Direct Incorporation Labeling of RNA on homemade arrays

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Modified from various Brown and DeRisi lab protocols

Throughout the protocol, I combine replicate reactions where possible. For example, I would do all the reference labeling in 1 scaled up reaction. Split the samples back into 1X reactions for the zymo purification. Once you elute, recombine the samples if appropriate. This method helps make sure the reference is as standardized as possible.

Unless otherwise noted, all spins are at full speed. Also note that you will lose 1 μl of volume in the zymo elutions.

Use RNeasy-purified RNA for best results.

RT

Bring 30-50 μg total RNA to 14.4 μl with water.
Add 1 μl 5 μg/μl anchored oligo dT ($T_{20}VN$).
Incubate 70C 10 minutes.
Ice 5 minutes.

Add 3 μl appropriate Cy-dUTP (or Cy-dCTP, just make sure the dNTP mix is modified appropriately).
Add 11.6 μl RT cocktail:

6 ul	5X superscript buffer
0.6 ul	50X dNTP mix (25 μl each 100 mM dATP, dCTP, dGTP; 10 μl 100 mM dTTP, 15 μl water)
3 ul	0.1 M DTT
2 ul	200 U/μl Superscript II (or III)

Incubate 42C 2 hours.
Incubate 95C 5 minutes.
Ice.

RNA Degradation

Add 15 μl 0.1N NaOH and 1 μl 0.5 M EDTA pH 8.
Incubate 15 min 67C.

Add 15 μl 0.1N HCl.

Cleanup

Purify sample with Zymo Clean and Concentrator 5 columns:
Add 0.5 ml binding buffer. Load on the column and spin 20 seconds. Discard flowthrough.
Add 200 μl wash buffer. Spin 30 seconds. Discard flowthrough.
Add 200 μl wash buffer. Spin 1 min.
Transfer column to a new Eppendorf tube with the lid cut off.
Add 22 μl water directly to the matrix.
Incubate RT 1 minute.
Spin 1 min to elute.
Check yield with nanodrop. You should get in the neighborhood of 1 μg cDNA at 20 pmol dye/μg cDNA.

Hybe

Mix Cy3 and Cy5 reactions. Add 120 μl water.
Add 40 μl 10X Agilent block.

Stagger the following steps if you are doing a lot of arrays.
Incubate 95C 5 minutes.
Incubate RT 5 minutes. Add 200 μl Agilent 2X hybe buffer. You can start adding the hybe solution while the samples are cooling.
Place a single array backing slide, Agilent side up, in a hybe chamber.
Pipet the whole volume of probe, avoiding bubbles, onto the center of the gasket area. Don't eject the last μl or two in order to avoid bubbles. Spread it around as you pipet, but not too close to the gasket.
Remove a crosslinked and blocked array from the box. The barcode side is the array side, and so should face down, onto the probe.
Carefully lower the array over the gasket slide, keeping it flat.
Once the array is resting on the gasket slide, place the top of the hybe chamber, and slide the screw over the assembly. Tighten the screw all the way down, finger tight.
Look through the back of the chamber and quickly rotate the slide to wet all the gaskets. There should be one big bubble that moves freely. There may be one big bubble and a couple of little ones stuck to the sides. If they are small and isolated, don't worry too much about them. You will probably do more harm than good trying to remove them. If they seem like they'll interfere, you can try knocking the array with the heel of your hand to dislodge them.

Put the array in the hybe oven. Make sure to balance the rotisserie.
Hybe 65C overnight at rotation setting 4.
Prepare for the washes by placing a bottle with 500 ml Wash 1 in the 65C waterbath.

Washes

Prepare your wash solutions. All solutions should be filtered.

Wash 1 (1 L)

940 ml	MilliQ water
50 ml	20X SSC
10 ml	10% SDS

Wash 2 (1 L)

950 ml	MilliQ water
50 ml	20X SSC

Wash 3 (1 L)

995 ml	MilliQ water
5 ml	20X SSC

Set up a glass chamber with enough Wash 1 to submerge a slide and a 500 ml beaker with the vertical rack with the bent hook and enough Wash 1 to cover the racked slides.
Pour the warmed Wash 1 into a 500 ml beaker in the 65C bath. Place it on the submergible stirplate and add a stir bar. Keep it covered with aluminum foil.
In the plastic tubs with lids, pour ~500 ml Wash 2 plus a horizontal rack and another with ~500 ml Wash 3.

Disassemble each hybe chamber one at a time. Use the plastic tweezers to gently wedge open the sandwich while submerged in the chamber of Wash 1. Transfer slide to the rack in RT Wash 1 beaker, with a space between each slide (maximum 6 if you place them back to back).
Once all the slides are in the rack, hook the rack on the side of the 65C Wash 1 beaker.
Stir 10 minutes with enough agitation that the solution is visibly turbulent.
Transfer slides to the rack in Wash 2.
Shake 10 minutes on the orbital shaker.
Transfer rack to Wash 3.
Shake 5 minutes on the orbital shaker.

Spin the slides dry (~15 seconds) one at a time in the little benchtop slide spinner.

Load the slides into scanning holders, barcode side up and barcode sticking out. The scanner scans through the back of the slide. Don't touch anywhere but the edges and the barcode.
Scan no more than 6 slides at a time to avoid ozone in the scanner.
Stagger the washes if you're doing multiple batches.

Slide crosslinking and blocking

Crosslink Corning slides at 700 energy on Donna's Stratalinker.
Make 600 ml 5X SSC, 0.1% SDS, and 6 g Roche Blocking Reagent (1096176).
Warm 65C with occasional agitation until dissolved.
Pour into a 500 ml beaker and warm until 65C.
Add the crosslinked slides in a vertical hanging rack and stir 35 minutes at 65C on submergible stirplate.
Move slides one by one into a new rack in RT water in a tupperware.
Wash on orbital shaker for 5 minutes.
Spin dry.
Use the same day, maybe the next only if absolutely necessary.