SYNTHESIS OF FLUORESCENTLY LABELLED cDNA PROBE FOR MICROARRAYS

DAY 1

- ***Note the reverse transcriptase, coupling and hybridization protocols have been adapted from those posted on www.microarrays.org/protocols.html by Joe DeRisi.***
- ***Blocking protocol has been adapted from those posted at the Erie Scientific website.***
- ***Protocol adapted from Gross Lab (Virgil Rhodus)***

Block Microarray slides

(Adapted from Erie Scientific)

A/ Stock BSA solution and blocking (remember to *etch* arrays on back, label side down, before proceeding.

1) **Make stock BSA solution**. In 1L beaker add 10 g Fraction V BSA to 840 ml MilliQ h_20, stir at RT until dissolved (takes several hours).
1) Add 150 ml 20x SSC and filter sterilize with 22 μm filter (can be stored at 4 ºC for up to 3 months).
1) **Blocking.** Fill dish with RT BSA solution and set on rotator with slides for 10 min.
1) Transfer slides to MilliQ h_20, plunge ~30x. Repeat 4x.
1) Transfer slides to boiling (>95 ºC) MilliQ h_20, incubate 2.5 min on bench.
1) Spin slides ~1 (400k)
1) Stores slides in slide box for up to 1 week.

Reverse transcriptase reaction

(Adapted from Joe De Risi and Holly Baxter, and developed by Rosetta Inpharmatics, Kirkland, WA)

A/ Primer annealing and cDNA synthesis

Note – continue to use RNase free tubes, pipette tips and solutions until the end of step A

1) **Annealing step**. In 0.5 ml microfuge tubes, mix 20 μg RNA sample with 10 μg random hexamer (10 μl of 1 μg/μl) in H_20 (DEPC treated, RNase free) to give a final volume of 20 μl.

1) Incubate mixture of RNA and hexamer at 70 °C for 10 min.

1) Chill on ice for 10 min.

1) **cDNA synthesis reaction:** Make up following master mix:

Vol. per reaction (μl)	Reagent	
3	**10× StrataScript RT Buffer**	Stratagene
0.6	**50× aa-dUTP/dNTP mix**	50× mix = 25 mM each dA/dC/dG, 15 mM amino-allyl dUTP, 10 mM dTTP
3	**StrataScript RNase H-RT**	Stratagene; catalog #600085-51
0.4	**RNase Inhibitor (40 U/μl)**	Boehringer Mannheim; catalog #799 017
3	**0.1 M DTT**	
10	**Total vol/tube**	

5) Add 10 μl of the Master Mix to each RNA/hexamer mixture (20 μl) to give 30 μl final volume.

5) Incubate at **37 °C for 10 min.**

5) Incubate at **42 °C for 1 hr 40 min.**

5) Incubate at **50 °C for 10 min.** (you can freeze samples @ -20ºC here)

B/ RNA Hydrolysis

1) Add **10 μl 0.5 M EDTA (pH 8.0)** to the 30 μl RNA/cDNA reaction, mix and spin, then add **10 μl 1 N NaOH** (freshly prepared or from unopened frozen aliquots).

1) Incubate at 65°C for 1 hr.

1) Add **25 μl of 1 M Hepes pH 7.5** to neutralize the reaction (or 10 μl 3M NaOAc pH 5.2).

C/ Cleanup using Microcon-30 filters

Note – When using the Microcon filters, try not to let sample spin dry: if this occurs, the sample can be recovered simply by adding ~30 µl H_2O to the membrane, incubating for a few minutes and then eluting the sample. The spin times are approximate and will vary from batch to batch and sample to sample.

1) Fill microcon-30 tube with 350 μl H_2O, add sample (~75 μl), and rinse reaction tube with 100 μl H_2O. (Total amount of H_2O added is 450 μl).

1) Spin at 10,000 g for 8.5 min using Beckman centrifuge rotor, F2402.

1) Check filter/vol. in upper chamber. Should be between 10-50 μl; if not, spin for additional 1-2 min. Recheck volume. Discard flow-through.

1) **Wash 2 times** by adding 450 μl H_2O to upper chamber and recentrifuging at 10,000 rpm for 8.5 min. Each time ensure the volume has reduced to 10-50 μl before proceeding.

1) **Elute sample** by placing the microcon **inverted** into a fresh microfuge tube. Centrifuge at 5,000 rpm for 30 sec.

1) Dry the sample in a speed vac. (approx. 20 min.) or ~3 μl remains . **Do not over dry**.

1) Store dried samples at –20 °C.

NB – the dried samples can be stored at –20 °C for at least 1 month.

DAY 2

Coupling reaction of Alexa dyes to aadUTP-cDNA sample and overnight hybridization to microarray

Note – the Alexa dyes are light sensitive. Therefore minimize light exposure where possible during the following procedures. In addition, the Alexa dyes degrade over a few days. Only perform the coupling reaction if it is possible to directly proceed to the hybridization step and then on to scan the microarrays.

A/ Coupling of Alexa dyes to the aadUTP-cDNA

Note – the Alexa fluor 555 dye appears pink, but scans as 'green' (comparable to Cy3), and the Alexa fluor 647 dye appears blue, but scans as 'red' (comparable to Cy5). By convention, the 'wild type' or control sample is labeled with 555 and the experimental sample is labeled with 647.

1) Dissolve cDNA in 5 μl dH_20, warm @ 42ºC ~5'.

2) Add 3 μl labeling buffer (25 mg sodium bicarbonate, 1 ml dH_20) to each sample, mix well.

3) Add 6 μl RT DMSO to tube of dye and resuspend (1 tube of dye per 3 samples). Add 2 μl DMSO/dye to sample.

4) Mix cDNA and dye together and incubate at room temperature for 1 hr in the dark.

B/ Cleanup with QIA-quick PCR kit.

1) Add 90 μl H_2O to each sample to make up to 100 μl.

2) **Add 500 μl PB** buffer from kit.

3) Apply to QIA-quick column. Spin at 13,000 rpm for 30-60 s.

4) Dump flow through. **Add 750 μl PE** buffer and spin at 13,000 rpm for 30-60 s.

5) Dump flow through. **Repeat PE** buffer wash step 4x.

6) Dump flow through. **Spin for 1 min** at 14,000 rpm. (Filters should look pink for Cy3 and blue for Cy5 reactions at this point).

7) Transfer to a fresh eppendorf tube. **Add 30 μl Tris pH 8.5 (EB buffer)**. Let sit 1 min. Spin 13,000 rpm for 1 min.

8) **Add an additional 30 μl** to column. Let sit 1 min. Spin 13, 000 rpm for 1 min.

9) Final volume 60 μl. The solutions should be clearly pink for Cy3 and blue for Cy5 at this point. If they are not, the labeling reaction did not work.

10) Pool sample pairs to give 120 μl of purple solution.

11) Apply samples to microcons and spin for 1.5-2 min to reduce sample to 2-5 μl. The flow through will be clear and the sample will be strongly visible on the membrane.

12) Invert microcons and elute samples into fresh tubes.

13) Dry samples in Speed Vac. Cover the lid with foil to avoid exposing the sample to light. (Approximately 5-15 min. to dry).

14) Store the dried sample in the dark (wrapped in foil) at 4°C. Stable for 1-2 days**.**

Day 3

A/ Hybridization step

(Hybridization conditions: cDNA from 16 µg total RNA, 15 µg poly(dI-dC), 3× SSC, 25 mM Hepes (pH 7.0), 0.225% SDS)

1) Set up the following hybridization mix in a 0.5 ml microfuge tube.

37.8 µl	resuspended cDNA in H_2O (dissolve at 65°C, 1-2 min.)
7.1 µl	20× SSC
1.2 µl	1 M Hepes pH 7.2
1.0 µl	10% SDS
47.1 µl	**Total volume**

Add SDS last after mixing the cocktail; do not chill samples after adding SDS, this will cause the SDS to precipitate.

2) Incubate samples at 95°C in dry heating block for 2 min.

3) Allow samples to cool 5–10 min at room temperature and spin down briefly.

B/ Slide preparation

Use fresh or less than 2 weeks old post-processed slides.

1) Whilst samples are cooling, place slides in hybridisation chamber and remove any dust using compressed air briefly.

2) Clean coverslips using EtOH soaked Kimwipes. Dry and dust with compressed air and carefully place over the top of the microarray using forceps such that the **dull white strips (rough side down)** are on the long axis of the slide and touching the glass.

3) Add a total of 6-10 2 µl drops of 3× SSC at the two ends of the slides removed from the coverslip.

C/ Sample application

1) After the samples have cooled, apply to the array by placing a pipette tip at one end of the coverslip and allow the sample to move up underneath the coverslip by capillary action. Move the pipette tip repeatedly along the length of the coverslip to avoid any bubbles. Add sample to the other end of the coverslip once completely full underneath, to 'top up' both ends.

2) Place cover on the hybridization chamber and tighten the lid screws carefully to make water tight. Keep the chamber horizontal at all times so as not to disturb the 3× SSC droplets.

3) Carefully lower the hybridization chamber onto a plastic holder in a water bath.

4) Hybridize at 63-65 °C for at least 5-6 hrs, or overnight (12 hrs max.).

D/ Rinse Step

1) Prepare wash solutions in glass slide dishes, with each dish having its own rack.

Wash solution 1:	340 ml Milli-Q water 10 ml 20× SSC 1 ml 10 % SDS
Wash solution 2:	350 ml Milli-Q water 1 ml 20× SSC
Wash solution 3:	350 ml Milli-Q water 100 μl 20× SSC
Wash solution 4:	350 ml Milli-Q water 10 μl 20× SSC

2) Remove array carefully from the water bath, keeping the chamber level. Dry the chambers with paper towels and 'wick' any water from the chamber seems.

3) Unscrew the chamber and remove array slide.

4) **First Rinse:** Rinse slide in Wash solution 1. Use forceps to move slide gently up and down in the solution until the coverslip is dislodged. Avoid allowing coverslip to scratch the surface of the array. Once coverslip is off and all the slides are in place, shake in solution by plunging rack up and down 10-20 times. Let incubate for 1 minute.

5) **Second Rinse:** Individually transfer slides to Wash solution 2, blotting the base of the slide on a paper towel to avoid carrying over too much SDS. Shake gently in solution a few times. Let incubate for 1 minute. Repeat for washes 3 and 4.

6) Remove excess liquid by blotting the rack on a paper towel, and then dry array at room temperature by centrifuging at 600 rpm for 5 min.

7) Scan array soon as the dyes are unstable and degrade differentially.