

## Microarray Protocol

### *Annealing Reaction*

1. Prepare primer-annealed RNA reactions; one for each Cy dye in a dye-swap experiment:

RNA (25 µg**) plus H <sub>2</sub> O	12.4 µL
Oligo dT (2 µg/µL)	2.5 µL
Random Oligo (2 µg/µL)	0.5 µL
<b>Final volume</b>	<b>15.4 µL</b>

- Where possible, prepare master mixes. For example, make a stock mix of oligo-dT and random oligos, and use 3 µL for the labeling reaction.

2. Annealing for gene-specific priming if using a gene specific sequence:

RNA (25 µg**) plus H <sub>2</sub> O	11.9 µL
Gene Specific RT Primer (2 µg/µL)	1.0 µL
Oligo dT (2 µg/µL)	2.0 µL
Random Oligo (2 µg/µL)	0.5 µL
<b>Final volume</b>	<b>15.4 µL</b>

3. Heat for 10 minutes at 70°C. Quick-chill on ice.

- Use at least 15 µg total RNA (up to 100 µg), or 2 µg polyA mRNA.
- Aim to use the same amount of RNA in all experiments. RNA labeling reactions are more efficient when the RNA is free from protein contamination. An A260/A280 ratio of 1.8-2.0 is acceptable. Rather than try to clean unacceptable RNA preps, repeat the RNA isolation.

**RT Reaction**

1. Prepare reverse transcription master mix:

5x First Strand Buffer	6.0 $\mu$ L
0.1M DTT	3.0 $\mu$ L
50x dNTPs*	0.6 $\mu$ L
Superscript II	2.0 $\mu$ L
<b>Final volume</b>	<b>11.6 <math>\mu</math>L</b>

\*50x dNTPs = 25mM dA, dC, dGTP; 10mM dTTP

2. Add 3.0  $\mu$ L Cy3 or Cy5 labeled nucleotide to respective primer-annealed RNAs.
3. Aliquot 11.6  $\mu$ L of the master mix to each reaction. This brings the total volume to 30  $\mu$ L.
  - Alternatively, prepare a master mix RT cocktail that does not contain enzyme; add 9.6  $\mu$ L of the cocktail to the chilled annealed primer/mix; add 3.0  $\mu$ L of the appropriate Cy dye; then add 2.0  $\mu$ L of Superscript II enzyme.
  - When making a master mix for several reactions, prepare enough to account for pipetting error (e.g., 20% more of each component).
4. Incubate 2 hours at 42°C. Place on ice.
5. Hydrolyze the remaining RNA by adding 1.5  $\mu$ L of 1N NaOH and 1.0  $\mu$ L of 60mM EDTA. Incubate at 65°C for 10 minutes. Return to ice.

**Labeled Target Clean-up****STEP 1**

1. Prepare one Microcon-30 filter for each reaction. Place 500  $\mu$ L TE (pH 7.4) into each filter retentate cup.
2. Add the RT reactions to the TE and centrifuge for 7 minutes at 10,000 rpm.
3. Repeat the TE wash by adding 400  $\mu$ L to remove unincorporated dyes.
  - Although it is possible at this step to recover the flow-through in order to re-purify unused labeled nucleotides, base hydrolysis of the RT reactions prevents good recovery.
4. Inspect the filters. Centrifuge in short intervals (30–45 seconds) until the volume is 10–20  $\mu$ L.
  - At this point a small amount of "flocculent" or "stringy" Cy dye-labeled material should be evident on the membrane. If you can see it, this is a good sign that cDNA synthesis was efficient.
5. Invert the filters into fresh tubes. Centrifuge 1 min (8000 rpm-soft) to harvest labeled cDNA.

## STEP 2

1. Combine Cy3- and Cy5-labeled cDNAs to be hybridized together, and clean up using the QIAquick kit:
  - If labeling has been efficient for both Cy dyes, then this mixture should look purple. Differing labeling efficiencies shift the color to red or blue.
2. Bring the volume to 100  $\mu$ L with water.
3. Add 5 volumes (500  $\mu$ L) PB to labeled cDNAs and apply to a QIAquick column.
4. Spin the column at 13,000 rpm for 30-60 seconds.
5. Aspirate off and discard the flow-through.
6. Add 750  $\mu$ L of PE to the column.
7. Spin at 13,000 rpm for 30–60 seconds.
8. Aspirate off and discard the flow-through and spin for 1 minute to dry the column.
9. Transfer the column to a clean Eppendorf tube.
  - At this stage the column membrane should be a shade of purple, purple-red, or purple blue.
10. Add 25  $\mu$ L of EB directly to the membrane and allow it to sit for 1 minute.
11. Spin at 13,000 rpm for 30–60 seconds.
12. Repeat the elution, combining the eluates.
  - Elutions should contain the labeled cDNA, and its color should be easily detected. If there is little or no color at this point of the purification, the labeling reaction has probably failed.

### ***Concentrate/Dry Down Labeled Targets***

1. Reduce the volume of the QIAquick-purified RT reaction (typically 60  $\mu$ L) by evaporation in a Speed Vac.
  - Do not precipitate with ethanol.
  - Heating the Speed Vac to 42°C accelerates drying.
  - Protect the labeled cDNA from light during the drying.
2. When the sample is dry, a colored patch of labeled cDNA material should be evident in the bottom of the tube.
3. If you have a small-volume spectrophotometer (such as the NanoDrop ND-1000), you can measure the incorporation of Cy dye into cDNA by re-suspending the labeling reaction in 10–20  $\mu$ L of water (see volume specifications below) and measuring incorporation using 1  $\mu$ L of sample.

## Hybridization Set Up

### Make 2X Hybridization Buffer:

HYB BUFFER FINAL	2X HYB BUFFER	200 $\mu$ L 2X HYB BUFFER
4x SSC	8x SSC	80 $\mu$ L
0.1% SDS	0.2 % SDS	4 $\mu$ L
0.1 mg/mL PolyA	0.2 mg/mL PolyA	4 $\mu$ L (10 mg/mL)
	dH <sub>2</sub> O	112 $\mu$ L

- All solutions should be filtered through a 0.45-micron filter.
- Do not use old solutions of SDS.
- PolyA RNA is used as a blocking agent in the hybridization.

### Hybridization Mixes:

1. Resuspend samples in 10  $\mu$ L or 20  $\mu$ L of dH<sub>2</sub>O (see below). Pipet to dissolve samples.
2. Add an equal volume of 2X HYB BUFFER (recipe above).
  - Use a minimum of 2.5  $\mu$ L target per cm<sup>2</sup> of cover slip.
  - Use a total hybridization volume of 20  $\mu$ L for a 22 x 25 mm coverslip; use 40  $\mu$ L for a 25 x 44 mm cover slip.
  - The hybridization mix should show some color.

### Filter Hybridization Mixes For Particulates:

1. Pre-wet a 0.45- $\mu$ m Millipore Filter by pipetting 5  $\mu$ L H<sub>2</sub>O and 5  $\mu$ L of 2X HYB BUFFER onto the membrane. Centrifuge for 1 minute at top speed.
2. Remove the eluted HYB BUFFER with a pipet.
3. Add the hybridization mixture to the filter. Pipet the hybridization mixture onto the filter wall, not directly onto the membrane.
4. Centrifuge for 2 minutes at top speed.
5. Discard the filter.

### **Set Up Hybridizations:**

1. Heat the filtered hybridization mixture to 94°C for 2 minutes.
2. Spin for 30 seconds to cool the mixture.
  - Don't apply hot hybridization mixture to the slide.
3. Carefully apply the mixture to the surface of a processed microarray on the array area.
  - If using a regular coverslip, gently "roll" over the hybridization mixture with it (avoiding bubbles).
  - If you are using a "Lifterslip": place the Lifterslip over the array first and pipet the hybridization mixture underneath, starting at one corner; capillary action will draw the hybridization mixture across the entire coverslip surface.
4. Place the slide into a hybridization chamber.
5. Place a small piece of filter paper at one end of the slide (e.g., on the label). Pipet 3x SSC onto the paper until it is saturated.
  - This will keep the chamber humidified and is critical for preventing evaporation of the hybridization mixture during the overnight incubation.
6. Close the chamber and place it into a pre-warmed, sealed, water-filled Tupperware box; submerge in a 65°C water bath.
7. Incubate overnight (range of 4–24 hours; typically for 12–16 hours).

### ***Wash and Scan***

1. Disassemble the hybridization chambers quickly and immediately place the slides in a Coplin jar containing room temperature 2x SSC / 0.1% SDS. Allow the coverslips to fall off; agitate gently if necessary.
  - Do not allow the slides to dry while moving them between Coplin jars.
2. Wash the slides as follows:
  - a. Wash in 2x SSC / 0.1% SDS at hybridization temperature (65°C) for 5 minutes.
  - b. Repeat.
    - Hybridization temperature washes are critical to prevent background noise.
  - c. Wash in 0.2x SSC at room temperature (25°C) for 1 minute (no SDS).
  - d. Wash in 0.05x SSC at room temperature (25°C) for 1 minute (no SDS).
3. Remove the slides from the last wash and place them in a slide rack. Put the slide rack on a plate rotor and immediately spin dry for 2–4 minutes at 1,200 rpm.
  - Do not allow the slides to dry before centrifugation.
4. Scan each slide. If the pre-scan indicates background noise from poor washing, you can rewash the slide at least a couple of times.