

**Microarray Protocols
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Introduction

Microarray technology has been developing rapidly over the last several years. The AGAC microarray website (www1.umn.edu/agac) and protocols have been compiled to facilitate the application of microarray methods at the University of Minnesota. The following pages are by no means meant to be all-inclusive, so we encourage researchers to consult other sources of information outside of the University in order to determine which protocols are ideal for their specific system.

The basics parts of a microarray experiment are:

The RNA

- An experiment is performed in which RNA (total or messenger) is collected
- Two or more samples of RNA are collected in order to compare gene expression levels

The Array

- Glass slide or membrane on which spots representing genes, ORFs, ESTs, etc. are placed.

The Probe

- Fluorescently labelled cDNA is prepared from the harvested RNA samples by reverse transcription. The two RNA populations to be compared are labeled with different fluorescent markers.

The Hyb

- The probe is allowed to hybridize to the array.

The Scan

- The array is washed and scanned twice with lasers specific for the fluorescent markers used.
- Labelled cDNA will fluoresce and “light up” the spot it has hybridized to.

The Analysis

- Fluorescent intensities are extracted from both of the scanned images. The ratio of these intensities is used to determine the relative expression levels between the two populations of cells.

PROTOCOL FOR PREPARING POLY-L-LYSINE SLIDES FOR MICROARRAYS

(Some people have had success using Corning GAPS-coated slides as an alternative - you must treat them slightly different than lysine-coated slides, but they essentially behave the same)

Basics:

- The following protocol is for coating 180 slides with poly-l-lysine.
- Be sure slide racks are bent slightly inwards in the middle to hold slides more securely.
- DO NOT use powdered gloves at any time during this protocol.
- To avoid dust, try to keep slides covered or submerged in solution at all times.
- Once coated, slides are usually stable for 4 months.

1. Prepare wash solution: 180 slides: 200g NaOH pellets
 1200 ml 95% EtOH
 800 ml H₂O
2. Mix wash solution until NaOH pellets are completely dissolved.
3. Rinse slide dishes completely with H₂O. Add one rack of GoldSeal Microslides per slide dish. Pour wash solution over slides and cover.
4. Shake slides gently for 2 hours in wash solution. Although the slides come "pre-washed" the are still significant amounts of oils and debris. This basic wash is necessary to completely clean the slides before applying the poly-l-lysine.
5. Rinse slides with several liters of Milli-Q H₂O. Fill a large container with H₂O so that you can vigorously dump the rinse over the slides. Let the slides sit in the water while you prepare the poly-l-lysine solution.
6. Prepare poly-l-lysine solution (**use only plastic-ware when preparing poly-l-lysine):
 180 slides: 1500 ml H₂O
 205 ml 1X PBS (filtered)
 352 ml poly-l-lysine

Mix ingredients on stir plate in plastic beaker in order listed above.

7. Before adding poly-l-lysine, dump H₂O from slides. Immediately after adding poly-l-lysine, pour solution over slides.

8. Let slides shake gently in poly-l-lysine for 30 minutes.
9. Rinse slides again with Milli-Q H₂O in same fashion as described above.
10. Immediately spin slides in tabletop at 600 rpm until dry (approx. 5 min).
11. To ensure slides are completely dry, place racks in 50°C vacuum oven for at least 5 min. If a vacuum oven is not available, place the slides in a standard oven for a longer amount of time.
12. Store slides in a clean plastic slide box. **Slides MUST be stored for at LEAST 14 days** before spotting DNA. We do not recommend using slides that are more than 4 months old as degradation of poly-l-lysine has been observed.

**Poly-l-lysine solution can be reused if you expect to prepare a large volume of slides. To do so, filter lysine solution after step 9 and store at 4°C in a plastic container. When ready to use, distribute solution to slide dishes and add an additional 10-15ml of new poly-l-lysine to each dish. This should be repeated a maximum of 6 times.

PROTOCOL FOR AMPLIFYING PRODUCTS TO PRINT ON ARRAY

There are several methodologies for preparing products for spotting on arrays. Amplification options are determined by many factors, including sequence availability and availability of commercial primers. For sequenced genomes, specific primers may be designed to amplify from a genomic template. Or, conversely, amplification can occur using common primers on sequenced clones or on synthesized templates which contain common 5' and 3' regions. In general, it is advisable to invest in primer sets rather than template synthesis due to the greater shelf-life of primers vs. templates. It has been observed that over time long template stocks can hydrolyze during storage and usage, leading to unsuccessful subsequent amplifications.

For unsequenced genomes, random genomic libraries can be constructed and common primers can be used in colony PCR. After hybridizations, clones can be sequenced as desired.

If you are using genomic DNA as a template for the PCR it is advisable to first run a 25 ul reaction, then use 0.5 ul of this PCR product for the template in your 100 ul reaction. This minimizes genomic DNA contamination in your final product.

All PCR reactions are performed in a 96 well format with 100ul reaction volume. Individual template and primer input should be optimized for each system.

PCR reactions are performed in 96-well thermocyclers (*we use Perkin-Elmer 9700s*)

Reactions are assayed via 96 well agarose gel (4 ul run per sample).

The concentration of the final PCR products is NOT critical. If you run a 100 ul reaction and can observe a band on your gel, there should be enough product to array.

PROTOCOL FOR CLEANUP AND 384 WELL ARRAYING OF PCR PRODUCTS

Notes:

- We have had success using Millipore microscreen 96-well filter plates for the purification of PCR products. They cost more than precipitation, but they save time and effort.
- The Biorobotics TAS array printer at the AGAC is set up to use Nunc 384 well flat-bottom plates.
- If you do not have a full plate of samples, they must be loaded onto the plate in 4X4 blocks starting with wells A-D 1-4 and moving down the plate (next E-H 1-4, then I-L 1-4, etc.)

For ethanol precipitation:

1. After running 96 well reactions on 1% agarose gel and documenting results, add 10ul Sodium Acetate, pH 5.5 and 110 ul room temperature Isopropanol.
2. Transfer reactions to a Costar 96-well U-bottom Polypropylene Plate. To save on pipette tips, first add IPA to Costar plates and then transfer PCR + NaOAC to IPA. This transfer to new plate allows centrifugation of 18 plates simultaneously in an RC3B centrifuge using 6 plate carrier adapters. To do this, plates must be taped together correctly. Please see experienced users for a demonstration.
3. Spin plates at 4500 rpm for at least 2 hours.
4. Carefully aspirate solution using a 12 channel Wheaton Aspiration Adapter (*You can also use an 8 or 12 channel pipette if you're doing a small number of plates*)
5. Add 100ul of 70% EtOH. Spin plates for another hour at 4500.
6. Aspirate again and let air dry or dry in 96 well speed-vac.
7. Allow PCR products to resuspend in 20ul of H₂O for at least 18 hours.
8. Transfer products to 384-well printing plates.
9. Dry plates down in speed-vac, resuspend products in an appropriate volume of 3X SSC. (*To print with the Biorobotics spotter, you need a final volume of at least 20ul in the Nunc plates*)
10. Let plates resuspend at least overnight before printing.

PROTOCOL FOR POST PROCESSING MICROARRAYS

Before beginning:

- Start boiling water on heat plate or in microwave, enough to cover slide rack.
- Turn on heat block (highest setting) to be used for snap drying.
- Use powder free gloves throughout protocol.
- DO NOT splash any solutions on array during hydration and heat fixing.

HYDRATION/ HEAT FIXING:

1. Pick out about 20-30 slides to be processed.
2. On back of slide, etch two lines above and below center of array to designate array area after processing. Post processing causes the array to disappear.
3. Pour 100 ml 1X SSC into hydration tray and warm on slide warmer at medium setting. (*Around 37-40°C - a heat block can also be used to warm the SSC*)
4. Set slide array side down and observe spots until proper hydration is achieved. Hydration times will vary depending on printing. You may want to standardize this time for yourself. Underhydration will cause spots to be too small for proper quantitation and over-hydration will cause spots to run together. The purpose of hydration is to "fill in" the spotted DNA/SSC which immediately dries in a ring after spotting. Good hydration will cause the DNA to spread out evenly over the entire area of the spot. (*Over-hydration is generally not a problem - when in doubt, let the slides continue to hydrate*)
5. Upon reaching proper hydration, immediately snap dry slide by setting the slide array side up on the heat block set at approx. 140°C for a few seconds until the array is completely dry.
6. Place slides in rack.

SURFACE BLOCKING:

(Steps 1-5 should be performed in a fume hood)

-Store succinic anhydride in vacuum dessicator until ready to use.

1. Measure 335 ml 1-methyl-2-pyrrolidinone into designated clean, dry slide dish with stir bar. *(Don't use 1-methyl-2-pyrrolidinone if it appears yellow - it should be colorless)*
2. Dissolve 5.5 g succinic anhydride completely.
3. **IMMEDIATELY** after succinic anhydride dissolves, add 15 ml 1M NaBorate (pH 8.0) and submerge slides in solution. Plunge slides rapidly in blocking solution and shake evenly under level of solution for at least 1 min. (NaBorate is made with boric acid and pH is adjusted with NaOH. The succinic anhydride reacts with the poly-l-lysine coating and prevents non-specific hybridization on the slide.)
4. Soak slides in solution on shaker for 15 min.
5. Before 15 min. incubation is done, reduce heat on boiling water so that temp is approx. 95°C. Drain excess blocking solution off slides briefly and transfer slide rack to the almost boiling water, plunging a couple times, and incubate for 90 seconds. The boiling water denatures the spotted DNA and makes it accessible for hybridization.
6. Transfer rack to dish of 95% EtOH and plunge 5X. Spin down in tabletop centrifuge.
7. Arrays may be used immediately or stored for future use.

PROTOCOL FOR TOTAL RNA ISOLATION

- This procedure will vary, depending on your system. You may be able to use an RNA isolation kit.
- It is important to remove all DNA contamination with DNase.
- **MAKE SURE YOUR RNA IS CLEAN**
- Commonly used systems include Qiagen kits, Trizol, and hot phenol. The method used is not critical as long as you obtain good RNA.
- Poly-A RNA can be isolated from eukaryotes, but total RNA seems to work as well if not better for the reverse transcription.

PROTOCOL FOR REVERSE TRANSCRIPTION AND AMINO-ALLYL COUPLING

(Derived from a protocol developed at Rosetta Inpharmatics, Kirkland, WA)

1. RT Reaction

Mix:	Amount	uL
Oligo dT / pdN6	10 ug each	
Total RNA	At least 5 ug	
		15.5 ul total volume

- The above calculation is for total eukaryotic RNA. For poly-A RNA omit the random hexamer(pdN6) from the priming and input at least 2ug of RNA. Optimizations may be required

- *For bacterial total RNA, we have had success using 10ug RNA primed with 30 ug of hexamers*

- Incubate RNA and oligo dT at 70°C for 10 min

(use PCR tubes and thermocycler for these steps, if available).

- Chill on ice 10 min.

- Set up cDNA synthesis

	Concentration	uL per one	2
5X buffer	Supplied with SSII	6	12
50X aa-dUTP/dNTP	see below	0.6	1.2
DTT	0.1M (w/ SSII)	3	6
SuperScript II	200 U/uL	1.9	3.8
Water (DEPC)		3	6
			14.5 ul aliquots

50X recipe: For 2:3**
10uL each 100 mM dA, dG, dC
4uL 100 mM aa-dUTP
6uL 100 mM dT

**A ratio of 2 aa-dUTPs: 3 dTTP's was optimized for yeast chips. Altering the ratio to 3:2 or 4:1 may help increase signal in other systems. Optimizations are encouraged.

- **Mix RNA-primer mix (15.5 ul) with RT mix (14.5 ul)**
- **Incubate reaction mixture at 42°C for 2 hours**

2. Hydrolysis

- Add and Mix: 10ul 1 N NaOH
10ul 0.5M EDTA
- Incubate: 15 min. at 65°C.
- Neutralize with addition of 25ul 1M Tris-HCl pH 7.4, mixing well.
- Samples may be stored at 4°C overnight at this point.

3. Cleanup

- To continue with the amino-allyl dye coupling procedure all Tris must be removed from the reaction to prevent the monofunctional NHS-ester Cy5-dyes from coupling to free amine groups in solution.
- Fill one Microcon 30 concentrator with 450 ul water.
- Add neutralized reaction.
- Spin at 12K for 8 minutes.
- Dump flo-thru.
- Repeat process 2X, refilling original filter.
- Elute (turn microcon upside down in clean tube and spin at 4000 rpm 4 min). At this point a volume over 150ul should be concentrated again with a second microcon filter.
- Dry eluate in speed vac.
- Samples may be stored at -20°C indefinitely.

4. Coupling

- NOTE: Cy-dyes are light sensitive. Avoid overhead fluorescent lighting as much as possible. Also note that monofunctional Cy-dyes should be stored at 4°C.
- Resuspend cDNA pellet in 9 ul NaBicarbonate Buffer (pH 9.0). Let sit for 10-15' at room temperature to ensure resuspension.
- Transfer entire 9 ul volume into tube containing the dried Cy-dye aliquot (see below). Use Cy3 for one sample and Cy5 for the other.
- Mix by pipetting
- Let incubate 1 hr at RT in the dark

Making dye aliquots:

- If using fresh tube of Cy3 or Cy5, resuspend entire tube in 32 ul DMSO.
- Aliquot 4 ul x 8 tubes and immediately dry in speed vac. Aliquots can be stored indefinitely at 4°C.
- Note: Decreasing the number of aliquots/dye tube may increase your signal strength.

5. Quenching and Cleanup

Before combining Cy3 and Cy5 samples for hybridizations, the reactions must be quenched to prevent cross-coupling.

- Add 4.5ul 4M hydroxylamine. Let reaction incubate 15 min. at RT in dark.

To remove unincorporated/quenched Cy dyes proceed with Qia-Quick PCR Purification Kit (QIAGEN).

- Combine Cy3 and Cy5 reactions.
- Add 70ul water.
- Add 500ul Buffer PB.
- Apply to Qia-quick column and spin at 13,000 rpm in for 30-60 sec.

- Aspirate off flo-thru.
- Add 750ul Buffer PE and spin 30-60 sec.
- Aspirate (dump) off flo-thru and repeat.
- Aspirate flo-thru and spin for 1 min. at high speed to dry column.
- Transfer to fresh centrifuge tube
- Add 30ul Buffer EB to center of filter and let sit 1 min. at RT.
- Spin at 13,000 rpm for 1 min.
- Repeat elution step again.

7. Hyb. Prep.

- Dry down Qia-quick eluate in speed vac. Bring volume to 15 ul with water.
- Add: 3ul 20X SSC
1.5 ul polyA (10mg/ml)

(You will want to use an alternative blocker such as salmon sperm DNA if you're using total bacterial RNA)

Optional:

- Filter probe in Millipore 0.45um membrane: Pre-wet with 10ul water and spin through at 8K in microcentrifuge. Remove flo-thru and deposit samples on inside tube as drop, not on filter. Spin at 10K and remove sample to 0.5 ml tube.

At this point reactions may be stored for up to 48 hours at 4°C before hybridization at **DO NOT** store at -20°C.

PROTOCOL FOR ARRAY HYBRIDIZATION

1. Prepare probe as described at end of labeling protocol.

Cy3+Cy5 samples	15.0ul
20X SSC	3.0ul
Blocker(10mg/ml)	1.5ul
2. Add 0.45ul 10%SDS.
3. Boil probe for 2 min. at 100°C. Let cool 5-10 min. at room temp. Boiling denatures the sample and makes it accessible for hybridization.
4. Label slide with etcher and set in hybridization chamber. Put a 10ul drop of 3XSSC at one end of the hyb chamber and another at the other end of the chamber on top of the slide. This is to ensure a constant humidity in the chamber during hybridizations. If the 3XSSC is not applied, the array will dry out.
5. Clean a lifterslip or coverslip with EtOH and Kimwipes. Place slip on array using forceps.

Lifterslip: Should be applied with dull white strips on the long axis of the chip touching the glass. This creates a platform, which allows even distribution of the hybridization solution across the array. Slowly pipette the probe under one cover of the slip until the entire array surface is covered.

Coverslip: Put the probe onto one edge of the array in a drop (avoiding bubbles). The coverslip should be placed on the array starting at the edge next to the probe while you slowly lower the other end onto the slide - practice with water first to avoid trapping bubbles under the coverslip. If bubbles do get trapped under the coverslip, most will move out once the slide is placed in the water bath.
6. Tightly screw down hyb chamber lid and carefully place chamber in a 63°C water bath. Take caution to keep array completely flat during transfer and hybridizations.
7. Allow hybridization to run for at least 5 hours but not more than 16 hours. Other systems may benefit by different temperatures and times.

PROTOCOL FOR ARRAY WASHING

- It is recommended that all wash solutions be filtered before using.
- Prepare wash solutions in glass slide dishes, with each dish having its own rack.

Wash Soln. I

340 mL millipure water
10 mL 20X SSC
1 mL 10% SDS

Wash Soln. II

350 mL millipure water
1 mL 20X SSC

1. Carefully remove array from water bath, making sure to keep chamber level. Dry array with paper towels and attempt to "wick" any water away from chamber seams.
2. Unscrew chamber and remove array. Some water may enter chamber and pool under slide at this time. If so, it is helpful to have a pair of forceps to pry array away from chamber.
3. Keep array level when submerging in Wash I. Once submerged tilt array and gently dump off coverslip. It may be necessary to lightly swish array under solution to dislodge the slip.
4. Once slip is off and lying on bottom of slide dish, put array in rack and remove any additional hybs from water bath. **DO NOT** allow the slide to dry out at any point during the washes. When all the slides are in Wash I, plunge rack up and down 10-20 times (at least 1 min).
5. Individually transfer slides to slide dish containing Wash II, do not transfer entire slide rack as this will cause too much SDS carryover. Plunge 10-20 times again.
6. Dry array in room temperature table top centrifuge at 600 rpm for 5 min.
7. Try to scan array within hours of washing as the Cy dyes are instable and degrade differentially.