Agilent yeast cRNA arrays for 44k platform

Maitreya Dunham September 2007

modification of the Agilent low RNA input fluorescent linear amplification kit and Agilent 60-mer oligo microarray processing (SSPE wash) protocols

Use RNase free solutions and plastics throughout.
Remember that the arrays come 4/slide.

RNA

If starting with crude total RNA, clean up an aliquot with a Qiagen RNeasy column.
QC on bioanalyzer. Measure concentration with nanodrop.
Make a 100 ng/µl stock of total RNA.

RNA labeling

The amplification/labeling is done per Agilent instructions with half volume reactions and a quarter amount of recommended dye.

Aliquot 3.25 µl (325 ng) total RNA into a PCR tube.
Add 2.5 µl T7 Promoter Primer.
65C 10 min
ice 5 min

Warm 5X first strand buffer at 80C, with occasional vortexing, until it completely dissolves (3-4 min).
Prepare cDNA master mix, in this order at RT:

1X

2 µl	5X first strand buffer
1 µl	0.1 M DTT
0.5 µl	10 mM dNTP mix
0.5 µl	MMLV RT
0.25 µl	RNaseOUT

Add 4.25 µl to each reaction.
40C 2 hours
65C 15 min
ice 5 min

Add 0.6 µl appropriate Cy CTP to each reaction.

Warm 50% PEG at 40C until it's resuspended and easy to pipet.
Prepare transcription master mix, in this order at RT:

1X

8.25 µl	water
10 µl	4X transcription buffer
3 µl	0.1 M DTT
4 µl	NTP mix
3.2 µl	50% PEG
0.25 µl	RNaseOUT
0.3 µl	inorganic pyrophosphatase
0.4 µl	T7 RNA polymerase

Mix by pipetting.

Add 29.4 µl to each reaction. Mix by pipetting.
40C 2 hours in the dark

Purify with an RNeasy column. All spins are full speed:

Bring reaction to 100 µl with 60 µl water.
Add 350 µl Buffer RLT and mix.
Add 250 µl ethanol and mix by pipetting.
Add mix to column.
Spin 30 sec. The filter should be tinted.
Move column to new collection tube.
Add 500 µl Buffer RPE.
Spin 30 sec. Discard flowthrough.
Add 500 µl Buffer RPE.
Spin 60 sec.
Move column to a new eppendorf tube with the lid cut off.
Add 30 µl water directly to the membrane. Let sit RT 1 min.
Spin 30 sec.
If any color remains on the filter, repeat with another 30 µl water.
Nanodrop 1 ul to check yield and dye incorporation.
Store RNA at –80C.

Hybridization

Find the amount of sample that gives 2.5-5 pmol dye. Determine how many ng are in that amount. The kit says you should get between 10-20 pmol dye/ug cRNA. Mix the red and the green reactions such that there is at least 2.5 pmol dye in each channel and there is the same amount of cRNA in each channel. This means that one channel with

have more than 2.5 pmol dye. Make sure that the amount of cRNA is no more than 1000 ng per channel.
Bring total volume to 41.8 µl with water.

You may want to randomize the arrays that are neighbors on the arrays. A simple way to do this in Excel is to list the samples in one column and the function =RAND() into each cell in the neighboring column. Copy the random number column, then, with the column still selected, paste special -> values so that the cells won't recalculate. Then, sort both columns by the random number. List the arrays in a third column next to the sorted list. A01 is nearest the barcode and A04 is farthest from the barcode.

Final probe will consist of:

Cy3-labeled DNA	at least 2.5pmol
Cy5-labeled DNA	at least 2.5pmol
Water	to 41.8µl
10X Agilent Blocking Agent	11µl
2X Hi-RPM hybridization buffer	55µl
TOTAL VOLUME	110µl
VOLUME LOADED	100µl

Prepare 10X Agilent blocking agent per tube directions.
Add 11 µl 10X blocking agent to each tube. Mix.
Add 2.2 µl 25X fragmentation buffer. Mix.
60C 30 min in the dark
You may want to stagger the fragmentation step if you are doing multiple samples. Do at most 4 samples/1 slide at a time.
Add 55 µl 2X Hi-RPM hybridization buffer to stop the reaction. Mix by pipetting. Try to spin out any bubbles that form.
Place a backing slide, Agilent side up, in a hybe chamber.
Pipet 100 µl of probe, avoiding bubbles, onto the center of one gasket area. Don't eject the last µl or two in order to avoid bubbles, but don't skimp on the volume or you'll get a hole in the center of the array. Spread the probe around as you pipet, but not too close to the gasket. Do the same for the next 3 samples.
Remove the array from the box. The Agilent side is the Array side. 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 rotate the slide. 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 with the array, you can try knocking the array on the bench to dislodge them.
Put the array in the hybe oven. Make sure to balance the rotisserie.
Hybe 65C for 17 hours at 10 RPM.

Washing

Prepare your wash solutions. Be aware of array materials that may be for RNA only use.

Wash A (1 L)
add in this order:

700 ml	Water
300 ml	20X SSPE
0.25 ml	20% N-lauroylsarcosine

Filter. Shake to mix.

Wash B (1 L)
add in this order:

997 ml	Water
3 ml	20X SSPE
0.25 ml	20% N-lauroylsarcosine

Filter. Shake to mix.

Rinse the wash chambers, racks, and stirbars with water.
Set up:
two Wash A chambers, one with a rack and a stirbar on a stirplate.
one Wash B chamber with a stirbar on a stirplate.
one acetonitrile chamber with a stirbar on a stirplate.

For all stirring steps, the wash liquid should be visibly turbulent. Make sure the entire slide is submerged at all times.

Disassemble each hybe chamber one at a time. Use the plastic tweezers to gently wedge open the sandwich while submerged in Wash A. Transfer slide to the rack in the other Wash A chamber. Leave a gap between each slide and between the slides and the wall.
Once all the slides are in the rack, stir for 1 min.
Start stirring Wash B.
Transfer the rack into Wash B and stir for exactly 1 min. Don't worry about transferring some Wash A into Wash B.
Start stirring the acetonitrile.

Quickly transfer the rack into the acetonitrile, draining off some of the Wash B as you go.
Let stir 30 sec. Slowly and evenly pull the rack out of the acetonitrile. If you see droplets remaining on the slides, submerge them and try again.
Set the rack on a kimwipe.
Load the slides into scanning holders, Agilent side up and barcode sticking out, blotting excess acetonitrile if necessary. The scanner scans through the back of the slide. Don't touch anywhere but the edges and the barcode.
Scan no more than 5 slides at a time to avoid ozone in the scanner.
You can reuse the wash buffers for more slides, replacing the first Wash A for every batch.

Scanning

Open the Agilent scan control program. If the lasers refuse to warm up, power cycle the scanner.
Place the slides in the scanner, noting the slot numbers.
Select the appropriate slot numbers from the pulldown menus on the upper left.
Select the directory column and click edit values. Browse to find the directory you want to save in. Hit set. The column values should change.
Check the default preferences for the correct scanning area (61 x 21.6 mm), resolution (**5 µm**), laser power (100% each) and with the split and rotate box not checked.
Scan.
Open the scanned tif with the Agilent feature extraction software.
Run the appropriate protocol.
Check the visual results to make sure it looks ok. I usually check that it's aligned properly, and that the flagged spots make some sense. If you get a larger than usual number of outlier spots, make a note of it