

MicroArray Protocol For Total RNA

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This protocol requires 50 µg of each type of RNA to be compared for each microarray. The word probe will be used to describe the labeled cDNA that binds to the DNA on the microarray.

Materials

The following is a list of supplies required to perform cDNA microarray hybridizations. Most items are available in the Invitrogen Freezer Program.

Company	Cat. Number	Description
Amersham Pharmacia Biotech	27-7836-01	poly d(A) sodium salt or use Sigma poly d(A)
Perkin Elmer	NEL576	Cy3-dCTP
	NEL577	Cy5-dCTP
Ambion	9915G	DepC treated water
	9480	7.5 M LiCl solution
Brinkmann/Eppendorf	0032 005.330	Phase Lock Gel-Heavy, 50 mL tubes
Qiagen	28104	QIAquick PCR purification Kit (50 columns)
Roche	799-017	Rnasin (2000 U)
	1467140	ss Salmon Sperm DNA
PGC Scientifics	62-6504-04	22 X 40 mm hybridlip, pack of 100
Fisher	42416	Microcon-50 microconcentrators (Amicon no. 42416)
	2551	CMT Hybridization Chamber (Corning no. 2551), 5 chambers
	08-812	Histology staining dish (horizontal)
	08-953-E	Jewelers Microforceps, 10.8 cm (straight)
	13-812-14	Kelly Forceps, curved, 140 mm
	12-548-5J	24 X 40 mm glass coverslips, 1 oz pack
	05-669-39	1.5 mL microfuge tubes with screw cap O-ring seal, 500 tubes
Gibco/BRL Invitrogen	15279-011	Human Cot-1 DNA, 500 ug
	18064-014	SuperScript II RT.
	18440-016	Mouse Cot-1 DNA (if required)
	10297-018	100 mM dNTP Set
	15596-026	TRizol Reagent, 100 mL (200 mL bottle is also available)
	15634-017	Herring Sperm DNA, 5 X 1 mL or below
	15632-011	Salmon Sperm DNA, 5 X 1 mL or above
Promega	PRM-4261	Rnase One
Sigma	D-2532	Denhardt's Solution 50X concentrate, 5 mL
	S-2015	20X SSPE, 1 L
	S-6639	20X SSC, 1 L
	F-9037	Formamide, 100 mL
	P-9403	Poly d(A) or use Pharmacia Amersham.
	R-7876	2500 U tRNA (type V wheat germ)
Ambion	9906	10 X 50mL DepC treated water
	9480	100 mL 7.5 M LiCl solution for RNA purification, 100 mL

Reverse transcription of probes.

1. Start with 50 µg RNA of each type in 9 µL water.
2. Have the following temperatures available:
 - a) 37°C, 50°C water baths
 - b) 42°C, 68°C and 95°C heating blocks
 - c) Thaw 5X 1st strand buffer, 0.1M DTT, oligo d(T)₂₀VN, Cy3-dCTP, Cy5-dCTP, 10X Low dC/dNTP, microarray pre-hybridization and hybridization solution.
3. Anneal oligo dT primers to RNA.
 - a) Add 1 µL dTVN primer (0.5 µg/µL) and incubate 5 minutes at 68°C
 - b) Give a quick spin to collect condensation. Keep at room temperature or solution will get too viscous.
4. Prepare 2 separate tubes of each fluorescent mix.

	(X4.5)	(X 5.5 reactions)
a) 4 µL 5X 1st strand buffer	18	(22 µL)
b) 2 µL 0.1 M DTT	9	(11 µL)
c) 2 µL 10X low dC dNTP	9	(11 µL)
d) 2 µL Cy3 or Cy5 dCTP	9	(11 µL)
e) 0.5 µL RNAsin	2.2	(2.7 µL)
f) This should give a total of 10.5 µL.		

5. Mix annealed RNA with appropriate fluorescent mix.
6. Add 1 µL SuperScript II RT per RNA reaction and incubate at 42°C for 45 minutes.
Using another pipettor at ~15 µL, pipette up and down to mix. This mixing step is VERY important. Since Cy5 and Cy3 are light sensitive, cover tops of tubes with foil during reaction. Do not let the temperature exceed 44°C.
7. Add another 1 mL SuperScript II RT per RNA reaction, mix with 15 mL pipette, and incubate at 42°C for another 45 minutes. Subsequently place at 94°C for 2 minutes, quench on ice and then combine reactions in one tube.

GO TO STEP 10 FOR MICROARRAY PREHYBRIDIZATION, THEN COMPLETE STEPS 8 AND 9.

8. Clean up cDNA.

a) Get rid of RNA

- i) Add 50 μL water
- ii) 10 μL 10X RNase One buffer
- iii) 2 μL RNase One
- iv) Incubate at 37°C for 30 minutes.
- v) This should give a total of 99 μL

b) Remove unincorporated label and small cDNA fragments using the QIAquick PCR Purification Kit.

- i) Add 500 μL Qiagen Buffer PB to probe and mix.
- ii) Apply the sample to QIAquick spin column and centrifuge at Room Temperature 45 seconds, 10 000 g.
- iii) Discard flow through, add 750 μL Buffer PE (wash buffer) to spin column and centrifuge for 45 seconds, 10 000 g.
- iv) Discard flow through and spin again for an additional minute to remove residual Buffer PE.
- v) Elute probe by adding 50 μL Buffer EB (elution buffer) to the center of the QIAquick membrane, let sit one minute and then centrifuge for one minute.
- vi) Place probe eluted from QIAquick column on a MICRON YM 50 column and centrifuge at Room Temperature for 1.5 minutes, 12400g. This should concentrate the probe to less than 15 μL .
- vii) To collect purified probe, invert column into new tube. Set centrifuge to 3000 RPM and spin for 15 seconds.
- viii) Check the volume and bring up to 15 μL .

9. Probe pre-hybridization.

- a) To purified probe, add 28.8 μL probe hybridization and 2.3 μL 20X Blocking solution to probe. This gives a total of 46 μL (extra is due to evaporation during prehybridization).
- b) Place in 94°C heating block for 2 minutes.
- c) To remove any particulate matter, centrifuge at 14K g for 5 minutes and transfer to a clean tube with a rubber o-ring cap to prevent excessive evaporation.
- d) Place probe at 50°C for one hour (minimum 45 minutes) to pre-hybridize. Do not exceed 1.5 hours due to evaporation.
- e) Once probe has pre-hybridized, centrifuge 5 minutes at 14K g and use 32-36 μL per microarray for hybridization in step 11.

10. Prehybridize microarrays.

- a) Add 34 μL of microarray prehybridization buffer solution (frozen aliquots at -20°) over microarray. This is accomplished by slowly ejecting the solution to form a drop on the pipettor tip and gently touching the drop (NOT the tip) to the middle of the microarray.
- b) Place coverslip over the prehybe. Use Plastic Coverslips or you'll have a difficult time removing it later! Coverslips are sandwiched between two plastic films. Break one film by bending coverslip to one side and remove coverslip with forceps. This gives a coverslip with the plastic film on one side. Using 90° forceps, lay so that tips are on line defining array. Coverslips will use this as a fulcrum to prevent sliding. While holding the corner of the coverslip, place the edge of the coverslip at the border of the micro array at $\sim 45^{\circ}$ angle, against the forcep. A line of prehybridization solution will form at the contact. Gently lower the slide until its almost down and then let go with the forceps. Do not drop the slide from too high a height or it will move. Once the coverslip is positioned, do NOT move it.
- c) Place the microarray with coverslip into hybridization chamber. Add 10 μL of 50% formamide/50% water to each dimple at the end of the CMT hybridization chamber.
- d) Place cover on chamber and place in 50°C water bath. Allow prehybridization of microarray to proceed for at least two hours.

11. Remove prehybridization and add hybridization solution.

- a) Rinse hybridization chamber and dry the outside of the chamber well. Make sure to keep chamber level at all times. Remove microarray from chamber and place on paper towel.
- b) Remove coverslip and microarray prehybridization solution. Using the left hand, tilt the slide so that its long edge remains in contact with paper towel and an acute angle forms between the coverslip side of the slide and the paper towel. Grasp the top corner of the coverslip with forceps and while holding coverslip in place, gently tilt the slide away. The prehybe solution will be seen to separate from the coverslip just like a acrylamide gel pulling away from a glass plate. Make sure the slide and coverslip maintain contact at the bottom edge where all the solution will gather. Once the coverslip touches the paper towel, the prehybe will blot all over.
- c) Add 32-36 μL hybridization solution to each microarray and hybridize overnight. Add 10 μL of 50% formamide/50% water to each dimple at the end of the CMT hybridization chamber.

The next day, wash and scan the microarrays and scan them.

12. Prepare 500 mL of the following wash solutions:
 - a) 1X SSC/0.1% SDS (25 mL 20X SSC/2.5 mL 20% SDS)
 - b) 0.2X SSC/0.1% SDS (5 mL 20X SSC/2.5 mL 20% SDS)
 - c) 0.2X SSC (5 mL 20X SSC)
 - d) 0.1X SSC (2.5 mL 20X SSC)
13. Rinse hybridization chambers making sure to keep chambers level so that the glass coverslip does not shift. Remove microarray from the chamber and place slides in a histology staining dish filled with ~200 mL 1X SSC/0.1% SDS and shake gently so that the cover slip falls off. Prewarm the dish in tissue culture incubator and place 1X SSC/0.1% SDS in 50C water bath.
14. Place slides in a slide holder and put holder into slide dish with ~200 mL of 0.2X SSC/0.1% SDS. Shake on orbital shaker 5-45 minutes. Shake with sufficient vigor that slides are moving in their holder. Make sure to cover the slide dish with a cardboard box because the dyes are light sensitive and the ceiling is glass.
15. Transfer slides only (not the entire slide rack) to dish with 0.2X SSC, then 0.1X SSC. Each wash is for 5-45 minutes.
16. Place slides in 50 mL Corning tube and centrifuge in Eppendorf 5819r at 550 g for 7 minutes. Make certain that the back of the slide is dry and spot free.
17. Scan the microarray and analyze data.

Solutions

Low dCTP/dNTP Solution

25 μ L dGTP (100 mM)

25 μ L dATP (100 mM)

25 μ L dTTP (100 mM)

10 μ L dCTP (100 mM)

415 μ L DepC water

MicroArray Prehybridization Buffer Solution

5 mL deionized formamide

3 mL 20X SSPE

500 μ L 10% SDS

1 mL 50X Denhardt's

200 μ L ss Salmon sperm DNA (10 mg/mL)

930 μ L dd water

Assemble solution and keep 100 μ L aliquots at -20C.

Probe Hybridization Solution

X 7

14 μ L formamide 98 μ L

1 μ L 20% SDS 7 μ L

2 μ L 50X Denhardt's 14 μ L

8 μ L 20X SSPE 56 μ L

Assemble solution and keep 100 μ L aliquots at -20C.

20X Hybridization Blocking Solution

40 μ L poly dA (1 μ g/ μ L)

8 μ L tRNA (10 μ g/ μ L)

200 μ L Human (or mouse) CoT 1 DNA (1 μ g/ μ L)

28 μ L 3M Sodium Acetate (pH 5.2)

Ethanol precipitate with 700 μ L ethanol.

Wash 2 X 1mL X 75% Ethanol and dissolve in 20 μ L water.