Microarray Protocols

Version 1

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August 17, 2006

Genomic DNA Labeling Protocol

This protocol was developed for microarray-based comparative genomic hybridizations.

Genomic DNA can be labeled with a simple random-priming protocol based on Gibco/BRL's Bioprime DNA Labeling kit, though nick translation protocols work too. The BioPrime labeling kit (Gibco/BRL) serves as a convenient and inexpensive source of random octamers, reaction buffer, and high concentration Klenow (do not use the dNTP mix provided in the kit). Other sources of random primers and high concentration Klenow work as well.

1. Add 2 ug DNA of the sample to be labeled to an eppindorf tube.

   Note: For high complexity DNAs (e.g. human genomic DNA), the labeling reaction works more efficiently if the fragment size of the DNA is first reduced. I routinely accomplish this by restriction enzyme digestion (usually DpnII, though other 4-cutters work as well). After digestion, the DNA should be cleaned up by phenol/chloroform extraction / EtOH precipitation (Qiagen PCR purification kit also works well).

2. Add ddH_20 or TE 8.0 to bring the total volume to 21 ul. Then add 20 ul of 2.5X random primer / reaction buffer mix. Boil 5 min, then place on ice.

   2.5X random primer / reaction buffer mix:
   125 mM Tris 6.8
   12.5 mM $MgCl_2$
   25 mM 2-mercaptoethanol
   750 ug/ml random octamers

3. On ice, add 5 ul 10X dNTP mix.

   10X dNTP mix:
   1.2 mM each dATP, dGTP, and dTTP
   0.6 mM dCTP
   10 mM Tris 8.0, 1mM EDTA

4. Add 3 ul Cy5-dCTP or Cy3-dCTP (Amersham, 1 mM stocks)

   Note: Cy-dCTP and Cy-dUTP work equally well. If using Cy-dUTP, adjust 10X dNTP mix accordingly.

5. Add 1 ul Klenow Fragment.

   Note: High concentration klenow (40-50 units/ul), available through NEB or Gibco/BRL (as part of the BioPrime labeling kit), produces better labeling.

6. Incubate 37 degrees C for 1 to 2 hours, then stop reaction by adding 5 ul 0.5 M EDTA pH8.0

7. As with RNA probes, purify the DNA probe using a microcon 30 filter (Amicon/Millipore):

   Add 450 ul TE 7.4 to the stopped labeling reaction.
   Lay onto microcon 30 filter. Spin ~10 min at 8000g (10,000 rpm in microcentrifuge).
   Invert and spin 1 min 8000g to recover purified probe to new tube (~20-40 ul volume).

February 17, 2006

Post-Processing of Oligo Arrays on Epoxy and Amine Coated Slides

Cross-linking

Mark boundaries of array on back of slide using diamond scriber. Array will become invisible after post-processing. Use these etches for cover slip positioning during hybridization protocol.

Expose slides to 65mJ of UV irradiation (Place slides inside, face up, on a paper towel. Set the Stratalinker (or equivalent UV cross-linker) to Energy and type in 650 x 100).

Blocking

The blocking procedure blocks the remaining reactive groups on the slide surface surrounding the spotted DNA. This step is necessary to prevent unspecific binding
of the labeled target, thereby reducing the background of the final microarray.

Stock BSA Solution

Reagent	Volume
Fraction V BSA	10 g
20xSSC	150 ml
Ultra-pure Water	840 ml

1. Dissolve 10 g of BSA into 840 ml of ultra-pure water on a heated stir plate.
2. When the BSA is completely dissolved, add 150 ml of 20X SSC and filter solution.
3. This solution can be stored at 4°C for up to 3 months.

Procedure:

1. Bring appropriate volume of stock BSA solution to 42ºC (enough to completely immerse slides).
2. Place the slides in the solution and incubate at 42ºC for 1 to 2 hours.
3. Pour off solution into appropriate waste container, add ultra-pure water and dip 15—20 times.
4. Rinse slides vigorously in 4 additional changes of ultra-pure water. Carry the slides to the centrifuge in the last water wash and load slides quickly and evenly onto the carriers to avoid streaking.
5. To dry the array slides, centrifuge slides for 5 min. @ 500-700 rpm, at room temperature.
It is recommended that slides should be used for hybridization shortly after completing this procedure, but can be stored, light protected and desiccated, for a couple days if needed.

October 2006

RNA Extraction Protocol

Tissue Harvest

Whatever the method of storage when harvesting, it is **CRUCIAL that the tissue be stored immediately following sacrifice and extraction!!

Snap Freezing in Liquid Nitrogen:

1) Following immersion, keep the tissue in the Nitrogen until the procedure is completed.
2) Upon completion of the harvest procedure, transfer the tissues to empty falcon tubes stored on dry ice.
3) Keep the tissue frozen until the homogenization procedure is ready to be performed.

Immersion in RNA Later:

1) Upon extraction from the animal, immediately slice the tissue into pieces no wider than 0.5cm and drop into RNA Later. (The volume of RNA Later should be at least ten times the volume of tissue)
2) Store the tissue (until homogenization) according to the following: Initially - overnight at 2-8 C, Then –indefinitely < -20 C, up to four weeks at 2-8 C, up to 7 days at 2-8 C, up to 1 day at 37º C.

***** For cultures of cells, pellet out of growth media, wash 3 X PBS, and resuspend in RNA Later. **(Do Not Freeze!)**

Homogenization

1) For tissues that are snap frozen or slightly in excess, the homogenization of the tissue should be done by mortar and pestle (cooled to temp in a liquid nitrogen bath).
2) At the same time, transfer at least 1mL TRIZOL / 100mg tissue to be homogenized into a falcon tube
3) Transfer the tissue to the pestle and grind until a layer of very fine dust is all that is left.
4) Use an RNase free spatula to transfer the dust to the TRIZOL solution. Be sure to get as much dust as possible.
5) Vortex mixture thoroughly.

* For tissues that are very small or highly precious, a hand-held tissue grinder is recommended. The homogenization is performed in the presence of the 1mL TRIZOL / 100mg tissue until the tissue is completely dissolved in solution.

* For cultures of cells (suspended in solution), quantify, pellet the cells, and resuspend in TRIZOL at a volume of 5 x 10^6 cells / 1mL TRIZOL.

6) Once homogenized, aliquot the solution to eppendorf tubes.
7) Normally at this point, leave in TRIZOL at room temp for five minutes, and then continue on with phase separation. As and alternate step, we have found that freezing the homogenized solution at -80 C overnight (min of 6 hours) increases the yield of total RNA. Allow samples to completely thaw to room temperature and then proceed to phase separation.

Phase Separation

1) Add 200ul chloroform / 1mL TRIZOL (originally used), vortex for 15 seconds, and leave at room temp for 2-3 minutes.
2) Centrifuge samples at 12,000g for 15 minutes at 2-8 C.

RNA Precipitation

1) Following centrifugation, there will be three phases visible within the tube. Transfer the aqueous phase (top) to a fresh tube, being careful not to contaminate the solution with the other phases. Contamination will be obvious by the presence of any flakes or unclear liquid.
2) Add 500ul isopropanol / 1mL TRIZOL (originally used) to the new tube and incubate at room temp for 10 minutes.
3) Centrifuge samples at 12,000g for 10 minutes at 2-8 C.

RNA Wash and Re-suspension

1) Following centrifugation, remove the supernatant.
2) Wash RNA pellet with 80% EtOH / 1ml TRIZOL (originally used) and vortex.
3) Centrifuge samples at 7,500g for 5 minutes at 2-8 C.
4) Remove supernatant. Allow remaining EtOH to air dry for 2-3 minutes.
5) Transfer tubes to 70 C heat block and let sit for 2-3 minutes.
6) Dissolve the pellet in 81ul of DEPC water.

DNase Treatment

1) Add 8ul of 10X DNase I Buffer.
2) Add 2ul of DNase I Enzyme.
3) Vortex, quickly spin and incubate at 42 C for 25 minutes.

RNeasy Column Purification (Using Qiagen's RNeasy Protocol)

1) Add 350ul Buffer RLT (with BME-10ul/ml Buffer RLT).
2) Add 250ul 100% EtOH.
3) Apply entire volume to RNeasy column and spin full speed for 1 minute.
4) Reapply entire volume to RNeasy column and spin full speed for 1 minute.
5) Transfer column to new 2ml collection tube.

6) Add 750ul Buffer RPE and spin full speed for 1 minute.
7) Discard flow-through and add 750ul Buffer RPE and spin full speed for 1 minute.
8) Discard flow-through and spin full speed for 1 minute.
9) Transfer column to new labeled 1.5ml Eppendorf tube.
10) Add 56ul DEPC H2O and let sit for 2 minutes.
11) Spin at full speed for 2 minutes.
12) Discard column and transfer tube to ice.

Quantification and Quality Control

1) Quantify each sample using Nanodrop (or equivalent spectrophotometer).
2) Run 5ul of each sample on Agarose gel.

cDNA Labelling Procedure

1. Dilute the RNA sample with DEPC-H_2O to a final volume of 11.5 μl. A typical labelling reaction require 5-8 μg total RNA for bacterial arrays and ~ 50 μg total RNA for Mouse Genome arrays. Keep the RNA and other reaction components on ice until instructed otherwise.
2. Add 1.5 μl random primers (stock concentration of 3μg/μl). For the mouse genome arrays Oligo dT primers can be used in place of random primers, remember to keep the concentration of 3μg/μl the same. Also, when using oligo dT primers, the 25°C 10 minute incubation at step 6 is no longer necessary.
3. Heat samples to 70 ^{o}C for 5 min., then snap-cool on ice.
4. Make up master mix of the following reaction components, excluding the reverse transcriptase (see Notes 1 and 2):

	Per Reaction
5X First-Strand Reaction Buffer	5.0 μl
100 mM DTT	2.5 μl
dNTP mix (5 mM each of dATP, dGTP, dCTP, 0.5 mM dTTP)	2.5 μl
Cy3-dUTP (control) or Cy5-dUTP (sample)	1.0 μl
Reverse Transcriptase (SUPERSCRIPT™ II)	1.0 μl

   Protect reaction mix from light throughout process, while maintaining on ice.

5. Add 12 μl of master mix including reverse transcriptase to each sample. Mix, then centrifuge briefly to collect contents at the bottom of the tube.
6. Incubate at 25 ^{o}C for 10 min. and then 42 ^{o}C for 90 min.
7. Degrade RNA by addition of 5 μl 1M NaOH and incubate at 65-70°C for 15 minutes. Neutralize by adding 5 μl 1M HCl (can wait for samples to cool before adding HCl, if necessary.)
8. Add 400μl TE to a Microcon30 and let sit for 10 minutes.
9. Combine the appropriate Cy3- and Cy5-labelled samples (If you will be performing a co-hybridization on the array).
10. Add the combined probes to the Microcon30 filter. Centrifuge at 12000 x g until nearly all of the volume is pushed through the filter (see note 3). Repeat the buffer exchange once by adding a fresh 400 μl filtered TE and centrifuge as before. Check the volume periodically until there is only about 5-10 μl of sample remaining in the filter unit.
11. Recover the labelled cDNA by inverting the Microcon30 filter into a new 1.5 ml collection tube and centrifuge at 10000 x g for 2 minutes.
12. Adjust volume to approx. 11 μl (see note 4).

Notes

1. Cy3-dCTP and Cy5-dCTP could be substituted making the appropriate adjustments to the dNTP mix.
2. The Reverse Transcriptase should be added to the master mix just prior to the samples finishing the 70 ^{o}C heating in step # 4.

3. A purple/dark blue colour should be visible on the filter or around the rim. If not, discontinue process – there is no labelled cDNA.
4. If access to a NanoDrop Spec is available this volume could be adjusted to 13 μl in order to take 2 μl and check the amount of cDNA recovered and the dye incorporation values.

Reagent	Company	Catalog number
Cy-3 dUTP	GE health care	PA53022
Cy-5 dUTP	GE health care	PA55022
Superscript II	Invitrogen	18064-022
Microcon YM30	Fisher (Millipore)	42409
dNTPs	Invitrogen	dATP 10216-018, dCTP 10217-016, dGTP 10218-014, dTTP 10219-012
random primers	Invitrogen	48190-011

RNA Isolation Protocol

Materials

50-ml screw cap tubes (Falcon)
2-ml Screw-cap tubes with O-ring
Crushed dry ice
1.5-ml microcentrifuge tubes
MiniBead Beater (BioSpec Products, 3110BX)
Trizol (Gibco-BRL), stored at 4°C
Glass beads (Sigma 100-125 uM; or BioSpec Products, 0.1mm silica/zirconium)
Phase Lock gel, heavy (Eppendorf)
Chloroform
Isopropanol
70% ethanol
10X DNaseI buffer (250mM Tris-HCl, pH 8.3, 375 mM KCl, 15 mM $MgCl_{2,}$, 10mM $CaCl_2$)
DNaseI (Ambion #2222 = 2000 units at 2 U/μl)
Phenol, equilibrated to pH 5.2 in sodium acetate (20mM)
Chloroform

RNA isolation procedure for *Mycobacterium Cells*

1. Freeze 15-ml PBS on a slant in a 50-ml screw-cap tube to prepare for the bacterial harvest.

2. Prepare tubes for each crude RNA sample:
   2-ml screw-cap with 0.3-4 ml dry glass beads;
   2-ml tube containing 200 μl chloroform and Phase Lock gel;
   1.5ml tube for isopropanol precipitation

3. Centrifuge 20-30ml culture 5 min at 3500 rpm. Pour off supernatant and proceed immediately to the next step. If the pellet is to be stored frozen, plunge the tube in a bed of finely crushed dry ice. ([Note 1])

4. Add 1ml cold Trizol to the pellet, vortex to suspend the cells.

5. Transfer the suspension to a 2 mL screw cap tube containing 0.3-4 ml glass beads

6. Homogenize 30 sec at 5000 rpm in bead beater. Continue to periodically invert samples for 5 min. and repeat the bead-beating step twice more during this period.

7. Centrifuge samples 45 sec at max speed.

8. Transfer Trizol solution that is above the bed of beads and cellular debris to a 1.5 ml tube containing 200 μl chloroform and Phase Lock gel. Invert rapidly for the first 15 sec, and continue inverting periodically for an additional 2 min.

9. Centrifuge 5 min. in the microfuge at top speed, transfer the aqueous, top layer (about 500 µl) to a fresh 1.5 ml tube.

10. Add an equal volume isopropanol (typically 0.6 ml) and mix. (Typically stored at –20C at this step until all the samples are processed. Samples are kept at –20C for 1-3 hour on average.)

11. Precipitate the nucleic acids by centrifugation (20 min, 12,000 X g at 4°C).

12. Rinse the pellet once with 1 ml 70% ethanol; invert several times and centrifuge 5 min.

13. Aspirate off the ethanol and dry the RNA pellet.

14. Dissolve RNA in dH2O (Table1,[Note 2]). Dilute 2 µl of the sample 1:100 in TE and measure the 260 nm and 280 nm to estimate the concentration and purity of the sample.

DNase-treatment

1. Create a master mix of DNaseI buffer, water and DNaseI according to the table:

Reagent	1X volume	Scaled volumes (factor =)
RNA	70	NA
10X DNase buffer	10	
dH_2O	19	
DNase I (2 U/µl)	1.0	
Total volume	100	

2. Combine the RNA, water and DNase buffer and enzyme mix as indicated by the table below. (Add 1 µl DNaseI for samples up to 50µg RNA per 100 µl reaction.) Save back a sample (minimum 300 ng) of the pre-treated crude RNA for evaluation by agarose gel electrophoresis.

3. Incubate 10 min at 37°C.

4. Extract the sample in phenol:chlorofomr
- add 100 ml TE (200 ml total volume)
- extract in 200 ml phenol:chloroform (4:1)
- extract again in 200 ml phenol:chloroform (1:4)

5. Precipitate in ethanol.
- add 1/10th volume 3M sodium acetate, pH 5.2
- add 2.5 volumes ethanol
- chill
- precipitate by centrifugation (12 –14,000 rpm for 30 min)

6. Wash the pellet once in ice cold 70% ethanol and dry the pellet.

7. Dissolve the pellets to 1 μg/μl

8. Evaluate 300ng of each the crude RNA and DNaseI-treated RNA by 2% agarose gel electrophoresis. Take spectrophotometric readings at 260/280 to evaluate purity and concentration of RNA.

Note 1 The initial harvest by centrifugation is probably sub-optimal because it exposes the cells to 5 minutes of new stimuli (*i.e.* centifugal force, compacting of the cells, and possible temperature changes) that are not part of the experimental treatment. We try to control for this by treating the all the samples in a similar manner. Indeed, replicate samples treated in this way show few differences when compared directly on the microarray. We have settled on this step, for now, because it is an approved biosafety procedure and we prefer sampling larger volumes (20-30 ml) of early-log phase cultures to obtain sufficient RNA yields.

Note 2 Average yields for log-phase cells are 200 μg of nucleic acids. The sample at this point in the protocol is refered to as "crude RNA" because it typically contains a mixture of RNA and genomic DNA. Reverse transciptase will use the genomic DNA as a template in the labeling reaction, interfering with the results of the experiment. Therefore, additional steps are needed to purify the RNA from the DNA. Several options exist for this purpose. There are several comercial RNA purification kits (For example, two kits sold by Qiagen Corp., RNeasy and Total RNA, have performed well for us, although there can be a significant loss of RNA at this step.)

October 2006

Microarray hybridization (Amine coated slides)

1. Pre-hybridization of arrays

The pre-hybridization of arrays takes place during the Post Processing Procedure. It is best to continue from Post processing/pre-hybridization directly to step 2.

2. Preparation of 2X Hybridization solution: (May need to adjust volume appropriately for the number of samples and the size of cover (lifter) slip need: 22 x 22 = 15μl, 25 x 25=24μl, 24 x 60 = 45μl)

2.1 Make up the following 2X Hybridization Solution

Formamide	50 μl
20X SSC	50 μl
10 % SDS	2 μl

Again, add the 10 % SDS solution last to avoid precipitation.

3. Mix labelled targets with blocking reagents (see note 2)

Cy-3 and Cy-5 labelled cDNA/RNA	11 μl
Yeast tRNA, 4 μg/μl	1 μl
TOTAL	12 μl

4. Hybridize arrays

4.1 Add appropriate amount of 2X Hybridization Solution to nucleic acid mixture from step 3 to achieve proper amount for cover (lifter) slip size in step 2.

4.2 Heat to 98 ^{o}C for 2 min. Snap cool on ice (briefly since SDS should not precipitate).

4.3 Centrifuge at 12000 x g briefly to collect sample in bottom of tube.

4.4 Place new cover (lifter) slip over region of array on slide and apply mixture under cover (lifter) slip. (see note 1)

4.5 Apply 15 μl H_2O to each well in the hybridization chamber to add humidity during incubation.

4.6 Place array slide in microarray hybridization chamber, seal and incubate submerged under water or in hybridization oven at 42 ^{o}C overnight (minimum of 15 hours).

4.7 Notes:

1. Once the cover slip has been applied, slide should be kept level. Minimize exposure to air until drying step. Minimize exposure to light throughout.
2. Use thin-walled 0.2 μl PCR tubes. The volume is not critical, but ideally should not exceed ~15 μl. Volume would still be 11 μl of labeled target if you are doing a single color hybridization to the array.

5. Washing

1. Remove lifter slip while slide is submerged horizontally in the first wash solution:

   1X SSC + 0.05% SDS

20X SSC	30ml
10% SDS	3 ml
ddH_2O	567 ml
Total	600 ml

   Wash for 2 minutes in first wash

2. Transfer slides to the second wash solution (This solution should be split into two 250 to 300 ml washes):

   0.06X SSC

20X SSC	1.8 ml
ddH_2O	598.2 ml
Total	600 ml

   Wash for 2 minutes in first of the second wash.

3. Transfer slide rack to fresh 2nd wash and wash for an additional 2 minutes. Carry slides to centrifuge in final wash to prevent evaporation, and then transfer to the plate rack lined with a paper towel and spin @ 500-800 rpm for 3 minutes to dry.

4. Scan slides as soon as possible. If not right away, store desiccated and protected from light.