Fluorescent labeling of yeast RNA for use with Agilent cRNA arrays

This protocol converts isolated yeast RNA into corresponding complementary DNA, then to fluorescently-labeled coding RNA (cRNA), which is hybridized to an array containing complementary DNA probes representative of the yeast genome.

The Botstein lab has custom-printed arrays from Agilent containing 8 arrays per slide with 15,000 probes per array (8 x 15k)(Among the 15,000 probes, there are two separate 60-mer probes for each gene and also a number of control probes)(Each spot on the array contains many, many copies of a single probe species). Labeling is performed using the Agilent Technologies Quick Amp Labeling Kit, using half of the manufacturer-recommended volumes (transforming a 20 reaction kit into a 40 reaction kit!)(all reaction components listed below are provided in the kit – except for RNAse-free water).

In general, the Botstein lab uses the Cy3 dye for labeling reference RNA (red) and Cy5 for labeling experimental RNA (blue). One RNA sample, the reference, will be labeled with both Cy3 and Cy5.

Materials:	Agilent Technologies Quick Amp Labeling Kit (part no. 5190-0424)	(~$638)
	(20 reactions according to Agilent, 40 reactions using our protocol)	
	Cyanine 5-CTP (blue) (10 μL (100 nmoles) per tube)	(~$240)
	Cyanine 3-CTP (red) (10 μL (100 nmoles) per tube)	(~$240)
	Order dyes from microarray facility 1 week in advance.	
	QIAGEN RNeasy Mini Kit (cat. no. 74104)(50 reactions)	(~$163)
	Prices are from 02.19.2010	

Turn on 40°C, 65°C, and 80°C water baths.

1. If starting with crude total RNA, clean up with Qiagen RNeasy column. Prepare clean RNA stock solutions with a concentration of 100 ng/μL.

2. Aliquot 4.15 μL (415 ng) cleaned RNA into an eppendorf tube.

3. Add 0.6 μL of T7 promoter primer. Mix well by pipetting up and down.

4. Place at 65°C for 10 minutes (*to denature RNA secondary structure and allow the primer to bind*).

5. Quick spin to bring liquid to the bottom of the tube, then chill on ice for 5 minutes.

6. Prepare cDNA master mix (in the order listed below) at room temperature:
   *Warm buffer before use at ≥80°C for 3-4 minutes with occasional vortexing.
   **Moloney Murine Leukemia Virus Reverse Transcriptase
   NOTE: Make sure to use the dNTP mix and *NOT* the NTP mix for this reaction!

	1X	10X
5X First Strand Buffer*	2.0 μL	20.0 μL
0.1 M DTT	1.0 μL	10.0 μL
10 mM dNTP mix	0.5 μL	5.0 μL
MMLV RT**	0.5 μL	5.0 μL
RNaseOUT	0.25 μL	2.5 μL

7. Add 4.25 μL of cDNA master mix to each reaction. Mix well by pipetting up and down.

8. Incubate at 40°C for 2 hours.

9. Incubate at 65°C for 15 minutes.

10. Chill on ice for 5 minutes, then do a quick spin to bring the liquid to the bottom of the tube.

11. While performing some of the above incubations, prepare the Transcription master mix below at room temperature in the order listed (add dye in microarray facility with lights dimmed, since it is light and ozone sensitive).
    *Warm the 50% PEG at 40°C for a couple minutes before use (for ease of pipetting)
    **By degrading the pyrophoshpate by-products of nucleotide chain synthesis, this reagent strongly promotes the forward reaction of nucleotide chain synthesis.
    ***Add in the ozone-free environment of the microarray facility (with the lights dimmed).
    NOTE: Cy3 (red) is generally used for reference RNA, Cy5 (blue) for experimental RNA
    NOTE: Make sure to use the NTP mix and *NOT* the dNTP mix for this reaction!

	1X	10X
Water (nuclease-free)	8.25 μL	82.5 μL
4X Transcription Buffer	10.0 μL	100 μL
0.1 M DTT	3.0 μL	30.0 μL
NTP mix	4.0 μL	40.0 μL
PEG (50%)*	3.2 μL	32.0 μL
RNaseOUT	0.25 μL	2.5 μL
Inorganic pyrophosphatase**	0.3 μL	3.0 μL
T7 RNA polymerase	0.4 μL	4.0 μL
Cy3-CTP or Cy5-CTP***	0.6 μL	6.0 μL

12. Add 30 μL of Transcription master mix to each reaction. Mix by pipetting.

13. Incubate at 40°C for 2 hours (in the dark).

14. Purify labeled RNA with a QIAGEN RNeasy Mini Kit as follows (all spins at full speed):
    - Bring reaction to 100 μL with 60 μL of nuclease-free water
    - Add 350 μL of Buffer RLT and mix by pipetting
    - Add 250 μL of ethanol and mix by pipetting
    - Add mix to column
    - Spin 30 seconds (the filter should now have a colored tint – that is the labeled RNA)
    - Move column to new collection tube
    - Add 500 μL of Buffer RPE
    - Spin 30 seconds and discard flow-through
    - Add 500 μL of Buffer RPE
    - Spin 2 minutes
    - Move column to a new collection tube
    - Spin 1 minute
    - Move column to a microcentrifuge tube for elution
    - Add 30 μL of nuclease-free water directly to the membrane, let sit at room temp. for 1 minute
    - Spin 1 minute (eluted solution should have a colored tint – that is the labeled RNA)

15. Use the Nanodrop Spectrophotometer to check RNA yield and dye incorporation.
    (Nikolai Slavov suggests that ~4 pmoles/μL is a typical dye yield at this point)

16. Store labeled RNA in the -20°C freezer downstairs until use.

Hybridization

Add 1250 μL water to lyophilized 10X Blocking Agent. Gently vortex. If it doesn't go into solution, heat at 37°C for a 4-5 minutes. Centrifuge. Can be stored at -20 for up to 2 months.

1. Make sure 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 will have more than 2.5 pmol of dye.

2. Get eppendorfs for mixing Cy3/Cy5 channels (1 eppendorf for every microarray)

3. Add appropriate amount of reference (Cy3 = red) to each tube. Mix red and green reactions in eppendorf such that there is at least 2.5 pmol of dye in each channel and the same amount of cRNA in each channel.

4. Final probe will consist of:

Cy3-labeled RNA……	at least 2.5pmol
Cy5-labeled RNA……	at least 2.5pmol
Water……	to 20.9 μL
10X Blocking Agent……	5.5 μL
25X Fragmentation Buffer……..	1.1 μL
2X Hi-RPM hydibridization buffer……..	27.5 μL
TOTAL VOLUME	**55 μL**
VOLUME LOADED	**50 μL**

5. Prepare 10X Agilent Blocking Agent (1250 μL water to lyophilized pellet)

6. Add 5.5 μL 10X Agilent Blocking Agent to each tube. Mix by pipetting.

7. Add 1.1 μL 25X Fragmentation Buffer. Mix by pipetting.

8. 60°C 30 minutes in the dark.

9. Add 27.5 μL 2X Hi-RPM hydridization buffer to stop the reaction. Mix by pipetting. Do not vortex!

10. Hard spin for 1 minute to reduce bubbles.

11. Place backing slide, Agilent side up, in a hybe chamber.

12. Pipet 50 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 volume, or array won't work. Spread the probe around as I pipet, but not too close to the gasket.

13. Do the sample for the next 7 samples.

14. Remove the array from the box. The Agilent side is the Array side. Carefully lower the array over the gasket slide, keeping it flat.

15. Once the array is resting on the gasket slide, place the top of the hybe chamber, and slide the screw over the assembly. Tighten all the way down. **TIGHTEN A LITTLE EXTRA BEFORE BANGING IT AGAINST THE TABLE! OTHERWISE, IT WILL LEAK!**

16. Look through back of chamber. Rotate slide. Should be one big bubble that moves freely.

17. Put array in hybe oven. Make sure to balance.

18. Hybe at 65°C for 17 hours at 20RPM.

Washing

Using Gene Expression Buffers 1 & 2 + 0.005% Triton X-102. Prewarm GE2 at 37°C overnight.

Rinse wash chambers, racks, and stirbars with water.

Set up:
- Two GE1 chambers, one with a rack + stirbar on a stirplate.
- One GE2 chamber, with a stirbar on a stirplate.
- One acetonitrile chamber with a stiarbar on a stirplate.

Liquid should be visibly turbulent and the entire slide should be submerged.

Disassemble hybe chamber.

Use **plastic tweezers** to wedge open the array/gasket sandwich while submerged in GE1.

Transfer slide to rack in other GE1 chamber. Leave a gap between each slide.

Once all slides are in the rack, stir for 1 min.

Start stirring GE2.

Transfer rack to GE2, and stir for exactly 1 min.

Start stirring acetontrile.

Quickly transfer rack to acetontrile, draining off some GE2 as you go.

Stir for 30 sec. **Slowly** and evenly pull the rack out of acetonitrile. If you see droplets remaining on the slides, submerge them and try again.

Set rack on a kimwipe.

Load slides into scanning holders, Agilent side up and barcode sticking out, blotting excess acetontrile if necessary. The scanner scans through the back of the slide. Don't touch anywhere but the edges and the barcode.

Scanning

Open Agilent scan control program.

Place slides in the scanner, noting the slot numbers.

Select appropriate slot numbers from the pulldown menus on the upper left.

Go to **edit.**

Select the directory column and click values. Browse to find directory you want to save in (D drive)

Check the default preferences for the correct scanning area (61 x 21.6 m), resolution (5 microns), laser power (100% each OR do the Extended Dynamic Scan/check the box in default settings + the scan screen), and with the split and rotate box NOT checked.

Scan.

Open the scanned tiff with the Agilent feature extraction software.

Check the image to ensure everything looks alright.

Use Tiff Splitter, split the Tiff.

Run the appropriate feature extractor protocol.

Extract files and put on PUMA.

Array/Sample tracking on a 8-pack array slide

Use the form below to make notes to track your samples on a 8-pack array slide.

Arrays

	Array 1_1	Array 1_2	Array 1_3	Array 1_4
BARCODE	Sample: _____ _____ _____	Sample: _____ _____ _____	Sample: _____ _____ _____	Sample: _____ _____ _____
	Sample: _____ _____ _____	Sample: _____ _____ _____	Sample: _____ _____ _____	Sample: _____ _____ _____
	Array 2_1	Array 2_2	Array 2_3	Array 2_4

Barcode Number _____

PUMA

My data → Enter my data → Enter new experiment into database

Search → Data Retrieval and Analysis