Protocol #2-Indirect Labeling Method

Preparation of Cy-3 or Cy-5 Targets for Micoarray Hybridization

Note: All the solutions that actually touch the printed microarray slides are filtered through 0.2 µm membrane.

Note: change made in **bold**

I. RNA Isolation

Total RNA (recommended) or poly A+ RNA is isolated from tissues or cells using any existing RNA isolation kit. We have good experience with Trizol reagent (BRL) followed by Qiagen RNeasy column or Qiagen RNeasy column alone on this application. Since good quality of RNA is essential to make good targets, I recommend taking an OD ratio of 260 to 280 in 10mM Tris buffer (pH 7.5) and running a RNA gel to determine the quality of your RNA preparation. The OD ratio should be close to 2.0 (however, 1.9 to 2.1 also worked for me). Two sharp bands reflecting rRNAs for total RNA prep or a nice smear (from 0.5 – 2.0 Kb or higher) for poly A+ prep is expected on gel electrophoresis. If you fail to get good quality of RNA as described, you may want to repeat the isolation again.

II. Target Preparation

1. cDNA synthesis

- 10 µg of total RNA is used per reaction.

You can use as low as 5 µg at an extreme case.
You can scale up the component for multiple reactions in a tube.

- Resuspend RNA in 21 µg of DEPC-dH2O.

If you include the control RNA in your sample, adjust the volume accordingly.

- Add 3 µl of oligo-dT_{18} (50 µM)

Total RNA (10µg)	21.0 µl
Oligo-dT at (50µM)	3.0 µl

- Heat at 65°C for 5 min.
- Quick spin and put the tube on ice for 2 min.
- Incubate @ 42°C for 5 min.
- In a separate tube, add

5X First strand buffer	8.0 µl
DTT (0.1M)	4.0 µl
dNTPmix*	1.0 µl
aa-dUTP*	1.0 µl
powerscript	2.0 µl

total final volume per reaction	40 µl

- Preheat the mixture @ 42 °C for 5 min
- Transfer 16µl per reaction to RNA-oligo mix.
- Incubate @ 42°C for 1.5-2 hrs.
- At the end add 1 µl of 0.5M EDTA.

You can stop here and store the target @ –20°C over night.

- Add 5 µl of strata clean resin per reaction and vortex gently for 30 sec.
- Spin at 5K for 1 min (personal quick-spin microfuge is also fine).
- Transfer the aqueous layer to a different tube– discard pellet.
- Add another 5 µl of strata clean resin per reaction and vortex gently for 30 sec.
- Spin at 5K for 1 min (personal quick-spin microfuge is also fine).
- Transfer the aqueous layer to a different tube– discard the pellet.

you can stop here and store the target @ –20°C over night.

- Heat targets at 95°C for 3 min.
- Quick spin and cool on ice for 2 min.

2. RNA Hydrolysis

- Add to each tube (per reaction).

1 M NaOH	15 µl
0.5M EDTA	14 µl

- Mix and incubate at 65°C for 15 min.
- Quick spin & transfer the tube on ice
- Add 15 µl of 1M HCl to neutralize the pH.
- Add 8 µl of 1M Tris-HCl, pH 7.5

3. Target Clean-Up

Note: speed and time required for spin may vary with different centrifuges.

- Add 350 µl of ddH2O to the targets.

For multiple reactions in a tube, add appropriate volume of ddH2O to make final volume up to 450 µl.

- Apply the target to Microcon-50
- Spin at 12,000 rpm for 8 min.

For one reaction sample size, there should be liquid left on the membrane after spin. If not, you should reduce the speed or spin time.
For multiple reactions, you may need longer spinning time.
After 8 min spin, spin additional 30 sec or 1 min each time until you can see the membrane of the microcon column (usually you'll see the membrane shines when it dries).

- Discard the flow through.

- Add 450 µl of ddH_2O on the membrane.
- Spin at 12.000 rpm for 8 min.
- Repeat the washing one more time.

For multiple reactions, you may need longer spinning time.
After 8 min spin, spin 30 sec or 1 min each time until you can see the membrane of the microcon. It's necessary to allow the next step to obtain appropriate volume to continue.

- Recover the DNA in a new tube by turning the top column upside down and spinning at **4,000 rpm** for 3 min.
- Adjust the target volume to 4 µl.

You expect to obtain samples ~2-4 µl.
If the volume is more than 4 µl, bring down the volume with speed-Vac.

4. Dye-Coupling Reaction

- Add 1 µl of 1 M $NaHCO_3$, pH 9.0 to 4 µl of targets solution or dissolve the dried target-DNA in 5µl of 0.2M $NaHCO_3$, pH9.0.

$NaHCO_3$ buffer needs to be replaced every 2-3 weeks.

- Add 5µl of Cy3 or Cy5 dye-solution* to each target.
- Mix and incubate for 45 min to 1 hour in dark @ RT.

5. Clean-up after Coupling (QiaQuick PCR purification Kit)

pre-heat the EB buffer (60-65°C) in the kit.

- Bring the sample volume to 100 µl with ddH2O.
- Add 500 µl of PB buffer to the sample and spin @ 10,000 rpm for 15 sec.
- Reload the flowthrough to the column one more time and spin @ 10,000 rpm for 15 sec.
- Discard the flow-through.
- Add 500 µl of PE buffer and stay for 1 min.
- Spin @ 10,000rpm for 15 second.
- Discard the flow-through.
- Repeat the PE buffer wash one more time.
- Spin again @ max speed (~13K) for 3 min
- Add 50 µl of EB buffer to the column, stay for 2 min and spin @ 12,000 rpm for 2 min.
- Repeat the elution one more time.
- Analyze your labeling reaction by OD.

Note: protocol is available on the website.
Total volume = 100 µl for each target
You may store the labeled target @ 4 °C for a couple of days if necessary until ready for hybridization.

6. Mix of Labeled Targets (Microcon-50)

- Combine Cy-3 & Cy-5 targets.
- Add 250 µl of ddH_2O to the mixture.
- Transfer to a Microcon-50.

Note: if you are going to use LucidiaPro hybridization automation setup, spin for a short time until the total volume of labeled mixture is 100 µl. Then add desired volume of blocking solution (at least 1µl or more, formula see below) into the mixture. Follow the instruction for sample submission to the facility (see SlidePro hybridization form).

For manual hybridization, continue the following procedure:

- Spin @ **10,000** rpm for 8 min.
- Spin additional 1 min each time until the membrane is seen.
- Reverse the top column on a new tube and spin @ **4000 rpm** for 3 min.
- Adjust volume to 6.5 µl.

~2-4 µl in volume is retained.

III. Preparation of Hybridization Cocktail & Prehybridization

- To each hybridization reaction, add the following component:

			Final
Formamide	30	µl	50%
50 X Denhardts Solution*	3	µl	2.5X
20 X SSPE	18	µl	6X
20% SDS	1.5	µl	0.5%
Hyb blocking solution*	1.0	µl	
Target mix	6.5	µl	
Total	60	µl	

- Heat the cocktail @ 95°C for 3 min.
- Incubate @ 42°C for 30-60 min.

IV. Preparation of Denatured Microarray Slide for Hybridization

Note: Slides should be kept in a slide box all the time during storage or transportation. All printed arrays have been baked @ 80°C for 90 min.

- Before starting, place slide on a piece of paper and draw an outline around it. Then, mark the area of the actual prints where you can see the dried salt left on the slide.
- Dissolve 1.3 g of succinic anhydride (Sigma) in 82 ml of n-methyl-pyrrolidinone (Aldrich). Shake until dissolved.
- To this, add 9.1 ml of 0.2 M NaBorate, pH8.0 (pH'd with NaOH) & mix well before use.

Solution turns yellow upon time.

- Arrayed DNA is vapor moistened (?1 sec) over boiling water.
- (Optional) Moistened slide is quickly exposed to 300 miliJoule total energy in a Stratalinker.

DNA site up

since printed DNA has been immobilized on slide by baking, it's not necessary to crosslink again. However, we find that this step can further increase a little bit on the hybdizatioin signal.

- (Optional) Remove slide and moisten again over boiling water.
- Heat snap slide on a hot plate (mild heated) (3 seconds or they break).
- Soak arrays in the pre-prepared succinic solution for 15-30 min with shaking.
- Rinse slide in 0.1% SDS for 1 min. (You will no longer see the prints after this step).
- Rinse in water for 1 min with one time change of water.
- Boil in 95^0 C water bath for 5 min.
- Dunk into ice-cold ethanol.
- Remove excess ethanol from slides by spinning the slide in a 50-ml conical tube at 1500 rpm for 5 min (Beckman) or by clean air spray.
- Slides are now ready for prehybridization.

V. Prehybridization and Hybridization of Microarray Slides

- Make a master mix of prehybridization buffer (scale up as needed)

			Final
dd H2O	6.5	µl	
Formamide	50.0	µl	50%
20 X SSPE	30.0	µl	6X
20% SDS	2.5	µl	0.5%
50 X Denhardts Solution	10.0	µl	5X
ss Salmon sperm DNA (10 mg/ml)	1.0	µl	0.1µg/µl

heat @ 95°C for 5 min and quickly chill on ice before adding to the mix).

Total	100	µl

- Add 45-60 µl of prehybridization buffer over the array.

45 µl for 24x50 mm coverslip; 60 µl for 24 x 60 mm coverslip.

- VERY CAREFULLY place coverslip (pre-cleaned with 0.1% SDS and ddH2O) on top of array using a fine forceps (make sure that the edges of the oversleep never touches the array area outlined earlier).
- Place slide in a humidified hybridization chamber (Telechem International) and incubate in a 42°C water-bath for at least 30 to 1 hour (3-hour prehyb is fine).
- After prehybridization, very carefully slide the covership to the edge of the slide and using the forceps to lift off the array.
- Tilt the slide and use a kimwipe to blot any prehybridization solution that flows to the slide edge.
- As soon as possible, pipette hybridization cocktail (45-60 µl) over the array and place a new cleaned coverslip over slide as before.

Do not allow the slide to dry on prehyb buffer at any time.

- Hybridize at 42°C overnight.

VI. Washing Arrays

- Place slide in a glass slide holder containing 1 X SSC, 0.1% SDS at RT# and shake so that the solution causes the coverslip to fall off. Shake for 1 min.
  # For a more stringent wash, you may heat up this wash solution to 42°C.
- Remove slide and place in another slide holder containing 0.2 X SSC, 0.1% SDS. Shake slide and change the washing buffer at least 1 time over 10 min @RT.
- Repeat this in another chamber but containing 0.2 X SSC only (NO SDS) for 5 min. Note: it is important to remove all SDS as it causes background florescence @RT.
- Immediately put slide in a 50-ml conical tube and spin for 5 min at 1500 rpm as before to remove all residual liquid.

#Gently take out the slide using a forceps. Do not reverse the tube, as the wash buffer will wet your slide again.

- Slide is now ready to scan in a laser confocal scanner.

Note:
If you process multiple slides at a time, be sure to have extra volume of each wash solutions.
Do not allow prehyb or hyb buffer to dry during the process any time. It gives high level of fluorescence background.

***Stock Solutions**:

1. dNTPmix

	dATP(100mM)	20	µl
	dCTP(100mM)	20	µl
	dGTP(100mM)	20	µl
	dTTP(100mM)	6.5	µl
	ddH2O	33.5	µl
Total		100	µl

Store aliquots @ -20°C

2. aa-dUTP (13.5 mM)

dissolve 1 mg of powdered aa-dUTP (90% purity) in 126 µl of RNAse-free water.

Store aliquots @ - 20°C

3. Cy-dye-solution

To a new vial of dye, add 72 µl of **provided DMSO**. Dissolve completely and mix well. Aliqout 12 µl in each tube.

Store aliquots @ -20 ^{o}C in dark.

4. 50X Denhatdts solution

Ficoll 400	1 g
Polyvinylpurrolidone	1 g
BSA	1 g
H_2O	to 100 ml

Store filtered aliqouts @ – 20°C

5. Preparation of 1X Hybridization Blocking Solution#

Combine the following components to a 1.5 ml tube

8 µl of Poly dA (Pharmacia, 5 µg/µl)	or 40 µg
16 µl of yeast tRNA (5 µg/µl)	or 80 µg
200 µl of (human or mouse) Cot 1 DNA (BRL	or 200 µg

Add 22.4 µl (1/10 volume) of 3 M NaAcetate pH5.2 to the mixture, then add 616 µl (2.5X total volume) of 100% ethanol to precipitate the DNA at 20° C.
Spin down the DNA pellet. Wash with 70% ethanol and air-dry. Dissolve the pellet in 20 µl of dH_2O.

#For some RNA samples, higher concentration of Poly dA and Cot1 DNA is required to lower the non-specific hybridization. For example, you may increase the concentration of poly dA to 8 µg/µl instead of 2 µg/µl as indicated here. Please adjust the quantity as needed. Control DNAs of complementary sequence are spotted on the array for your reference.

* For consistency and easy use, the Microarray Resource Facility has put up a kit together for this protocol at manufacturer's price. Please inquire the information.