Liu Lab protocol
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Preparation of Probe and Microarray Hybridization *Using Amplified* RNA

This protocol is designed for prints with a 40 × 22 mm^2 spot area

Reagents

(1)	Random primers		Invitrogen	Cat# 48190-011
(2)	Cy3-dUTP	1 ml	NEN Life Science	Cat# NEL999
(3)	Cy5-dUTP	1 ml	NEN Life Science	Cat# NEL999
(4)	SuperScript II	200 U/µl	GIBCO-BRL	Cat# 18064-014
(5)	RNAsin	20-40 U/µl	Promega	Cat# N2515
(6)	Yeast total tRNA	4 µg/µl*	Sigma	Cat# R8759
(7)	human COT-1 DNA	500µl (1µg/µl)	Roche	Cat# 1-581-074
(8)	polyA	8 µg/µl*	Sigma	Cat# P-9403
(9)	100 mM dNTP set	20X, low-dT^	Amersham-Pharmacia	Cat# 27-2035-01
(10)	Microcon	YM-30 columns	Amicon	Cat# 42410
(11)	BSA		Sigma	Cat#B4287

Other reagents: 20X SSC, TE pH7.4, 10% SDS, 500 mM EDTA, 1M NaOH, 1M Tris-HCl pH7.5, sterile dH_2O

* comes lyophilized, must be resuspended at specified concentration.

^ comes as 100 mM;
for 20X stock 10 mM each of dATP, dGTP, dCTP and 4 mM of dTTP.
For a total volume of 250 µl:
25 µl dATP + 25 µl dGTP + 25 µl dCTP + 10 µl dTTP + 165 µl H_2O

I. Probe Preparation

1. Prepare the following RT labeling reaction mix for each probe:

Component	*Vol (µl)*
Random primer (3 µg/µl)	2
5-6 µg amplified RNA + H_2O	17

Incubate at room temperature for 10 min.

2. Add the following mix to each probe:

Component	*Vol. (µl)*
5X first strand buffer	8
20X lowT-dNTP mix	2
0.1 M DTT	4
RNAsin	1
Cy-3 or Cy-5 dUTP	4
SuperScriptII enzyme	2

Incubate at 42°C for 60 min.

3. Add 5 µl of 500 mM EDTA. **STOP REACTION WITH EDTA BEFORE ADDING NaOH!!**

4. Add 10 µl of 1M NaOH.

5. Incubate at 65^{o}C for 15 min. to hydrolyze residual RNA.

6. Cool to room temperature.

7. Add 25 µl of 1 M Tris-HCl (pH7.5) to neutralize pH.

II. Probe Clean up

1. Add 500 µl of 1XTE to Microcon-YM30 column and spin at 13Krpm for 5-6 min. to wash the column. Check for membrane integrity by looking into the top insert. A thin film of TE (~50 µl) should just cover the membrane.

2. Add 400µl 1XTE to each of the sample tube (from step 7 in ***I***) and transfer all contents to the washed Microcon-YM30 column.

3. Spin at 13Krpm for 5-6 min. until ~50 µl is left on the membrane. Check for the "crystals of Sotiriou" (dye crystals along the edge of the column membrane), indicating the probe is likely to be good.

4. Add 450µl 1XTE to column; spin down to ~50µl as above. Again, check for the crystals. Invert the Cy-3 labeled probe into a clean tube, and spin at 14K for 1 min. to elute the probe.

5. Carefully add the Cy-3 labeled probe to the Cy-5 labeled probe in the column. Add ~450 µl 1XTE to column and spin at 13Krpm until ~13-14 µl of combined probe remains on the membrane (**check with pipette**).

6. Invert the combined probe into a clean tube, and spin at 14K for 1 min. to elute.

7. Transfer the probe (14 µl) into a clean eppendorf tube and store at 4 ^{o}C until ready to hybridize.

III. Probe Hybridization

1. Prepare Prehybridization Buffer (5X SSC, 0.1% SDS, 1% BSA), store in aliquots at -20 ^{o}C and **warm to 42^{o}C before use**:

Component	***Vol. (µl) for 1ml***
20X SSC	250
20% SDS	5
100mg/ml BSA	100
H_2O	645

   Add 20 µl of H_2O to each humidifying well in the Hybridization Chamber (to maintain humidity). Place 40 µl of prehybridization buffer to the center of the slide and quickly (but carefully!) place the cover slip on the slide, taking care to prevent bubble accumulation beneath slip. Firmly attach margin clamps of the Hybridization Chamber, and incubate at 42 ^{o}C for least 1 h.

2. Wash slide in distilled H_2O for 2 min. followed by isopropanol for 2 min. Dry slide in centrifuge (5804R, Eppendorf) at 705 rpm (~70x *g*) for 4 min. Proceed with step 4 and set up probe for hybridization. **It is advisable to hybridize the slide within 1 h of the prehybridization step**.

3. Make fresh 2X Hybridization Buffer (50% formamide, 10X SSC, 0.2% SDS):

Component	***Vol. (µl)***
20X SSC	50
formamide	50
10% SDS	2

   Keep 2X Hybridization Buffer at 42 ^{o}C.

4. Mix probe (from ***II***, step 7) with 2 µl COT1-DNA (1 µg/µl), 2 µl polyA (8-10 µg/µl), and 2 µl yeast tRNA (4 µg/µl). Denature for 1 min. at 100°C, and snap cool on ice and spin down.

5. Add 20 µl of 2X hybridization buffer to the denatured probe, mix well (take care to minimize bubble formation) and keep at 42 °C until ready to spot on slide.

6. Prepare hyb chamber as in the prehybridization step with 20µl of distilled water in each well. Place slides in chambers **face up**.

7. Hybridize slide with probe 14-16hrs at 42 °C.

IV. Slide Washes

1. Carefully remove margin clamps of Hybridization Chamber to prevent water from seeping in and contaminating the array.

2. Remove slide from chamber, hold slide with forceps and allow cover slip to fall off into the solution comprising 2X SSC, 0.1% SDS.

3. Wash for 4 min. in 1X SSC, 0.1% SDS.

4. Wash for 4 min. in 0.2X SSC.

5. Wash for 1 min. in 0.05X SSC.

6. Quickly spin dry in centrifuge at 705 rpm (~70x *g*) for 4 min. If water droplets can be seen on the slide spin for another 4 minutes.

7. Minimize exposure to light by placing the dried slides in a slide box until ready for scanning.

Washes:	2X SSC, 0.1%SDS	1X SSC, 0.1%SDS	0.2X SSC	0.05X SSC
20X SSC:	200 ml	100 ml	2 ml	0.5 ml
20%SDS:	10 ml	10 ml	-	-
dH_2O	←	Make up to 2 L	→	