cDNA & Biotinylated cRNA Synthesis Reactions for GeneChips

Date:_____

Samples:

Sample ID	Sample Descr.	RNA Input Vol

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C1	*1 ug of mRNA Control*	2 ul

Reagents/Materials needed:

Invitrogen Superscript Choice kit – includes:
5X 1st Strand Buffer
0.1M DTT
10mM dNTP
DEPC-H2O (variable)
SSII RT (variable: see table)
5X 2nd strand buffer
DNA ligase (lot #:_____)
DNA polymerase I (lot#:_____)
RNase H (lot # _____)

T7-(dT)24 primer (100 pmol/ul): 1 ul per sample
Optional: RNasin RNase Inhibitor (Promega)

Table 1

Input RNA (ug)	SSRT (ul)
5-8	1
8.1-16	2
16.1-24	3
24.1-32	4
32.1-40	5

1. cDNA synthesis

PCR PROGRAM = "ROB" => "CDNA"
ALL DONE ON ICE!! Keep enzymes in benchtop cooler at all times.

STEP 1: Primer hybridization

- Calculate amount of RT enzyme needed (see table above), **then** calculate H20.
- Total Volume of STEP 1+2+3 = **20** ml

							C1	
Total RNA							2	- 70 C, 10 mins.
DEPC-H20							9	- Trx to ice, pulse spin
T7 Primer							1	

STEP 2: Temperature adjustment

							C1	
5x 1 st St. Buff.	4	4	4	4	4	4	4	- Add reagents
0.1M DTT	2	2	2	2	2	2	2	- 42C, 2mins
10mM dNTPs	1	1	1	1	1	1	1	

STEP 3: First strand synthesis

RT used: supplier : _____, lot number: _____ Rnase Inh. Lot (Promega Rnasin):_____

							C1	
SSII RT							1	- Start:
RNase Inh.								- End:

-Trx to ice. Pulse spin, 4C

STEP 4: Second strand synthesis

							C1
DEPC-H20	91	91	91	91	91	91	91
5x 2nd S.. Buff	30	30	30	30	30	30	30
10mM dNTPs	3	3	3	3	3	3	3
DNA Ligase	1	1	1	1	1	1	1
DNA Pol I	4	4	4	4	4	4	4
Rnase H	1	1	1	1	1	1	1

- Mix gently. Pulse spin down, 4 C.
- 16 C, 2 hr.
  Start:
  End:
- Pulse spin down, 4C

STEP 5: T4 DNA Polymerase

							C1
T4 DNA Pol	2	2	2	2	2	2	2

- 16 C, 5 min

- Add 10 ul EDTA (0.5M, pH 8) to each vial to quench.
- Mix, place on ice, trx to new 1.5 ml eppendorf tubes. (If stopping here, store at –20.)

Clean-up of (ds) cDNA:

- Spin down PLG-I heavy tubes, 30 sec, max rpm, RT.
- To each sample, add equal vol. Phenol-chloroform-isoamyl alcohol (25:24:1) = **162 ul**. Vortex quickly.
- Trx to PLG-I tubes. **(DO NOT VORTEX.)** Spin 2 mins, max rpm, RT.
- Upper phase to new tube, labeled "G", dated: _____

  Volumes: 1: 3: 5:
  2: 4: C1:

Immediate Ethanol Preciptation:

							C1
CDNA							
0.5 V 7.5 M Rnase-free NH_4OAc							
2.5 V 100% EtOH							
Glycogen	2	2	2	2	2	2	2
Pellet Paint	1	1	1	1	1	1	1

- Spin, 20 minutes, max rpm, 4 C.
- Wash 2x 500 ul 70% EtOH. 5 mins each spin, max rpm, 4 C
- Dry pellet. (Pipette, syringe, swab, air 5').
- Resuspend pellet in 12 ul DEPC-H20

2. In Vitro Transcription (IVT) Reaction to generate biotinylated cRNA:

Reagents needed: cDNA from above steps and the following components from Enzo IVT kit.
Kit lot number: _____; date opened:_____
Has a control been run from this kit? If not, do IVT control (C2) using 2 ul of T7 IVT control (Ambion).
PCR Program = "ROB" => "IVTME"

							C1	***C2***
CDNA	12	12	12	12	12	12	12	12
DEPC-H20	10	10	10	10	10	10	10	10
10x HY Buff.	4	4	4	4	4	4	4	4
10x Bio Ribonucs	4	4	4	4	4	4	4	4
10x DTT	4	4	4	4	4	4	4	4
10x Rnase Inh.	4	4	4	4	4	4	4	4
20x T7 DNA Pol	2	2	2	2	2	2	2	2

Assemble @ RT.
5 hrs, 37 C
Start:
End:
Stir every 45-60 mins.
Trx to ice, spin down @ 4 C.

IVT Clean-Up (via the Chroma-spin method)

- Trx IVT product to 1.5 mL tubes and add 60 ul DEPC-H20 (to 100ul). Labeled "H", date:_____
- Invert columns to mix. Spin down to remove buffer: 2184 rpm, 5 mins, RT. Place column in new tube.
- Add 50 ul cRNA to CENTER of column. Spin 2184 rpm, 5 mins, RT.
- Add 50 ul DEPC-H20 to CENTER of column. LET STAND 1 MIN. Spin 2184 rpm, 5 mins, RT.
- Trx eluate to new 1.5 mL tube.

Volumes: 1: 3: 5: C1:
2: 4: 6: C2:

- Quantitate here via OD_{260}. If RNA is well concentrated here, **then do not proceed to EtOH ppt,** and store @ -80 C in DEPC-H20.

If low [RNA]…

IVT Product EtOH Precipitation:

							C1	***C2***
CRNA								
0.5 V NH_4OAc 7.5m								
2.5 V 100% EtOH								
Glycogen	2	2	2	2	2	2	2	2
Pellet Paint	1	1	1	1	1	1	1	1

- Spec samples (see below). Fragment ASAP.

Add ICE COLD EtOH last.
Vortex. Store O/N @ -80 C.

- Spin 30 mins, max rpm, 4 C.
- Wash 2x 0.5 ml 70% EtOH, 5 mins, max rpm, 4 C.
  Dry (pipette/syringe/swab/air).
- Resuspend pellet in 20 ul DEPC-H20.