA-6 detector and chart recorder. Collect the 70S ribosome peek into clean 15 ml FALCON tubes on ice. (Usually recover about 8 ml/gradient)

22. Pool 70S peeks. Raise the Magnesium chloride conc. to 10 mM final. Add buffer C to final 60 ml total volume. (This helps to dilute the sucrose percentage)

23. Spin at 40,000 RPM for 13 hours at 4 ºC in Ti-70 rotor. Use clean "Red-capped" 25 ml Ultra Centrifuge bottles (BECKMAN Cat# 355618).

24. Wash the 70S ribosome pellet with 5 ml of buffer C. This can be accomplished by adding the buffer to the centrifuge bottles and gently twrilling the bottles. Remove the buffer by decanting carefully.

25. Resuspend the 70S ribosomes in a TOTAL volume of 1.5 ml of buffer C. (Approximately 250 ul of buffer C per gradient).

26. Make 10 ul aliquotes and quick-freeze in liquid nitrogen. Store at –80 ºC.

BUFFERS FOR RIBOSOME PREP

Make buffers a day in advance and store them at 4 ºC. Add 2-mercaptoethanol to the buffers just before use.

Buffer A:
50 mM Tris-HCl pH 7.6
10 mM $MgCl_2$
100 mM NH_4Cl
6 mM 2-mercaptoethanol
0.5 mM EDTA

Buffer B:
50 mM Tris-HCl pH 7.6
6 mM $MgCl_2$
100 mM NH_4Cl
6 mM 2-mercaptoethanol

Buffer C:
50 mM Tris-HCl pH 7.6
10 mM $MgCl_2$
100 mM NH_4Cl
6 mM 2-mercaptoethanol

40% Sucrose in Buffer B:
70.6 Gram BRL sucrose in final vol 150 ml of buffer B. **NOTE: This is not a mistake, it is based on a Table from Beckman.**

10% Sucrose in Buffer B:
15.6 Gram BRL sucrose in final vol 150 ml of buffer B. **NOTE: This is not a mistake, it is based on a Table from Beckman.**

50% Sucrose in water for Syringe Pump:
92 Gram BRL sucrose in final vol 150 ml of water.