Method for preparing fragmented cRNA

Method: based on protocol in Expression analysis technical manual (Affymetrix) /Garvan Institute for medical Research - IARP

(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^{o}C 5min to denat). Run gel at ~100V (low) for 40-60min. Also quantitate RNA on spectrophotometer.
- Aliquot 20µg sample RNA, dry the RNA pellet down and resuspend in 10µl RNAse free H20

(B) First strand cDNA synthesis

1. Add 1µl 100pmol/µl T7-$(T)_{24}$ primer (Geneworks) to 20ug 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 70^{o}C, 10min
4. Quick spin and put on ice
5. On ice add to the RNA/primer mix
   4µl 5x 1st strand buffer
   2µl 0.1M DTT (fresh)
   1µl 10mM dNTPs (10mM each dATP, dCTP, dGTP, dNTP)
6. Heat at 37^{o}C, 2min (can do at 42^{o}C, if needed)
7. Add 2µl SuperScript II (400U total) (Life Technologies)
8. Mix and quick spin if needed
9. Heat at 42^{o}C, 1 hour
10. Proceed to second strand cDNA synthesis

(C) Second Strand cDNA synthesis

1. Ice all reagents and first strand cDNA tubes (from section B)
2. Add to first strand cDNA tubes
   90µl H_2O
   30µl 5x 2nd strand buffer
   3µl 10mM dNTPs (10mM each dATP, dCTP, dGTP, dNTP)
   2µl DNA polymerase I (40U)
   1µl E.coli DNA ligase (10U)
   2µl RnaseH (2U)
   Mix and spin if needed
3. Incubate 16^{o}C, 2 hours
4. Add 10µl (10U total) T4 DNA polymerase, incubate 16^{o}C for 5min (to finish off ends properly)
5. Add 10µl 0.5M EDTA, mix

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NOTE: Can store at –80°C if required

(D) Clean up of dsDNA

1. Spin Phase-Lock tubes at maximum speed for 30sec (so gel comes down to bottom of tubes)
2. Add all the cDNA reaction (170µl)
3. Add equal volume buffered saturated phenol (or phenol/chloroform) in fume hood
4. Vortex lightly (white ppt)
5. Spin at maximum speed ~13000rpm, 2 min at 4°C
6. Transfer upper phase to new tube (~170µl)
7. Add
   _ volume 7.5M NH_4OAc (85µl)
   2.5 volume 100% ethanol (415µl)
   1µl glycogen (20mg/ml)
8. Vortex
9. Spin at maximum speed ~13,000rpm for 20min RT or 4°C
10. Decant Supernatant, be careful of pellet
11. Wash pellet twice with 80% ethanol
12. Speed vacuum to dry
13. Resuspend in 23µl DEPC-H_2O, store at –20°C O/N

NOTE: Should take 1µl sample and check on EtBr gel to make sure cDNA is okay, and that the ribosomal bands are still present, before IVT.

(E) In vitro transcription

1. If not already done, take 1µl sample of ds cDNA to run on gel and check
2. To 22µl ds cDNA add:
   4µl 10x HY reaction buffer
   4µl 10x biotin-labelled ribonucleotide
   4µl 10x DTT
   4µl 10x RNase inhibitor mix
   2µl 20x T7 RNA polymerase
   Total volume 40µl
3. Carefully mix and spin liquid down
4. Incubate for 6 hours at 37°C. Mix every hour
5. 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 15sec-1min RT
6. Transfer columns to new collection tubes

7. Add 500µl RPE buffer
8. Spin at 13,000rpm for 2min
9. Add 500µl RPE buffer
10. Spin at 13,000rpm for 2min
11. Transfer spin columns to new collection tube
12. Add 50µl DEPC H_2O to membrane of spin column
13. Let soak for 4min
14. Spin at 13,000rpm, 1min
15. 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.
16. 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^{o}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
3. Need 20µg 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 4µl of 5xfragmentation buffer (200mM Tris-Acetate, pH8.1, 500mM KOAc, 150mMMgOAc)
2. Mix
3. Heat at 95^{o}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^{o}C