KH 19/10/06

Primer extension

· DEPC → use at 0.1% v/v

(A) Isolation of RNA

1. From a fresh transformation plate or restreak of your strain, grow up an overnight culture in 5 ml LB (plus antibiotic).
2. Inoculate 10 ml LB (plus antibiotic) with 200 µl overnight culture, and grow culture to OD_{650} of 0.4-0.5 (approx 2-3 h).
3. ~~Mix 2.5 ml culture with 4.5 ml RNAlater (Ambion) in 15 ml centrifuge tube. Prepare three of these tubes per culture.~~
4. ~~Incubate at room temperature for at least 5 minutes~~, then spin for 20 min, 4000 rpm, 20°C. Remove supernatant. Cell pellets can be used immediately for the RNA preps, or can be stored at -20/-70°C.
5. Resuspend all three pellets in a total of 200 µl TE buffer + 40 µg/ml lysoyzme. (i.e. resuspend first pellet in 200 µl TE, then use this suspension to resuspend second pellet etc.)

FRESH per reaction (1 µl/ml)

6. Incubate for 15 min at room temperature.
7. Prepare buffer RLT by adding 10 µl β-mercaptoethanol per 1 ml buffer RLT. Add 700 µl buffer RLT, vortex, then add 500 µl ethanol to each sample and mix by pipetting.
8. Split each sample between two RNeasy mini columns. Centrifuge 15 s at 10,000 rpm.
9. Discard flow-through and add 700 µl buffer RW1 to column. Centrifuge 15 s at 10,000 rpm. Discard flow-through and place column into new 2 ml collection tube.
10. Add 500 µl buffer RPE to column. Centrifuge 15 s at 10,000 rpm. MW tube
11. Add a further 500 µl buffer RPE to column and centrifuge 2 min at 10,000 rpm.
12. Place column into new collection tube. Centrifuge at full speed for 1 min to remove residual ethanol.
13. Transfer column to new microfuge tube. Elute RNA from each column in 30 µl RNase-free water. Combine the two eluates for each sample. You should have approx 60 µl in total for each RNA prep. Keep RNA samples on ice at all times.

Ut stand for 30s

14. To remove DNA contamination:
    a. To each RNA sample, add:
       3.5 µl 10x Turbo DNase Buffer
       1 µl Turbo DNase (Ambion)
    b. Incubate 30 min at 37°C.
    c. Add 6.1 µl DNase inactivating reagent and incubate at room temperature for 2 min, occasionally tipping/flicking the tube to mix.

d. Centrifuge 1.5 min at 10,000 rpm. Transfer supernatant to a clean tube and keep on ice.

15. Calculate RNA concentration using quantifier, and run on gel to check integrity of RNA (you should see two clear bands, corresponding to 16 s and 23 s rRNA). Make up the agarose and 1 x TBE in DEPC/treated water, and wash the tank thoroughly with Ethanol before use.
16. Freeze RNA in aliquots. It is advisable to aliquot the RNA such that each tube contains the required amount of RNA for a single primer extension reaction (20-30 µg, see section (C) below).

(B) Labelling of primer

1. Mix the following in an RNase-free microfuge tube:

0.5 µl	100 µM primer (see Note 1)
2 µl	10 x polynuclotide kinase buffer
15.5 µl	DEPC-treated water
1 µl	γ^{32}P-ATP
1 µl	T4 polynucleotide kinase (NEB)
20 µl	

2. Incubate 30 min at 37°C, then 10 min at 68°C to inactivate the enzyme.
3. Store labelled primer at -20°C.

(C) Primer annealing/extension

Day 1

+32P
D49274

1. Precool centrifuge to 4°C and set heating blocks to 50°C and 75°C
2. Mix 1 µl labelled primer with 20-30 µg RNA.
3. Add 1/10 volume 3 M sodium acetate, pH 7.0 and 2.5 volumes cold 100% ethanol. Vortex and incubate ~~10~~ 30 min at -80°C.
4. Centrifuge ~~10~~ 30 min at 13,000 rpm, 4°C.
5. Discard supernatant (hot!) and add ~~1 ml~~ 750 µl cold 70 % ethanol. CHECK! Centrifuge ~~5~~ 10 min at 13,000 rpm, 4°C.
6. Discard supernatant (hot!) and dry pellet for 5-10 minutes in the speedvac at 45°C.

7. Resuspend pellet in 30 µl hybridisation buffer (20 mM HEPES, 0.4 M NaCl, 80% formamide). Vortex for ~~5 min~~ 15 secs, then incubate 5 min at 50°C, vortex and spin briefly.
8. Incubate 15 min at 75°C, then 3 h at 50°C to anneal primer (see Note 2).
9. Add 75 µl cold 100 % ethanol, vortex and incubate overnight at -80°C. - 30 mins

Day 2

1. Precool centrifuge to 4°C and set heating blocks to 37°C and 72°C.
2. Pour sequencing gel. 20
3. Centrifuge annealed primer/RNA sample (stored overnight at -80°C) for 10 min at 13,000 rpm, 4°C. 10
4. Remove supernatant and add ~~1 ml~~ 750 µl cold 70 % ethanol. Centrifuge 5 min at 13,000 rpm, 4°C.
5. Remove supernatant and dry pellet (approx 20 min).
6. Resusupend pellet in 31 µl DEPC-treated water, then add:

   10 µl 5 x reverse transcriptase buffer (Promega)

   1 µl 50 mM DTT

   5 µl 10 mM dNTPs

   2.5 µl AMV reverse transcriptase (Promega)(see Note 3)

   0.6 µl RNasin (Promega)

   10µl Goscript
   5µl 10mM dN
   1µl Goscript
   0.6µl RNAsin
   MgCl2 3µl

7. Incubate 1 h at 37°C. Meanwhile, pre-run sequencing gel.
8. Incubate 10 min at 72°C to inactivate enzyme, then spin briefly.
9. Add 1 µl 10 mg/ml RNase and incubate 30 min at 37°C.
10. Add 6.7 µl 3 M Sodium ~~ammonium~~ acetate, pH 4.8 and 125µl cold 100% ethanol. Centrifuge 10 30 min, 13,000 rpm, 4°C. 10
11. Remove supernatant and add 1 ml cold 70% ethanol. Centrifuge 5 min at 13,000 rpm, 4°C.
12. Remove supernatant and dry pellet (approx 20 min).
13. Resuspend pellet in 4 µl stop solution from T7 sequencing kit.
14. Load 3 µl each primer extension reaction on a 6% polyacrylamide sequence gel, along with sequence reactions. Run gel at 60 Watts for approx 2 hours (until dark blue runs off bottom of gel, then a further 3-4 cm).

INCUBATE -80deg 30min. -overnight

=> Fix gel for 15 min