

# Target Preparation/Hybridization Using Total RNA

## I. cDNA Generation:

Prepare separate cDNA labeling reaction for each fluorescent dye you wish to use.

A "master mix" (step 4) can be made, and the reaction increased up to 5X if needed.

1. Make dilution of 5.0-20.0 µg of total RNA in 16.0µl of DEPC water.
2. Add 2.0µl of 2.5µg/µl anchored oligo d(T)<sub>20</sub> primer.
3. Incubate at 70°C for 5 minutes. Cool on ice for at least 1 min.
4. Combine the following components in a sterile, RNase/Dnase-free microcentrifuge tube:
  - a. 6.0µl of 5X First-Strand buffer
  - b. 1.5µl of 0.1 M DTT
  - c. 1.5µl of 10mM dNTP mix
  - d. 1.0µl of RNaseOUT™ (40 U/µl )
5. Add the mixture to the annealed primer and RNA.
6. Add 2µl of 400 U/µl SuperScript™ III RT and incubate at 48°C for 2hrs.
7. Incubate at 70°C for 5 minutes to stop reaction.
8. Cool down by spinning in a microcentrifuge at maximum speed for 1 minute.
9. Add 2µl of 2 U/µl RNase H and incubate at 37°C for 20 min.
10. Add 0.5 µl of 0.5M, pH 8.0 EDTA, mix well and proceed with purification.

## II. cDNA purification: (QIAGEN MINElute purification kit)

1. Add 100 µl of Binding buffer **PB** to RT reactions and mix well.  
**Note:** Can add a maximum of 2 reactions per column.
2. Apply to separate spin columns. Incubate for 1 minute.
3. Spin for 1 min at full speed.
4. Discard flow-through.
5. Add 500 µl of Wash buffer **PE** per reaction (Be sure that ethanol was added to **PE** buffer).
6. Spin for 1 min at full speed.
7. Discard flow-through.
8. Repeat wash step.
9. Discard flow-through.
10. Spin for 1 min at full speed to eliminate the possibility of carrying over Wash buffer.
11. Place columns in a fresh 1.5ml microcentrifuge tubes.
12. Add 10 µl of Elution buffer directly to the membrane. (20 µl of Elution buffer if 2 rxns were put on the column).
13. Incubate for 1 min.
14. Spin for 1 min at full speed.
15. Apply flow-through back on membrane.
16. Incubate for 1 min.
17. Spin for 1 min at full speed.
18. Discard columns, spec on Nanodrop to determine cDNA concentration.
19. Dry down in SpeedVac for 15 min at medium temp. DO NOT OVERDRY!

## II. Alternative procedure – cDNA precipitation:

1. Add 3µl of 3M sodium acetate, pH 4.5.
2. Add 1µl of 20mg/ml glycogen.
3. Add 100µl of ice-cold 95% EtOH.
4. Incubate at -20°C for at least 30 minutes. The reaction can be stored at this point for several days or up to 2 weeks.
5. Spin the reaction at 13-14,000Xg for 20 minutes at 4°C. Carefully decant supernatant.
6. Wash with 0.5 ml ice cold 70% EtOH and spin at 13-14,000Xg for 15 minutes at 4°C. Carefully decant supernatant and let to air dry. A vacuum dryer can be used but DO NOT OVERDRY!

### **III. NHS-ester containing dyes coupling reaction:**

1. Resuspend cDNA pellet in 5 $\mu$ l of 2x coupling buffer. (If pellet was over dried gently heat at 37 $^{\circ}$  C for 15 minutes to aid in the resuspension process.)
2. The first time a tube of dye is used, resuspend in 45 $\mu$ l DMSO. Use DMSO provided with the kit.
3. Add 5 $\mu$ l of the resuspended monofunctional reactive dye to cDNA.
4. Mix thoroughly by gently pipetting up and down.
5. Incubate for 30 minutes up to 1 hour at room temp in the dark, flicking the tubes occasionally.

### **IV. Dye-Coupled cDNA Purification:** (using QIAGEN MINElute purification kit)

1. Add 10 $\mu$ l of 3M Sodium Acetate, pH 5.2 to each RT reaction, mix well.
2. Add 100  $\mu$ l of Binding buffer **PB** to RT reactions and mix well.
3. Apply each RT reaction to separate spin columns.
4. Incubate for 1 min.
5. Spin for 1 min at full speed.
6. Discard flow-through.
7. Add 500  $\mu$ l of Wash buffer **PE** per reaction (Be sure that ethanol was added to **PE** buffer).
8. Spin for 1 min at full speed.
9. Discard flow-through.
10. Repeat wash step.
11. Discard flow-through.
12. Spin for 1 min at full speed to eliminate the possibility of carrying over Wash buffer.
13. Place columns in a fresh 1.5ml microcentrifuge tubes.
14. Add 10  $\mu$ l of Elution buffer directly to the membrane. (20  $\mu$ l of Elution buffer if 2 rxns were put on the column).
15. Incubate for 1 min.
16. Spin for 1 min at full speed.
17. Apply flow-through back on membrane.
18. Incubate for 1 min.
19. Spin for 1 min at full speed.
20. Can spec using Nanodrop to determine labeling efficiency and cDNA concentrations.

### **V. Pre-hybridization:** (should start approximately 2 hours before setting up hybridization)

Pre-hybridization buffer = 5X SSC, 0.1% SDS and 1% BSA. (Can make 10% BSA stock and filter before use or purchase pre-filtered BSA; store pre-hyb buffer at -20 $^{\circ}$  C and thaw only once, warm to 42 $^{\circ}$  C prior to use.)

1. Apply 40  $\mu$ l of pre-hybridization buffer to the array and incubate for 42 $^{\circ}$  C for at least 30 mins and up to 1 hour.
2. Wash off the pre-hybridization solution by rapidly plunging the slide in distilled water for 2 mins, then transfer slide to 100% isopropanol for 2 mins.
3. Allow slide to air dry completely prior to use. (Can spin dry if in a rush.) (NOTE: Do not exceed 1 hour after pre-hybridization/drying before setting up hybridization.)

### **VI. Setting up hybridization:**

1. Combine Cy3 and Cy5 labeled targets together (~9  $\mu$ l recovered for each).
2. Add 1 $\mu$ l COT-1 DNA (8-10  $\mu$ g/ $\mu$ l) and 1 $\mu$ l poly A (8-10  $\mu$ g/ $\mu$ l).
3. Denature target at 100 $^{\circ}$ C for 1 minute, then snap cool on ice. (Final volume should be about 20 $\mu$ l.)
4. Make fresh 2X Formamide hybridization buffer (50% formamide, 10x SSC, 0.2% SDS) and warm to 42 $^{\circ}$ C just before adding to samples.
5. Add 20 $\mu$ l of 2X F-hyb buffer to samples.
6. Load 40 $\mu$ l sample onto microarray.
7. Add 20 $\mu$ l of 3X SSC to wells in hyb chamber to maintain humidity.
8. Incubate overnight (12-16 hours) at 42 $^{\circ}$  C in water bath or hybridization oven.

**Wash:**

<b>a. <u>cDNA slides:</u></b>	<b>b. <u>Oligo slides:</u></b>
<ul style="list-style-type: none"> <li>• 2x SSC, 0.1% SDS for 2 minutes, with occasional plunging</li> <li>• 1x SSC, for 2 minutes, occasional plunging</li> <li>• 0.2x SSC, for 2 minutes, occasional plunging</li> <li>• 0.05X SSC, for 1 minute</li> <li>• Spin 3 minutes / 650 rpm to dry</li> </ul>	<ul style="list-style-type: none"> <li>• 2x SSC, 0.1% SDS for 2 minutes, with occasional plunging</li> <li>• 1x SSC, for 2 minutes, occasional plunging</li> <li>• 0.2x SSC, for 2 minutes, occasional plunging</li> <li>• <u>NOTE:</u> The last wash of 0.05X SSC is omitted</li> <li>• Spin 3 minutes / 650 rpm to dry</li> </ul>

<b>WASHES:</b>	<u>2XSSC+0.1%SDS</u>	<u>1XSSC</u>	<u>0.2XSSC</u>	<u>0.05XSSC</u>
<u>dH<sub>2</sub>O:</u>	179 ml	190 ml	198 ml	200 ml
<u>20XSSC:</u>	20 ml	10 ml	2 ml	0.5 ml
<u>20%SDS:</u>	1 ml	-	-	-

**Recommended Supplies for Microarray Probe and Hybridization***(January 2004)*

SUPPLIES	SUPPLIER	ORDERING INFO
SuperScript™ Indirect cDNA Labeling Core Kit	Invitrogen	L1014-03
Cy3 monofunctional reactive dye	Pharmacia	PA23001
Cy5 monofunctional reactive dye	Pharmacia	PA25001
Ribonuclease H	Invitrogen	18021-071
Poly (dA) 40-60	Pharmacia	27-7988-01
Human COT-1 DNA (for human arrays)	Invitrogen	15279011
Mouse COT-1 DNA (for mouse arrays)	Invitrogen	18440016
Lifterslips (25x 40 mm)	Erie Scientifics	25X40I-2-4772
Staining Dish/rack (10 slide)	Fisher	08-812
Slide Box (100 slide)	Thomas Scientific	6708-G28
Slide Box (25 slide)	Thomas Scientific	6708-G08
Hybridization chambers: (2 arrays at a time)	GeneMachines	www.GeneMachines.com
(1 array at a time)	Telechem Int., Inc. Corning	AHC-1 (phone: 408-744-1331) 2551 (800 -492-1110)
Centrifuge with microplate carrier assembly		
Hyb Oven	Fisher Scientific	
Forceps	Fisher Scientific	10-295
Mini-Elute PCR Purification Kit	Qiagen	28004
High Quality Pre-filtered BSA	Invitrogen	15561-020