CMB Microarray protocol 7/21/11

Rehydrate microarray slide as follows:

1. Hold microarray, face down, about 4-6 inches above a 50oC water bath for 7 seconds.
2. Snap dry on a 65oC heat block for 5 seconds.
3. Repeat steps 1 and 2, 4 to 6 times.
4. Examine slide under scope to verify re-hydration of all spots (spots go from cracked open circles to more solid circles).
5. If rehydration is not complete, repeat steps 1 and 2 until it is.

Crosslink spots onto the slide's surface as follows:

1. Place slide face up in UV linker.
2. Set Energy level to 180mJ and push start. Linker should light up and count up the energy level. When it reaches 180mJ it will stop and linking is done. This process takes about a minute.
3. Mark edges of slide with a pencil where spot grid is. Once wet, it will be hard to discern for cover slip placement.
4. Wash slides in 1% SDS dissolved in sterile ddH2O for 5 minutes at RT.
5. Rinse slides by plunging 10 times into sterile ddH2O.
6. Rinse in 100% EtOH for 2 minutes with gentle shaking.
7. Spin dry the slides in a centrifuge at 1000rpm for 2 minutes
8. Store slides in a light-proof box under cool dry conditions (we do a 50ml tube wrapped in foil, in a cupboard).

cDNA Synthesis –take all necessary precautions to eliminate RNase contamination (wear gloves, wipe down work area with 10% bleach and dry completely, use only nuclease-free tubes and ddH2O).

1. For each RNA sample to be labeled and hybridized to a microarray, prepare an RNA-Primer mix in a PCR tube as follows:

   1-9µl of RNA (3µg total)
   6µl Random RT primer (Vial 2) (2µl/µg RNA)
   1µl oligo DT (Vial 8)
   Bring up to 15µl with nuclease free-water (Vial 10)

2. Mix and spin each PCR tube.
3. Heat each RNA primer mix to 80oC for 10 minutes in a thermal cycler and place immediately on ice for 2 minutes.
4. While RNA primer mix is incubating, make a reaction master mix for each RNA-Primer mix as follows:

   5µl 5X Superscript II First Strand Buffer
   2µl 0.1M DTT (supplied with Superscript enzyme)
   1µl dNTP mix (Vial 3)
   1µl Superase-In RNase inhibitor (Vial 4)
   1µl Superscript II enzyme, 200units
   Final volume 10µl

5. **Gently** mix, spin, and keep on ice until ready to use.
6. Add the Reaction Master mix to its corresponding RNA primer mix (total volume now 25µl), **gently** mix and spin briefly.
7. Incubate at 42oC for 1.5-2 hours.

   - Stop cDNA Synthesis as follows:

1- Add 4.35µl of 0.5M NaOH/50mM EDTA to each reaction.
2- Incubate at 65oC for 15 minutes.

-Neutralize Stop Reaction as follows:

1- Add 6.25µl 1M Tris-HCl, pH 7.5.
2- Add 26.88µl 10mM Tris, pH8/1mM EDTA.
3- Mix gently and spin. Total volume now ~58µl.

Purification of cDNA with Qiagen Minelute Kit – Purify each reaction separately. Below is for a single 50 – 60µl reaction.

1. Add 250µl of Buffer PB to each sample and mix and spin (290µl for at 58µl reaction).
2. Apply cDNA/PB to the MinElute column. Centrifuge for 1 minute at 17,900 rcf. Discard flow-through.
3. Add 750µl Buffer PE to column. Centrifuge for 1 minute at 17,900 rcf.
4. Discard flow-through and centrifuge for an additional 2 minutes at 17,900 rcf.
5. Move column to a fresh 1.5ml eppie tube and warm both the tube and EB buffer to 60oC.
6. Add 18µl of Buffer EB to center of the column.
7. Incubate for 2 minutes at 60oC.
8. Centrifuge for 2 minutes at 17,900 rcf.
9. Reapply eluate to column and repeat steps 7 and 8.
10. Discard column, transfer 2µl of flow-through to a fresh tube and add 4µl of EB buffer for quantitation of 2 µl in duplicate, using the nanodrop spec with EB buffer as the blank.
11. Transfer remainder of each purified cDNA sample (measure but should be around 15µl) to a fresh PCR tube and bring up to 16.5µl with nuclease-free water. Can stop here and store at -20oC.

Perform Terminal Deoxynucleotidyl Transferase (TdT) Tailing Reaction on each cDNA sample as follows:

1. Heat each cDNA sample to 95oC for 10 minutes in the thermal cycler and place immediately on ice for 2 minutes.
2. Add the following components to each cDNA sample for a final volume of 25µl:

   2.5µl 10 Tailing Buffer (Vial 13)
   4µl 10mM dTTP (Vial 4)
   2µl Terminal Deoxynucleotidyl Transferase (Vial 15)

3. Mix gently, spin, and incubate at 37oC for 30 minutes.

Proceed immediately to Ligation of 3DNA Capture Sequences as follows:

1. Heat tailed cDNA sample to 95oC for 10 minutes in the thermal cycler and place immediately on ice for 2 minutes.
2. Determine which dye you will use (Cy3-Green or Cy5-Red) for each sample and write it down!
3. Add the following components to each tailed cDNA sample for a final volume of 32µl:

   5µl 6X ligation mix (Vial 11 either Cy5 or Cy 3)
   2µl T4 DNA Ligase (Vial 12)

4. Mix gently, spin, and incubate at 19oC for 1 hour.

- Stop ligation of 3DNA capture sequence to tailed cDNA as follows:

1- Add 3.5µl of 0.5M EDTA to each reaction and vortex thoroughly for 5 seconds.
2- Add 14.5µl of 1X TE buffer for a total volume of 50µl.

<u>Purification of tailed and tagged cDNA with Qiagen Minelute Kit</u> – Purify each reaction separately.

1. Follow steps 1-9 of <u>Purification of cDNA with Qiagen Minelute Kit</u> **<u>except</u>** in Step 6, add 13µl of warm buffer EB to the center of the column instead of 18µl, and in step 10, transfer 1µl for quantitation vs 2µl. Discard column and close tube. Can stop here and store at -80oC until ready to do primary hybe.

<u>Primary Hybridization of cDNA to Microarray:</u>

1. Thaw and resuspend 2X Enhanced Hybridization Buffer (Vial 5) by heating to 70oC for at least 10 minutes. Vortex to ensure that the components are resuspended evenly. Quick spin.
2. Thaw and combine the tailed and tagged (one Cy3 and one Cy5) cDNA samples for a total starting volume of 20µl and add the following:

7µl of nuclease-free water (Vial 10)
2µl LNA dT Blocker (Vial 9)
29µl 2X Enhanced Hybridization Buffer prepared above (Vial 5)
Final Volume 58µl

(*We have eliminated the Cot-1 for now since excessive hybe is not our current problem. If change, add 100ng Cot-1/µg starting RNA and make up difference with less water.*)

3. Mix gently, spin, and incubate in thermal cycler at 80oC for 10 minutes and then at 58oC til loading on array.
4. Preheat microarray, 24 x 60mm cover slip, and hybe chamber to 58oC by placing on a heat block for 10 minutes.
5. Lube gasket of hybe chamber and place a thin strip of filter paper in the groove in the bottom of the chamber and load with 60µl of nuclease –free to maintain moisture during the hybridization.
6. Place pre-warmed slide into place in the hybe chamber.
7. Mix hybe mix gently and spin briefly, then load entire contents onto slide and drop on the cover slip. Center if needed. Best to avoid bubbles but do not try to remove once cover slip is on.
8. Place pre-warmed top on hybe chamber and screw down each screw little by little.
9. Transport all on heat block to water bath and place all level in 58oC water bath on plastic tiptop or something non-conductive to keep it off the potentially hotter metal bottom.
10. Incubate in dark (foil cover) for 2 days at 58oC (Genisphere recommends O/N at 55-62oC).

(*Increase in temp and decrease in incubation time may reduce non-specific binding if needed.*)

<u>First Washes:</u> All wash solutions should be made with Ambion, Nuclease-free Water.

1. Pre-warm 2, 50ml tubes of 2X SSC, 0.2% SDS to 42oC.
2. Remove cover slip by placing microarray into the 1st tube of pre-warmed 2X SSC, capping tube and sliding it into a sleeve of foil to protect microarray probes from bleaching. Hold tube on its side so that the cover slip is facing down and gently agitate, sliding the tube out of the foil frequently to check to see if cover slip has fallen off (it should literally just drop off by itself). Once cover slip is off, hold tube upright but at an angle so that the microarray can be removed from the tube without touching the cover slip.
3. Place microarray in a fresh tube of pre-warmed 2X SSC, 0.2% SDS and wash for 15 minutes, providing gentle agitation to the tube every 5 minutes.
4. Place microarray into a fresh tube of 2X SSC at room temperature and wash for 10 minutes on its side with very gentle agitation.
5. Place microarray into a fresh tube of 0.2X SSC at room temperature and wash for 10 minutes on its side with very gentle agitation.

6. Immediately place microarray in a dry 50 ml tube, barcode/ID label down at bottom and spin at 1000 rpm for 2 minutes. Do this quickly to avoid streaky background.
7. May cap and store in dry, foiled, tube in the dark, at this point if necessary, or move directly to 3DNA Hybridization.

Secondary Hybridization of 3DNA to Microarray: Keep reagents in the dark as much as possible.

1. Thaw 3DNA Array 900MPX Capture Reagent (Vial 1 Red and Blue) in the dark at room temperature for 20 minutes. Meanwhile, heat 2X Hybridization Buffer (Vial 6) to 70oC for at least 10 minutes.
2. Vortex 3DNA (Vial 1 Red and Blue) at max setting for 3 seconds, and incubate at 55oC for 10 minutes.
3. Vortex 2X Hybridization Buffer (Vial 6) and be sure all components are resuspended. Place in 55oC heat block til ready to use.
4. Vortex 3DNA (Vial 1) at max setting for 5 seconds and spin briefly to collect all contents at the bottom.
5. Inspect solution for aggregates in the form of bubbles or flakes. Repeat steps 2 and 3 if aggregates are still present.
6. Prepare 3DNA Hybridization Mix as follows:

    2.5µl Capture Reagent 1 (Vial 1 Red)
    2.5µl Capture Reagent 2 (Vial 1 Blue)
    20µl Nuclease-free water (Vial 10)
    25µl 2X Hybridization Buffer (Vial 6)
    Final Volume 50µl

7. Gently mix and briefly spin 3DNA Hybridization Mix and incubate at 80oC for 10 minutes.
8. Place 3DNA Hybe Mix, microarray, 24x60mm cover slip, and hybe chamber onto a 60oC heat block to set up secondary hybridization.
9. Follow steps 7-9 of Primary Hybridization to set up Secondary Hybridization.
10. Incubate in the dark for 4 hours at 60oC.
11.

Secondary Washes (in the dark): All wash solutions should be made with Ambion, Nuclease-free Water.

1. Add DTT to all washes to minimize oxidation of Cy dyes. Use 50µl of 0.1M stock to each 50 ml wash.
2. Pre-warm 2, 50ml tubes of 2X SSC, 0.2% SDS to 60oC.
3. Remove cover slip by placing microarray into the 1st tube of pre-warmed 2X SSC, capping tube and sliding it into a sleeve of foil to protect microarray probes from bleaching. Hold tube on its side so that the cover slip is facing down and gently agitate, sliding the tube out of the foil frequently to check to see if cover slip has fallen off (it should literally just drop off by itself). Once cover slip is off, hold tube upright but at an angle so that the microarray can be removed from the tube without touching the cover slip.
4. Place microarray in a fresh tube of pre-warmed 2X SSC, 0.2% SDS and wash for 15 minutes, providing gentle agitation to the tube every 5 minutes.
5. Place microarray into a fresh tube of 2X SSC at room temperature and wash for 10 minutes on its side with very gentle agitation.
6. Place microarray into a fresh tube of 0.2X SSC at room temperature and wash for 10 minutes on its side with very gentle agitation.
7. Immediately place microarray in a 50 ml tube containing a 10µl drop of DTT, barcode/ID label down at bottom and spin at 1000 rpm for 2 minutes. Do this quickly to avoid streaky background.

SCAN IMMEDIATELY.
Choose small area to scan and check histogram for average ratio of 1 while adjusting PMTs before scanning all. Align blocks and features. Analyze. Normalize. Apply Fair Feature Flags. Save and backup all data.

Results can now be analyzed further with Gene Spring, MeV, or downstream analysis of choice.