Hybridization with cRNA Targets

Overview:

This hybridization protocol has been optimized for use with cRNA targets and 70-mer oligonucleotide probes. It is an adaptation of a protocol developed for cDNA arrays produced by the Potato Functional Genomics Project (http://www.tigr.org/tdb/potato/microarray_SOPs.shtml). With practice, this protocol yields very reproducible images with low back ground and high signal to noise ratio.

Materials

- 20X Saline-Sodium Citrate (SSC) (Sigma; Cat # S-6639)
- 10% Sodium Dodecyl Sulfate (SDS)(Life Technologies; Cat # 15553-035)
- Bovine Serum Albumin (BSA) (Sigma; Cat # A-9418)
- Formamide, redistilled (Life Technologies; Cat # 15515-081)
- Isopropanol (Fisher Scientific; Cat # A451-1)
- Ethanol
- Coplin jar (VWR; Cat # 25457-200)
- Microarray holder and wash station (Telechem International Cat# HTW, http://www.arrayit.com/)
- tRNA (10 mg/ml) (Invitrogen Cat# 15401-011)
- Salmon Sperm DNA (10mg/ml) (Invitrogen Cat # 15632-011)
- Microscope Lifter Slip (Erie Scientific Cat #24x60I-2-4733)
- Hybridization chamber (Corning Costar; Cat #2551 or Telechem International Cat# AHCXD)
- 1 L .22 µm CA (cellulose acetate) Filter System (Corning; Cat #430517)
- Pressurized air duster (Fellows; Cat # 99790) or clean in-house pressurized air source

Methods

DNA Probe Immobilization:

Re-hydration and UV cross linking can be done well in advance before microarray hybridization, and the slides can be stored at room temperature for several months. However we do not recommend storage of washed microarray slides for extended periods of time.

1. Mark the corner boundaries of the array on a separate glass slide. Once spots have been immobilized and the slide is washed, the spots will not be visible (the spots are only visible due to the presence of SSC crystals). One needs to know the boundaries of the array in order to correctly place coverslip over array during hybridization.
2. Re-hydrate slide over a 55°C water bath for ~5 seconds. Hold slide label side down over the water vapor. Watch spots carefully so that they do not over-hydrate and begin to merge together. In humid environments this is particularly important.
3. Snap dry the slide on a 45°C heating block for 5 seconds. Place slide label side up on heating block. Allow slide to cool for 1 minute or cool with compressed air by blowing over the back of the slide.
4. Repeat steps 1-3 a total of four times.
5. UV cross-link the slides by exposing them in batches, label side up, to 180 mJ in a commercial cross-linker (we employ a Stratalinker).
6. Wash the slide in 1% SDS for 5 minutes at room temperature in wash station with stir bar rotating at ~120 RPM. Remove SDS by dipping the slides ten times into DDH2O.
7. Immediately transfer the slides to 100% ethanol, dip five times, then incubate for three minutes with shaking.
8. Spin dry slide in centrifuge at no more than 200 x g for 2-4 minutes. Pack bottom of 50 mL centrifuge tube with Kimwipes. Using forceps carefully place slide into tube with label at the

bottom. Repeat spin if any liquid is remaining on the slide surface.

9. Repeat the ethanol wash if any visible streaks remain after spin dry. Store slide in a lint-free light-proof box at room temperature with low humidity.

Prehybridizaton

1. Prepare prehybridization buffer (5X SSC, 0.1% SDS, 1% BSA) and sterilize by filtration using a CA filter. Preheat ~50 ml to 42°C in a coplin jar for 30 minutes. The prehybridization buffer can be made up in advance and stored at room temperture
2. Place the printed slide(s) to be used for the hybridization in a Coplin jar containing prehybridization buffer preheated to 42°C. Incubate at 42°C in water bath for 45 minutes.
3. Wash the slides for 5 minutes in the wash station filled with room temperature ddH2O.
4. Repeat step 3 with fresh ddH2O for 5 minutes.
5. Shake the slides at room temperature in ethanol for 2 minutes.
6. Dry with pressurized air (Dust Off XL VWR# 21899-094), **OR** Dry down in centrifuge by placing slides in slide rack on a swinging plate tray (500rpm for 5 minutes). If you see white streaks on the slide repeat water/water/ethanol wash cycle.
7. Use slides immediately following pre-hybridization to ensure optimal hybridization efficiency.

Hybridization

1. Prepare 1X hybridization buffer (50% formamide, 5X SSC, and 0.1% SDS). To prepare 1.0 ml of 1x hybridization buffer: 250 µl 20X SSC, 10 µl 10% SDS, 500 µl formamide, 180 µl H20, 40 µl tRNA (10 ug/µl), and 20 µl Salmon Sperm DNA (10 ug/µl).
2. Resuspend labeled target (Cy3/Cy5 probe mixture) in ~60 µL of 1X hybridization buffer or 130 µl for the MOA and MOB slide set. The dried target can be prepared either by speed vacuum or ethanol precipitation. Use about 3 ug of each cRNA target per slide or 6 ug of each target for the two slide set. The target may be hard to get into solution but will go into solution after the heating step below.
3. To denature, heat the probe mixture at 95°C for 3 minutes and snap cool on ice for 30 sec.
4. Centrifuge the probe mixture at maximum angular velocity for 2 minute to remove any particulates. Keep at room temperature or warm slightly and use immediately.

Note: Expose Cy labeled probe to light as little as possible during the hybridization and washing process.

Applying the Labeled Probe Mixture

1. Rinse ArrayIt™ Hybridization Cassette with distilled water and dry thoroughly.
2. Make sure flexible rubber gasket is seated evenly in gasket channel.
3. Insert the microarray (1" x 3" or 25mm x 75mm slide) into cassette chamber with the DNA (labeled side) side up.
4. Place the lifter slip over the microarray slide (make sure the white stripe of the lifter slip is at the lower side)
5. Apply the PRE-HEATED sample slowly to the one end of the lifter slip and let it disperse. Use 50 to 60µl of sample for each slide being careful not to introduce air bubbles.
6. Add 25 µl water to the lower groove inside the cassette chamber.
7. Quickly place the clear plastic cassette lid on top of the cassette chamber.
8. Apply downward pressure and manually tighten (clockwise) the four sealing screws.
9. Check all four screws again to confirm a tight seal.
10. Place the cassette into a hybridization oven set at 42°C.
11. Allow the hybridization reaction to proceed for 12 to 14 hours.
12. After hybridization, remove cassette, manually loosen the four sealing screws (counterclockwise) and remove lid.
13. Remove the microarray slide from the cassette chamber using forceps and place the slides into the washing station containing first wash (see below).

Note: Do not flip the hybridization chamber upside down during hybridization; this may cause the lifter slip to shift from the slide and adversely affect the hybridization.

Microarray Washing

1. Wash slide in the following solutions for 5 min each:
    1. 2x SSC, 0.1% SDS @ 42°C
    2. 0.1x SSC @ room temperature
    3. 0.05x SSC @ room temperature
    4. 0.05x SSC @ room temperature
2. Washing is done by immersing the slides in a glass the Telechem wash station (Cat# HTW) containing approximately 450 ml of wash buffer, followed by placing it on a magnetic stir plate set at ~120 rpm. Pre-heat the first wash solution to 42°C, and make sure the slides are completely immersed in wash buffer. To ensure even washing, rotate the slide holder 90 degrees mid way through each wash.
3. After completion of the washes, spin dry the slide in the centrifuge at no more than 1000 rpm for 2-4 min.
    a. Pack bottom of 50 mL plastic disposable centrifuge tube with Kimwipes.
    b. Using forceps, carefully place slide into tube with label at the bottom.
    c. Repeat spin if any liquid remains on the slide.

    Note: Washing is a critical step and care needs to be taken not to over or under wash your slides. For consistent results it is **essential** to wash the slides the same way each time.
4. Scan slide immediately, or store in a light proof box @ room temp under dry conditions. Save the image as .TIFF file. Immediate scanning is recommended. However, we have observed that properly stored slides (light protected-dry- RT) can retain the signal up to a month. Some reports indicate environmental pollutants (ozone) can drastically affect fluorescence. Examine the scanned images immediately to determine the number of elements that are near zero or are saturated (for a 16-bit scanner, this represents a value of 65,400). The proportion of these elements should be acceptably low, since information is lost in either case. It is much more preferable to rescan with altered gain settings on the scanner than to proceed with analysis of images containing large proportions of zero or saturated elements.