Microarray Indirect Labeling Probe Synthesis Protocol
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The basis of this method is that the total RNA, containing polyA+ RNA molecules, is first annealed with OligodT (which is expected to anneal only with the mRNA having a poly A end) and cDNA is synthesized, with a reactive nucleotide (amino allyl dUTP) substituted for one of the regular nucleotides. No bulky dye is used in the cDNA synthesis, increasing yield. Afterwards, the cDNA is reacted with an activated form of the Cy dye and coupled to it, cleaned up from unincorporated dye, and hybridized to the array. One advantage to this method is that it is supposed to reduce dye asymmetry. The method is based on the method on the web site of Joseph DeRisi, as modified at Pomona College by Michelle Wu in Laura Hoopes' laboratory, in consultation with Charles Kang at UCSF.

In this protocol, use RNA paranoia: powder-free gloves, don't get any dust into the samples, work on an RNase free surface, use RNase free tips and tubes, etc.

Anneal Primers and Reverse Transcription:

Bring 50 μg Total RNA to 14.5μl (by SpeedVac or adding more volume using DEPC H_2O). Add 1μl oligo dT primer.

	Concentration	μl
Oligo dT	5 μg	1
Total RNA	50 μg	14.5

Mix, quick spin
Incubate at 70 °C for 10 min.
Chill on Ice 10 min.

cDNA Synthesis

To each reaction add 14.5 μl of the following mix:

	μl	10.5 Reactions
10X buffer (stratagene)	3	31.5
50X aa-dUTP/dNTPs	0.6	6.3
DTT 0.1M	3	31.5
Stratascript RT	3	31.5
DEPC water	5	52.5
	14.5	14.5 μl Aliquot

Mix, quick spin, then incubate at 42 °C for 2 hours.

This is a convenient place to stop in one laboratory period, since the unstable RNA has been copied into cDNA. The cDNA is not labeled with dyes yet so it is very stable and can be frozen for an indefinite period. The remainder of this protocol can also be done in one laboratory period of 3-4 hours.

RNA Hydrolysis and Cleanup of the cDNA:

1 µl 0.5 M EDTA pH 8 (add this first!)
3.2 µl 1M NaOH
Mix, quick spin, and incubate 10 min at 65°C

(Note: subsequent steps use the Zymo DNA clean and concentrator 5 column but differ from protocol included in Zymo kit!)

Add 1 mL of Zymo Binding Buffer to each tube, mixing well
Spin the solution through a Zymo DNA Clean and Concentrator-5 column at 6000 RPM, 30 sec.

(You will have load the column twice as it only holds ~600 µl; the collecting tube holds 1 ml so there is no need to empty the collecting tube after the first spin)

Remove flowthrough and wash the column with 600 µl of Wash Buffer (with EtOH added as per Zymo directions). Spin through at 14000 RPM (or Max speed), 30 sec.

Remove flowthrough and spin column again 1 min to remove any residual solution.

Add 8 µl of ddH_2O to the column and let sit for 30 seconds. Spin through at top speed.
Repeat with 6 µl of ddH_2O for a total of 14 µl (use it all in the next reaction, or if running some on a gel, use all that remains to couple to the dye. Make sure you are know which of the dyes you want to couple to which of the cDNAs).

The cDNA is now ready for the dye coupling reaction or it can be stored at –20°C indefinitely.
Optional quality control: to be sure your cDNA synthesis went well, run 3.5 µl out on a 1% agarose gel. You should see a smooth smear from 300bp to 1.5kb.

Indirect labeling by dye-coupling

Note: this protocol uses the Amersham CyScribe single reaction dye pack of the activated Cy3 and Cy5 dyes.

Add 1 µl of fresh (<1 month) 1M sodium bicarbonate pH 9.0 to the 10.5 µL of cDNA. (add 1.4 µl if you have 14 µl)

Resuspend CyDye in X µl fresh 100 mM sodium bicarbonate pH 9.0. (X = number of reactions _ 2 + 1; number of reactions for a dye pack has a maximum of 6)

Note to other users: Dyes are available in other formats and can in some cases be stored frozen in DMSO in small aliquots. We do not recommend that the remainder of the dyes prepared in the format we use be stored, but if you need to store them, follow this protocol: Dyes can be resuspended in same volume of DMSO as recommended for the water if excess dye is to be stored (in the dark at 4°C) but this is not recommended.

Quickly add 2 µl dye to each tube and mix thoroughly then spin down the volume.

Incubate at room temperature in dark for 1 hr (Wrap the rack with foil and place the rack in a drawer).

Removing uncoupled dye material:

The Zymo DNA Clean and Concentrator-5 column is also used for this purpose.

Add 1 mL of DNA binding buffer to each reaction and mix well.

Spin through zymo column at 6000 RPM. (As above)
Remove flowthrough.

Wash the column with 600 µL of wash buffer and spin at top speed.
Remove flowthrough.

Spin column at high speed to remove residual solution.

Apply 6 µl MQ H_2O to the column and tap down the droplet so that the water is in/on top of the filter. Allow to sit for 1 min before spin at top speed for elution.

Take the eluate and **apply to the same column again** for second elution. This will result in a properly concentrated dye sample for the hybridization without the necessity for Speed Vac treatment. You will not get 100% yield, but the higher concentration is worth the small loss in yield.

Combine the appropriate matching Cy5 and Cy3 labeled reactions so the total volume of the dye labeled cDNA is about 10 µl. (Add more MQ H_2O if less than 10 µl.)

Preparation for hybridization:

Add 20 µl 1.5X DIG Easy Hyb solution.
Add 1.5 µl poly-A RNA from xxxx(10 mg/ml).
Total volume will be ~30 µl.

Denature the probe for 2 minutes at 100°C.

Allow to cool at room temperature 10-15 minutes in the dark and spin down the probe.

In the meanwhile, clean the lifter type cover slip with 95% ethanol. Kimwipe the slip as little as you can to avoid accumulating too much static. Notice that the lifter cover slips have a side where the white lifter strip looks shiny and a side where it looks dull. The shiny side is the top of the coverslip, and you will need to recognize it in the next step in order to apply the coverslip right side up.

Blow off traces of lint and dust with Whoosh Duster. Always hold the canister upright and beware of dispensing air too strong, too fast. Tiny aerosols of particles could spread onto the cleaned cover slips!

Make sure you know where, on the microarray slide, your array is located (often marked with a diamond marker), and which side is up (usually the side where the etched number is readable).

Put the cover slip down over the microarray with tape (dull) side down.

Coming in from the side, over the edge of the bench, with the pipettor almost lying on the level of the bench and slanting upwards only slightly, load probe gently under the cover slip. Place microarray into a Corning array holder, and put 11 ul of water into each of the tiny wells for hydration, then seal up the holder.

Note to other users: it is possible to use a 50 ml tube with a trace of water on the side of the tube under the array slide for the hybridization; other GCAT users have used this method to save money and been successful. It is important not to have the hydration water touch the edges of the slide during incubation or transport to the incubator.

Hybridize at 37°C (in the dark) for about 15 hrs.

Notes on materials used in this procedure:

Materials

DEPC water
Milli Q water
OligodT primer
Stratascript II- Reverse transcriptase (Stratagene 600085)
dNTP set ultrapure (Amersham) ...see preparation notes below.
5MG 5-(3-aminoallyl)-2'-deoxyuridine 5' triphosphate sodium salt (Sigma A0410)... see preparation notes below
Zymo DNA Clean and Concentrator-5 (Zymo Research Catalog D4004) (200/pack)
CyDyes: Amersham Catalog #RPN5661 (each box contains 12 Cy3 and 12 Cy5 packs)...see notes above under the dye coupling protocol
Whoosh-Duster (VWR)

Preparation of reagents:

Note: How to Make 40 µl 50X 3aa-dUTP:2dNTPs:

dNTP mix:
10 µl Each of100 mM dATP dCTP,dGTP
2 µl 100 mM aa-dUTP
3 µl 100 mM dTTP

Concentraion of Nucleotide Triphosphates in the Final Reaction
500 µM each dATP, dCTP, dGTP
200 µM aa-dUTP
300 µM dTTP

*****Note to other users: A ratio of 3 aa-dUTP's:2 dTTP's was optimized for our yeast chips using total RNA. Altering the ratio to 3:2 will make the labeling become optimized for using polyA RNA. Ratios of, 1:4 or 4:1 may help increase signal using different conditions.*

Advantages and Disadvantages

Advantages:
This procedure breaks nicely into two laboratory periods with storage of stable DNA that is not labeled with unstable dyes between laboratories.
If no disasters occur (see notes on use of method in Seattle), the method gives strong signal from most spots on ISB arrays.
Students have learned this method well and used it successfully in their research in Hoopes lab; as a standard procedure it is quite robust and reliable.
The method does not have dye asymmetry effects due to bulky adducts present during the cDNA synthesis, although there can still be some asymmetries due to differential coupling of dyes to the activated amino allyl derivative in the cDNA. Still, one company markets a kit for it saying that it eliminates dye asymmetry and asymmetry is certainly reduced.
Use of base to degrade the RNA is cheaper than enzymes.
Zymo columns are cheaper than Qiagen columns.
Disadvantages:
The method takes a longer total time than the cDNA incorporation method; that is why it is suggested to put in into two laboratory periods.
The method does not amplify the signal as strongly as dendromers (Genisphere method) can.
It is important to use the correct concentration method (see notes on use of method in Seattle), but this can be handled by giving the student users ONLY the Zymo columns.

Notes on treatment of GCAT slide prepared by this method in Seattle:
The Zymo column binding buffer leaked during shipping to Seattle and only 1 ml of the 4 needed for the procedure remained at the time of the experiment. Almost all of the following problems stemmed ultimately from this one factor.
The first Zymo column was replaced by a Qiagen column and a Speedvac step had to be included to reduce the volume after this step, adding 30 minutes to the total time for the preparation.
The experimentor intended to use a Zymo column for the second purification, after dye coupling, and since only enough binding buffer was available for one column, she pooled together the two samples and added this binding buffer to it. But, the experimentor accidentally selected a Centricon concentrator column rather than a Zymo column to purify this pooled sample. It was some time before she realized that this had happened, during which about _ to 2/3 of the sample was lost by running through the centricon for 3 times at 10 minutes each under high speed conditions to try to get some of it prepared. This mistake added about 30 minutes to the total time of the preparation.
Once the Zymo column was discovered and used for the remaining unfiltered sample, it successfully retained a small amount of the colored cDNA sample on it and it was eluted successfully and very quickly.
Then the tube with the 1.5 x Dig Easy Hyb buffer was not found, and Steve offered to let the experimentor use the 1x or 2x Dig Easy Hyb used by ISB. The experimentor chose the 2x sample, in order to get the hyb mixture to have the correct concentrations of the denaturing and facilitating ingredients, but it was rather hard to get into solution. That step added about 10 minutes to the procedure.

Then, once the labeled cDNA and the Dig Easy Hyb were combined, the experimentor chose the wrong tip to take the entire 40 ul sample up with, and the filter barrier prevented release of the sample from that tip. Steve was able to rescue some of the sample from this time, but this step added about 10 minutes to the procedure.
As can be seen, the total time taken and the labeled cDNA yield in Seattle were both adversely impacted by problems and mistakes most of which could be avoided in a class by controlling student access to the incorrect materials.