# Agilent yeast cRNA arrays for 8x15k platform

Maitreya Dunham September 2007, updated for UW lab in 2010

This protocol was originally a modification of the Agilent low RNA input fluorescent linear amplification kit and Agilent 60-mer oligo microarray processing (SSPE wash) protocols. It is current for use with use the Quick Amp Labeling Kit, and the Hi-RPM Gene Expression Hybridization Kit.

Use RNase free solutions and plastics throughout.
Remember that the arrays come 8/slide.

**RNA**

If starting with crude total RNA, clean up an aliquot with a Qiagen RNeasy column.
QC on bioanalyzer (optional). 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 tube.
Add 0.6 µl T7 Promoter Primer and
1.9 µl water.
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:

First Strand Mix

| | |
|---|---|
| 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 of the First Strand Mix to each reaction.
40C 2 hours
65C 15 min

ice 5 min (start warming the PEG for the next step)

Add 0.6 µl appropriate Cy-CTP to each reaction.
Warm 50% PEG at 40C until it's easy to pipet.
Prepare transcription master mix, in this order at RT:

Transcription Mix

| 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 | Rnase Inhibitor |
| 0.3 µl | inorganic pyrophosphatase |
| 0.4 µl | T7 RNA polymerase |

Mix by pipetting.

Add 29.4 µl to each reaction for 40 µl total. Mix by pipetting.
Incubate 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.
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 on the microarray setting, to check yield and dye incorporation.
Agilent recommends the following:

If the yield is <825 ng and the specific activity is <8.0 pmol Cy3 or Cy5 per ug cRNA do not proceed to the hybridization step. Repeat cRNA preparation

Store RNA at –80C.

**Hybridization**

Since we use the Eichler Lab scanner, HYB chambers and oven, you'll need to coordinate with them, to be sure they will be available for you. Always be courteous.

Find the amount of sample that gives 1-2 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 1 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 1 pmol dye. Make sure that the amount of cRNA is no more than 400 ng per channel.
Bring total volume to 19 µl with water.

If you want to randomize the positioning of the arrays in Excel, 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.

Thaw 10X Agilent blocking agent (in –20C Labeling Box). If there is not a frozen aliquot, there should be a fresh tube in the RNAse free supplies. Add 1250µl* RNAse free water to the lyophilized pellet. Vortex and incubate at 37C until pellet is resuspended.
*We buy the Large volume Hi-RPM kit, the amount of water will be different if you're using the regular sized kit.

Add 5 µl 10X blocking agent to each tube. Mix.
Add 1 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 25 µl 2X Hi-RPM hybridization buffer to stop the reaction.
Carefully mix by pipetting to avoid making bubbles.
Spin 1min 13k rpm.

Final probe will consist of:

| | |
|---|---|
| Cy3-labeled RNA | at least 1 pmol |
| Cy5-labeled RNA | at least 1 pmol |
| Water | to 19µl |

| | |
|---|---|
| 10X Agilent Blocking Agent (-20C) | 5 µl |
| 25X Fragmentation Buffer | 1 µl |
| 2X Hi-RPM hybridization buffer | 25µl |
| **TOTAL VOLUME** | 50µl |
| **VOLUME LOADED** | 40µl |

Place a backing slide, Agilent side up, in a hybe chamber.
Pipet 40 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 7 samples.
Remove the array from the box. If it's a new slide, the Agilent side is the Array side. If there isn't an Agilent sticker on the slide, it's been stripped, and the array is on the side with the barcode. Carefully lower the slide, array side down over the gasket slide, keeping it level.
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, 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 hitting the chamber on the heel of your hand to dislodge them.
Put the array in the hybe oven making sure to balance the rotisserie.
Hybe 65C for 17 hours at 19 RPM.
You may want to make your washes in advance.

**Washing**

Before you prepare your wash solutions, make sure the Agilent scanner is on and that the Scan Control software can see the scanner and is warming up (it takes @20 min).
While preparing your washes, keep everything RNA safe, and use the filters with the bottles attached.

Wash A (1 L)
add in this order:

| | |
|---|---|
| 700 ml | Water |
| 300 ml | 20X SSPE |
| 0.5 ml | 10% N-lauroylsarcosine |

Filter. Shake to mix.

Wash B (1 L)
add in this order:

| 997 ml | Water |
|---|---|
| 3 ml | 20X SSPE |
| 0.5 ml | 10% N-lauroylsarcosine |

Filter.  Shake to mix.

Rinse the wash chambers, racks, and stirbars with RNAse free water.
Set up:
One Wash A chamber for disassembling the sandwich (a small jar or beaker works- just be sure that the sandwich can be submerged).
One Wash A chamber 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
One optional chamber with Agilent's Stabilization and Drying Solution. Only do this one if the ozone is above 30ppb.
Check by Going to this website (http://airgraphing.pscleanair.org/). Select 'Ozone West' as the parameter.  Then click 'clear grid', and add back the Beacon Hill/Seattle station.

All the washes should be stirring so that they are visibly turbulent (Setting 7).  Make sure the entire slide is submerged at all times.

Disassemble each hybe chamber one at a time using 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.
Transfer the rack into Wash B and stir for exactly 1 min.  Don't worry about transferring some Wash A into Wash B.
Quickly transfer the rack into the acetonitrile, draining off some of the Wash B as you go.
Let stir 30 sec (only 10 sec if using Stabilization and drying solution, then Quickly transfer to Stabilization and Drying solution, and stir for 30 sec).
Slowly and evenly pull the rack out of the final wash chamber (acetonitrile, or acetonitrile based Stabilization and drying solution).  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, Active side up (If the slide has 2 stickers, the Agilent side is the Active side.  If the slide has been stripped, there will only be one sticker and it will be on the active

side). 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 in the same day, replacing the first Wash A for every batch.

**Scanning**

Open the Agilent scan control program. Wait a minute to be sure it can find the scanner, so it can begin warming up. It takes about 20 minutes.

Place the slides in the scanner. Take note of which barcodes are in which slot.
Select the appropriate slot numbers from the pulldown menu 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.
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.
Run the appropriate protocol.

| Type of Array | Protocol |
|---|---|
| 8 x 15k Gene Expression | **GE**2**-v5_95_Feb07** |
| 4 x 44k chip-chip | cgh_Dec_105-cp |
| 2 x 99k bayanus (design id 017940) | |
| 1 x 244k S. cerevisiae (design id 019037) | |

If you get a larger than usual number of outlier spots, make a note of it.