## Small Scale Method for preparing fragmented cRNA

Method: based on protocol by baugh@fas.harvard.edu, Nucleic Acids Research 2001, vol 29, no 5 Baugh et al

(A) Isolating Sample RNA

- Use RNeasy Isolation Kit (Qiagen) to get RNA from sample
- Run 1-2µl RNA sample on 1% EtBr TBE agarose gel to make sure RNA is not degraded (use RNA loading buffer which contains formalin and heat 70°C 5min to denat). Run gel at ~100V (low) for 40-60min. Also quantitate RNA on spectrophotometer.
- Aliquot 1µg sample RNA into a 0.2ml PCR tube. If the volume is greater then 5ul dry the RNA down to a 3.5ul volume using pre-measured 0.2ml vials with varying volumes of water in them. If the volume of the 500ng is less then 3.5ul make it up with RNAse free H20.

(B) First strand cDNA synthesis

1. Add 1µl 5uM HPLC T7-$(T)_{24}$ primer (100ng) to the 1µg sample RNA (section A) (GGC CAG TGA ATT GTA ATA CGA CTC ACT ATA GGG AGG CGG-$(dT)_{24}$)
2. Mix, quick spin if needed
3. Heat at 70°C in a PCR machine for 4min
4. Place straight on compacted ice to cool to 4 °C.
5. On ice mix the following cDNA reagent mix if two samples make up 2.25X then aliqout in each tube:

| | 1X |
|---|---|
| 5x 1st strand buffer | 2ul |
| 0.1M DTT (fresh) | 1ul |
| 10mM dNTPs (10mM each dATP, dCTP, dGTP, dNTP) | 0.5ul |
| T4gp32 (2.4mg/ml) | 1ul |
| Rnase Inhibitor (40U/ul) | 0.5ul |
| Superscript II (200U/ul) | 0.5ul |

Mix and quick spin if needed

6. Add 5.5ul of cDNA reagent mix to the RNA/Primer tube, vortex and incubate at 42°C, 1 hour
7. Heat inactivate at 65 °C for 15 minutes (Note: take the cDNA tube out of PCR machine whilst it reaches 65 °C, then place it back in the PCR machine), place on ice.
8. Proceed to second strand cDNA synthesis

(C ) Second Strand cDNA synthesis

1. Ice all reagents and first strand cDNA tubes (from section B)
2. Make up the second strand reagent mix if have two samples make a master mix of

| | 1X |
|---|---|
| $H_2O$ | 43.2ul |
| 5x 2nd strand buffer | 15ul |
| 10mM dNTPs (10mM each dATP, dCTP, dGTP, dNTP) | 1.5ul |
| DNA polymerase I (5U/ul) | 4ul |
| E.coli DNA ligase (10U/ul) | 0.5ul |
| RnaseH (1U/ul) | 1ul |
| Mix and spin if needed | |

3. Add 65ul of second strand reagent mix to the first strand cDNA tube and mix by pipetting. Incubate at 16°C for 2 hours.
4. Add 10µl (10U total) T4 DNA polymerase, incubate 16°C for 15min (to finish off ends properly)
5. Heat inactivate by taking tube out of PCR machine, increase temperature to 70 °C, then place tube back in PCR machine for 10 minutes. Place tube on compacted ice to cool.

NOTE: Can store at –80°C if required

(D) Clean up of dsDNA

1. Spin Phase-Lock tubes at maximum speed for 1min @ 4°C (so gel comes down to bottom of tubes)
2. Add all the cDNA reaction (75µl)
3. Add equal volume buffered saturated phenol (or phenol/chloroform) in fume hood
4. Vortex lightly (white ppt)
5. Spin at maximum speed ~13000rpm, 5 min at 4°C
6. Transfer upper phase to new tube (~75µl)
7. Prepare BioGel P-6 Microspin column. Shake Biogel to get rid of air bubbles then snap off bottom of column and remove lid so liquid can run out, leave 5 min on bench till tube is empty. Spin at 3500rpm for 2 min. Place colomn into a fresh RNAse free tube.
8. Transfer aqueous phase from the Spin-Phase lock tube to the Bio gel P-6 and spin at 3500rpm for 4min collecting 75ul in the fresh tube.
9. Precipitate the dscDNA by adding 2.5V 100% Ethanol and 1ul glycogen (20mg/ml) leave at -20 °C for 2 hours (or O/N).
10. Spin at 13000rpm for 20 min @ 4°C . Remove S/N from pellet.
11. Wash 1x with 70% ethanol and spin down at 13,000rpm for 5min. Remove S/N and pulse spin and remove residual ethanol.
12. Air dry or use RNA desicator for 2-3min then take pellet up in 20ul of Rnase free water. Leave 5 min @ RT to make sure it dissolved.

(E) In vitro transcription (GeneChip kit)

1. To 2oµl ds cDNA add:
   4µl 10x IVT buffer
   4µl labelling enzyme mix
   12µl labelling NTP
   Total volume 40µl

2. Carefully mix and spin liquid down and transfer to a 0.2ml tube.
3. Incubate for 9 hours at 37°C in PCR machine. This can be done O/N.
4. Spin down liquid

(F) IVT clean up

1. To IVT reaction tube add:
   160µl DEPC $H_2O$
   700µl RLT buffer (need to add 10µl βME per ml RLT)
2. Mix
3. Add 500µl 100% ethanol
4. Transfer 700µl to two separate spin columns (RNeasy mini column)
5. Spin at maximum speed 13,000rpm for 1min RT
6. Transfer columns to new collection tubes
7. Add 500µl RPE buffer
8. Spin at 13,000rpm for 1 min
9. Add 500µl RPE buffer
10. Spin at 13,000rpm for 2min
11. Remove flow through and spin again to get rid of RPE on the column surface.
12. Transfer spin columns to new collection tube
13. Add 50µl DEPC $H_2O$ to membrane of spin column
14. Let soak for 4min
15. Spin at 13,000rpm, 1min
16. Repeat steps 12 to 14 using first the first elution as the second eluate solution i.e instead of adding 50ul of fresh water to column for the second elution use the first elution liquid, now in bottom of tube containing cRNA, to place back on column and run through again.
17. Take 2x1µl samples – one will be run on a gel and the other to measure cRNA concentration using the spectrophotmeter. Store all samples at –80°C O/N

(G) Quantitate cRNA

1. Take 1µl cRNA sample up in 75µl $H_2O$ (1:75 concentration) and take another tube with 75µl of dilution water as a blank
2. Determine spectrophotometer reading – require 260/280 >1.8
3. Need 10-15µg starting material to carry out affymetrix.
4. Fragment all cRNA – require a final concentration of 1µg/µl

(H) Fragmentation of cRNA

1. Take all cRNA up in $H_2O$ to get a concentration of 1.25µg/µl (eg 19.2µg IVT + 16µl $H_2O$) and add 5xfragmentation buffer to get a 1X concentration.
2. Mix
3. Heat at 95°C for 35min (no longer)
4. Take 1µl sample to run on gel (to check cRNA is completely fragmented) and freeze the rest at –80°C