6.3 RPFs from Free and Membrane Bound Ribosomes

This procedure was developed to determine the point during translation at which a secreted nascent protein chain became committed to the translocation machinery of the ER membrane. Variations of this procedure can be used to obtain free and bound polysomes and polysomal mRNA.

Buffers and Reagents

CBnuc - see section 6.2

CBTK

1.9M KAc	475uL of 4M stock
40mM MgAc2	40 uL of 1M
25mM EGTA	25 uL of 1M
20mM Hepes-K pH 7.5	20 ul of 1M
3.5mM DTT	3.5 uL of 1M
437 uL water---> 1mL	

5x BTK

2.3M KAc	575 uL of 4M
100mM Hepes-K pH 7.5	100uL of 1M
50mM MgAc2	50 uL of 1M
25mM EGTA	25 uL of 1M
10mM DTT	10 uL of 1M
240 uL water---> 1mL	

2.25 M sucrose

BTKS1.8

1X BTK	200uL of 5X

1.8M sucrose	800uL of 2.25M

1x BTK

BP

50mM NaCl	12.5 uL of 4M
50mM Tris-Cl pH 7.5	25 uL of 2M
5mM EDTA	10uL of 0.5M
953 uL water--> 1mL	

20% CHAPS (Boehringer)

other reagents as in section 6.2

Isolating RPFs

1. Perform steps 1 to 4 of section 6.2.
2. Add 10 uL of CBTK. Layer over 100 uL of BTKS1.8 in a 200 uL (7 x 20 mm) TLS-55 polycarbonate centrifuge tube. Spin in TLS-55 rotor at 55,000 rpm for 14 h at 4øC, accel=6, decel=6.
3. Remove top 120 uL and set aside. This fraction contains membranes and bound ribosomes.
4. Add 100 uL BP to the lower 30 uL and pellet. Resuspend the pellet and add 6.5 uL of 4 mg/mL proteinase K. Incubate at 37øC for 30 min. Phenol extract and ethanol precipitate free rpfs as in section 6.2.
5. To the membrane fraction, add 6 uL of 20% CHAPS. Incubate 15 min at 4øC to solubilize membranes.
6. Layer over 60 uL of BTKS1.8 containing 1% CHAPS, in a 200 uL TLS-55 tube. Spin in TLS-55 at 55,000 rpm for 7 h as above.
7. Remove the top 150 uL and discard. To the lower 30 uL, add 100 uL BP and 6.5 uL 4 mg/mL proteinase K. Incubate, extract and precipitate bound rpfs as above.
8. Analyse and map rpfs as above. There may not be equal amounts of rpfs in the free and bound fractions, so the toeprinting reaction and interpretation of data should be handled accordingly.

Notes:

The free and bound ribosomes are fractionated in 460 mM KAc. Wolin and Walter (1993) also report low salt conditions (150 mM KAc) with the appropriate buffers.

I use CHAPS instead of deoxycholate for ease of handling and with good results.

The original conditions include 0.5% SDS in the proteinase K buffer (BP). It is omitted here to avoid precipitation with the high K+ concentration. The use of a mixture of SDS and CHAPS (see section 6.2) should be further investigated here.

(JCY, may 1994)