

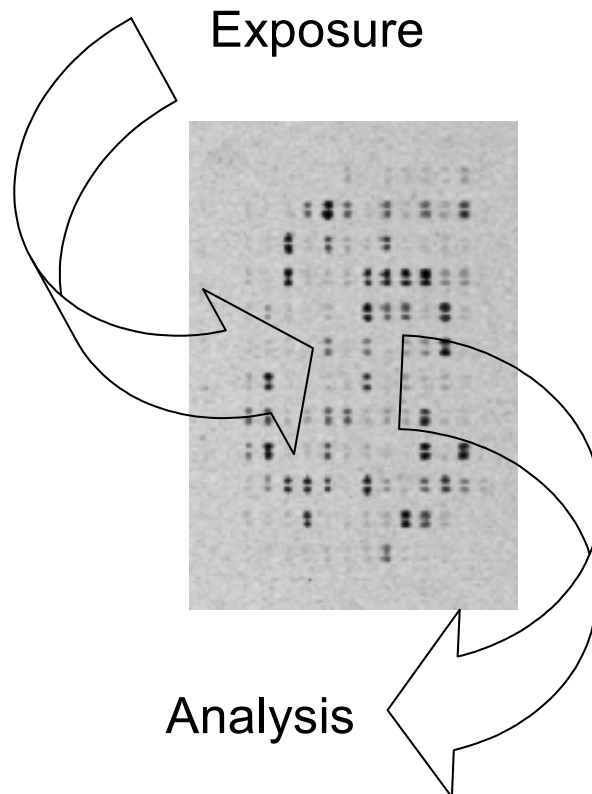


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Microarray Protocol

500-Gene Tox-BassChips[®]

(Catalog No. CH-LMB-500)





Manual Version 1.0

Warrant and Liability

EcoArray is committed to providing the highest quality products. EcoArray warrants that the products meet or exceed performance standards. If you are not completely satisfied with this product, our policy is to replace the product or credit the full purchase and delivery charge. No other warranties of any kind, expressed or implied, are provided by EcoArray. EcoArray's liability shall not exceed the purchase price of the product. EcoArray shall have no liability for direct, indirect, consequential or incidental damages arising from the use, results of use, or inability to use its products. The protocols listed in this manual are meant as a guide for array labeling and hybridizations. Researchers need to make sure that proper laboratory safety procedures are in place before proceeding with any of these protocols. This product is intended for research use only. This product is not intended for diagnostic or drug purposes.

Literature citation

When publishing scientific papers using this product, we would appreciate that you refer to the chips as BassChips. When referring to genes that were spotted on the arrays, please refer to the genes by their EcoArray gene ID#. This number, which does not change, can be queried in the BlastQuest gene database for gene identification.



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Introduction

A. Background

Gene sequences spotted onto a solid support matrix are called gene arrays (also referred to as microarrays, macroarrays, gene chips, or DNA arrays). An array can consist of hundreds to thousands of cDNAs or oligonucleotide gene sequences spotted in an organized pattern. The arrays can be probed with mRNA obtained from treated tissues or cells in order to obtain a semi-quantitative measure of expression for each of the genes present on the array. Thus, gene arrays can be used to simultaneously measure the expression level of all the genes that are affixed to the surface. Because thousands of genes can be queried at one time, this global analysis of gene expression can be quite useful in measuring the effects of toxicological exposure as well as other areas of research. The 500-gene tox-BassChip[©] contains PCR products from genes that were isolated by a variety of methods including shotgun sequencing of cDNAs from an E₂ treated largemouth bass (LMB) library, PCR amplification of target genes of the endocrine system using degenerate primers, and differential display (DD RT-PCR). Thus, many of these genes are known to be responsive to estrogens. The bass-gene chips contain many genes of toxicological importance including vitellogenin, choriogenin, several cytochrome P450s, estrogen receptor alpha, glutathione S transferase, among others.

The Basschip[©] has been optimized using a α -³³P label probe with minimal (2 μ g) total RNA as the starting material. Ten nanograms of PCR product is spotted per gene. The membranes can also be labeled with ³²P or a chemiluminescent label following manufacturer's recommendations.

B. Materials provided

- Four 500-gene tox-BassChips[©] (printed on nylon membranes).
- A complete list of genes that were spotted (file will be sent by email).
- Free access to BlastQuest, a searchable website database that contains pertinent information obtained for each gene spotted (access codes will be sent by email). The BlastQuest data includes expectation (e) values, bit scores, accession numbers, and a link to sequence information at PubMed, NCBI.
- Single-use aliquots of spiking RNA (one tube provided per membrane).



C. Materials not provided

For RNA extractions...

- **RNA extraction kits**
We recommend using either STAT-60 (Teltest, Friendswood, TX, Cat. # CS-110, 111, or 502) **or** NucleoSpin columns (Clontech # K3064-1) for RNA purification.
- **RNA re-suspension solution** (not required, but recommended)
We recommend using RNAsecure (Ambion, Cat. # 7010).
- **DNase treatment**
We recommend using DNA-free (Ambion, Cat. # 1906).

For probe preparation...

- **RNA labeling kit** (1 labeling reaction needed/membrane)
 - EcoArray Cat. # KT-0401...currently in beta testing, see alternate product below.
 - Alternate product -- We recommend Ambion Strip EZ RT kit, 20 reactions/kit, Cat. #1490.
- **Nucleotide removal kit** (1 clean-up column needed/membrane)
We recommend Qiagen nucleotide removal kit, 50 columns/kit, Cat. #4389.
- **Radiation** (~ 12.5 μCi [α - ^{33}P] dATP needed/membrane)
We recommend [α - ^{33}P] dATP, Perkin Elmer, 250 μCi , Cat. # NEG612H.

For hybridization...

- **Hybridization buffer** (~15mL buffer needed/membrane)
 - EcoArray Cat. # SN-0620...currently in beta testing, see alternate product below.
 - Alternate product -- We recommend Ambion ULTRAhyb, 125 mL, Cat. # 8670.

For washes...

- **Wash #1 (Low stringency wash solution)** (~125 mL buffer needed/membrane)
 - EcoArray Cat. # SN-0616, 500 or 1000 mL solution
 - (Protocol for solution provided in Appendix.)
- **Wash #2 (High stringency wash solution)** (~125 mL buffer needed/membrane)
 - EcoArray Cat. # SN-0617, 500 or 1000 mL solution
 - (Protocol for solution provided in Appendix.)

For detection and scanning...

- **Chromatography Paper, Grade 3MM** (Whatman # 3030-6461). We recommend Whatman # 3030-6461 (26 x 41 cm) paper purchased from Fisher, Cat. # 05-713-336.



Microarray Procedure

We recommend you read the entire protocol below before proceeding. The protocol is a compilation based on reagents and methods that are supplied by other manufacturers. The protocol has been optimized as described in Larkin *et al.*, 2002 and 2003. We further recommend that you read all catalogue material provided by any manufacturers whose products are used in this protocol before proceeding with any steps.

A. RNA preparation

RNA needed for labeling: 2 μg of high-quality total RNA that is DNase-treated. Prepare the total RNA with a final concentration as close as possible to 1 $\mu\text{g}/\mu\text{L}$.

Source of RNA

It is critical to harvest tissues from animals (or cells) under conditions that will preserve RNA. Methods of choice include freezing the samples in liquid nitrogen and then storing at -80°C , or using a preservative such as *RNAlater* (Ambion, Austin, TX, Cat. # 7020, 100 ml) that is easily transported to the field. Tissue samples should be cut into very small pieces for quick preservation. In the case of *RNAlater*, it is important to use the ratio of solution to tissue recommended by the manufacturer. Using less will cause improper preservation of tissue and will result in severe RNA degradation.

Preparation of RNA

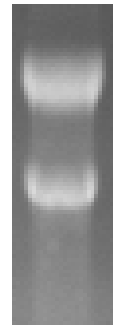
For array expression analysis, total RNA must be extracted from samples that are to be arrayed. We recommend using either STAT-60 (Teltest, Friendswood, TX, Cat. # CS-110, 111, 502) or NucleoSpin columns (Clontech, Cat. # K3064-1) for RNA purification. Both of these kits yield total RNA of consistently high quality. Some RNases accompany the RNA throughout the isolation procedure, surviving the strong denaturants that are routinely used. These RNases must be removed by treatment of the RNA with RNase inhibitors or with proteinase K, to remove all traces of proteins. An exceptionally good product for this purpose is *RNAsecure* (Ambion, Cat. # 7010), but several other supply companies have similar products.

DNase treatment of RNA

It is necessary to remove any contaminating DNA from the RNA samples prior to cDNA labeling since the samples will be labeled with random primers, and there is a slight chance that DNA could also be labeled in the reaction. For digestion of any DNA in the RNA samples the following kit is recommended: *DNA-free* (Ambion, Cat. # 1906). Follow the manufacturer's recommendations. Prepare the total RNA to have a final concentration as close as possible to 1 $\mu\text{g}/\mu\text{L}$.

Quality inspection of RNA

The importance of **high quality** RNA cannot be overstated. Total RNA should be examined by agarose gel electrophoresis to ensure that the rRNAs appear intact (2 bands should be observed with the upper band being approximately twice as intense as the lower band). The RNA also should be quantified by absorbance at 260 nm. The ratio of absorbances at 260 and 280 nm should be between 1.8 and 2.0. Lower values indicate contamination by proteins.



RNA run on a denaturing agarose gel showing two distinct bands.

Figure 1. RNA

B. Probe preparation

Total RNA samples will be random primer labeled with [α -³³P] dATP by a direct incorporation of the radio-nucleotides during first strand cDNA synthesis. Following cDNA synthesis, unincorporated nucleotides need to be removed.

For the probe synthesis and clean-up, the following two kits are recommended: Ambion's RNA labeling kit (Cat. # 1490) for labeling and Qiagen's nucleotide removal kit (Cat. # 4389) for removing unincorporated nucleotides. Follow the manufactures' recommendations.

Abridged version of labeling reaction

This is the procedure that EcoArray follows to label and hybridize our RNA samples to the microarrays. The procedure is based upon the manufacturer's recommendations for each of the kits. If any steps are unclear, refer to the manufacturer's recommendations for each of the kits.

Equipment needed:

- Rotor hybridization oven (Set at 64°C; 12-15 rpm.)
- Water baths (Three: set at 65°C, 37°C, and 100°C.)
- Phosphor-imager cassette
- Phosphor-imager
- Hybridization bottles

Prior to beginning any work:

- Remove radiation from freezer and keep behind a radioactive shield to thaw. (*Note: Let thaw at room temperature, not in a heat incubator since radio-nucleotides are somewhat temperature sensitive*).
- Pre-warm UltraHyb array hybridization buffer at 64°C.



1. RNA labeling (Ambion's RNA labeling kit, Cat. # 1490)

Single use aliquots (1.2 μL) of the spiking RNA are provided. Spiking RNA is exogenous (plant) RNA that is added to every RNA sample that is radiolabeled. PCR products corresponding to the spiking RNA samples have been added to the membranes. Data from the spiking RNA spots on the membranes can be used for normalization purposes.

- Add 2 μg of RNA, 2 μL random hexamers, and 1.2 μL spiking RNA to an RNase-free 1.5 mL microcentrifuge tube. (See Tables below for example calculations.)
- Heat tube at 65° for 5 minutes.
- Cool at room temperature for 2 minutes.
- Briefly spins the tubes(s) down and then add the following reagents in the following order:
 - X μL DEPC treated water
 - 2 μL 10X RT Buffer
 - 2 μL 10X dNTP solution
 - 1 μL RT enzyme
 - 2 μL radiolabel ($[\alpha\text{-}^{33}\text{P}]$ dATP)

EXAMPLE:

Membrane #	RNA Sample #	Avg Conc $\mu\text{g}/\mu\text{L}$	$\mu\text{L}/\mu\text{g}$	2 $\mu\text{g} = x$
7	15	1.23	0.81	1.63 μL
8	16	1.76	0.57	1.14 μL
10	17	1.2	0.83	1.67 μL

(Note: labeling reaction volume is 20 μL .)

RNA Sample #15	RNA Sample #16	RNA Sample #17
Add: 1.63 μL RNA (sample 15) 1.20 μL spike RNA + 2.00 μL Random Decamers 4.83 μL	Add: 1.14 μL RNA (sample 16) 1.20 μL spike RNA + 2.00 μL Random Decamers 4.34 μL	Add: 1.67 μL RNA (sample 17) 1.20 μL spike RNA + 2.00 μL Random Decamers 4.87 μL
<ul style="list-style-type: none"> • Heat to 65° (5 min) • Cool to room temperature (approximately 2 min) 		
Then add: 8.17 μL nuclease-free water	Then add: 8.66 μL nuclease-free water	Then add: 8.13 μL nuclease-free water
<ul style="list-style-type: none"> • Then add to each tube (will have 20 μL after all additions): <ul style="list-style-type: none"> ▪ 2 μL 10X RT Buffer ▪ 2 μL 10X dNTP solution ▪ 1 μL RT enzyme ▪ 2 μL $[\alpha\text{-}^{33}\text{P}]$ dATP radiolabel 		



- Incubate at 37° for 1.5-2.0 hours. (*Note: this is longer than the kit recommends.*)
- Spin down tubes briefly before proceeding to the next step below.

2. Removal of un-incorporated nucleotides (Qiagen's Nucleotide Removal Kit, Cat. # 4389)

- Add 10-fold (200 μ L) of PN Buffer to a 1.5 mL microcentrifuge tube that contains the cDNA. Gently mix solutions with pipette.
- Move solution to purification spin column and spin down for 1 min at 6000 rpm.
- Transfer purification spin column to a clean 2.0 mL collection tube. (*Note: We buy 2.0 mL tubes in bulk and simply cut off the lids to make additional collection tubes.*)
- Add 500 μ L of PE buffer to column.
- Spin down column for 1 min at 6000 rpm.
- Transfer column to a clean 2.0 mL collection tube.
- Add 500 μ L of PE buffer to column.
- Spin down for 1 min at 13000 rpm.
- Transfer column to a clean microcentrifuge tube (1.5 mL) and make sure to wipe excess fluid from outside of column.
- Add 50 μ L of EB Buffer directly onto filter in column and let sit for 1 min.
- Spin down for 1 min at 13000 rpm.
- Transfer eluted fluid to clean, labeled 1.5 mL microcentrifuge tube.

3. Check radio-nucleotide incorporation

- Add 1 μ L of probe to scintillation fluid that is in vial.
- Read CPMs of radiation in a Scintillation Counter.
- **Notes:**
 - *1 x 10⁶ CPM (or DPM) of radiation is needed per mL of hybridization buffer. (See below for example of calculations.)*
 - *The amount of hybridization buffer used is a reflection of the size of the hybridization bottles. A general rule of thumb is that more hybridization buffer is better than less. (In a 150 mm tall hybridization bottle we add 4-6 mL of array hybridization solution; 8 mL for the larger 300 mm tubes.)*

EXAMPLE: (Continued from page 7)

RNA Sample #	WIDE CPM (or DPM)	UltraHyb Array Buffer
15	279481.5	4 mL (6" tube)
16	196426.0	4 mL (6" tube)
17	194216.2	4 mL (6" tube)

C. Hybridization

1. Radiation calculations

- *Note: it is very important to **WAIT** to pre-hybridize the membranes until **AFTER** it is determined that there are sufficient counts of radiation required for the hybridization step. In a typical labeling reaction we get 250,000-500,000 CPM/1 μ L of probe.*
- **Formula for figuring out how much radiation to add to hybridization bottles:**

(1 μ L radiation / # of CPM) (1,000,000 CPM)(X mLs UltraHyb Array Buffer) = X μ L radiation to add to hybridization bottle with 4 mL buffer

(X μ L Radiation)(20fold) = Y μ L 10 mM EDTA
(To be added to probe right before heat denaturation step.)

Example (from Table on page 8):

Sample #15:

(1 μ L radiation / 279481.5 CPM)(1,000,000 CPM)(4 mL Ultra Array Buffer) = 14.3 μ L radiation

(14.3 μ L Radiation)(20 fold) = 286 μ L 10 mM EDTA

Sample #16:

(1 μ L radiation / 196426.0 CPM)(1,000,000 CPM)(4 mL Ultra Array Buffer) = 20.4 μ L radiation

(20.4 μ L Rad)(20 fold) = 407 μ L 10 mM EDTA

Sample #17:

(1 μ L radiation / 194216.2 CPM)(1,000,000 CPM)(4 mL Ultra Array Buffer) = 20.6 μ L radiation

(20.6 μ L Rad)(20 fold) = 412 μ L 10 mM EDTA

2. Pre-hybridization of membranes

- Add membranes to hybridization bottles; cDNA printed side should be on the **INSIDE** – not in contact with the bottle. Pre-hybridize (no radiation at this point) membranes by adding 4 mL (150 mm bottle) of pre-warmed UltraHyb Array Buffer. Place bottles in rotor hybridization oven (12-15 rpm) that is set at 64°C for a minimum of 1.5 hours.



Figure 2. Rotor Hybridization Oven



3. Hybridization

- Mix appropriate volume of radiation and 10 mM EDTA into labeled 1.5 mL microcentrifuge tube.
- Heat shock probe mixture in a 100°C water bath for 5 mins. (*Note: Make sure there is a lid cap on the 1.5 mL tube, otherwise the top will pop off due to the extreme heat.*)
- Quench tube in slushy ice mixture for 1-2 minutes.
- Spin down tube before opening.
- Add solution directly to pre-hybridization mixture (there is no need to dump out the pre-hybridization mixture). *Note: Do not pipette the probe onto the membrane, but **directly into** the pre-hybridization solution.*
- Agitate (shake) the hybridization tube briefly and then put in rotor hybridization oven overnight (~ 14 hours at 12-15 rpm).
- Put washes #1 and #2 in a 64°C water bath for use the next day.

D. Washing membranes (Day #2)

1. Begin washes

- *Note: The first pre-wash rinse is most important, make sure to agitate the membranes in the hybridization bottles for a few minutes.*
- Washes are to be done in 15 min intervals. Dump the wash solutions out of the hybridization bottles between each 15 minute wash cycle. Note that the wash solutions **MAY** be radioactive and should be monitored prior to disposal.
- Use this table to keep track of the required washes, if desired.

Wash # 1	Start time	End Time	Wash #2	Start time	End time
Pre-wash rinse			A		
A			B		
B			C		
C			D		
D					

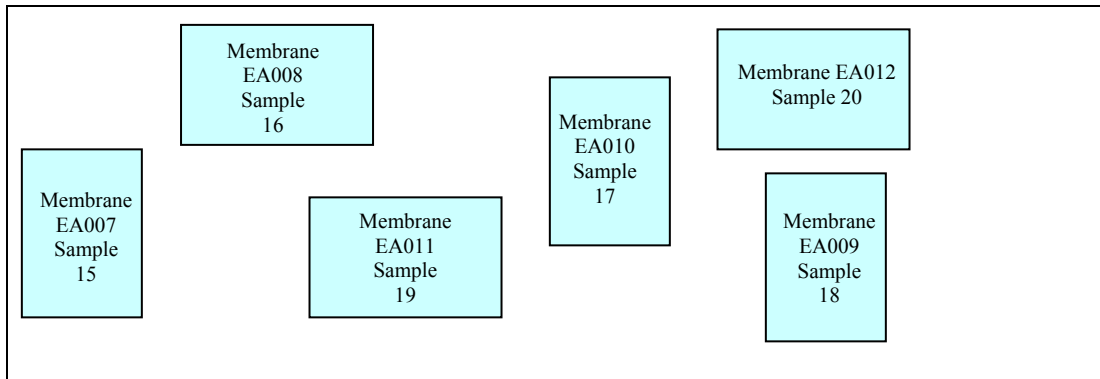
2. Membrane Layout

- After all washes are finished: Set membranes, cDNA spotted side up, on 3MM chromatography paper that has been **dampened** (do not cover the membranes) with DEPC treated water. This is critical if you want to strip of the probes and re-label the membranes. (*Note: The best method to strip the membranes is to boil them for ~5*

minutes in DEPC treated water. While this method does remove the radioactive probes, some of the PCR products will also be removed.)

- Arrange membranes in a nonsymmetrical pattern on the chromatography paper, but keep membranes very straight. See example below.
- Wrap the entire paper and membranes in plastic (Saran) wrap without making any wrinkles or folds.

Example of membrane lay-out:



E. Detection and scanning

- Expose the wrapped membranes to a phosphor screen overnight or longer. (May be exposed for as long as 4 days.)
- Scan the screen in a Typhoon (or other scanner) using the 100 micron setting.



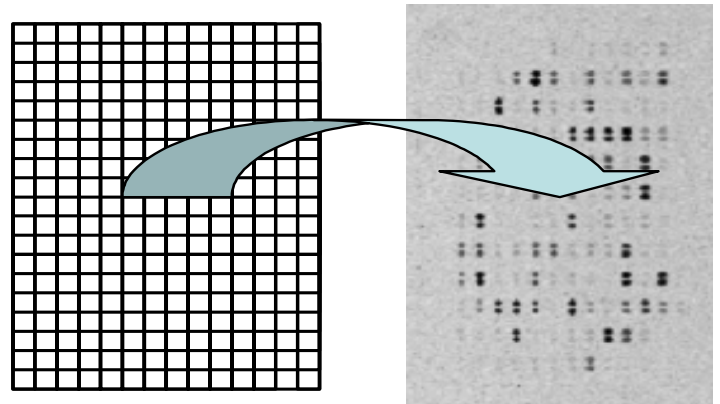
Figure 3. Phosphor screen



Figure 4. Typhoon scanner

F. Image analysis

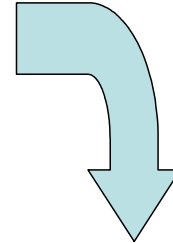
Quantification of the gene chips can be performed using software that usually comes with the scanner used for detection. In general, quantification of the arrays involves drawing boxes or grids around each of the spots and determining the intensity of each spot. Use the gene spot orientation (last page of manual) to correctly orient the membranes, prior to making the grids.



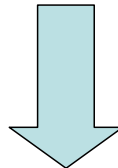


Customers are also provided via email an excel list that contains information for each gene.

Paste in intensity values
for each gene (data obtained from
scanner software)



Spot location	EcoArray gene ID #	Top hit_definition	Top hsp_evalue	Top hsp_score	Top hit_accession	Intensity values
1	EA-LMB-034	gene E protein	1.54103E-25	294	NP_040748	
2		splicing gene (CAD)				
3	EA-LMB-035	no significant match in GenBank				
4	EA-LMB-036	unknown gene				
5	EA-LMB-037	ceruloplasmin (ferroxidase)	4.91776E-31	341	AAH8037	
6	EA-LMB-038	apolipoprotein B	0	622	S65460	
7	EA-LMB-039	complement C3-H1	0	872	BAA36619	
8		splicing internal 5C spots				
9	EA-LMB-041	L-serine dehydratase/L-threonine deaminase	6.14236E-18	228	P09367	
10	EA-LMB-042	unknown gene				
11	EA-LMB-043	selenoprotein P0	6.69615E-28	314	AAG53689	
12	EA-LMB-044	unknown gene				
13	EA-LMB-220	unknown gene				
14	EA-LMB-221	no significant match in GenBank				
15	EA-LMB-222	unknown gene				
16	EA-LMB-223	macrophage stimulating 1 (hepatocyte growth factor-like)	0	636	NP_694512	
17	EA-LMB-224	unknown gene				
18	EA-LMB-225	alpha-2 collagen type VI, subunit	8.05864E-24	279	CAA44206	
19	EA-LMB-367	no significant match in GenBank				
20	EA-LMB-368	androgen receptor	0	1155	AAG8340	
21	EA-LMB-369	no significant match in GenBank				
22	EA-LMB-370	no significant match in GenBank				
23	EA-LMB-371	no significant match in GenBank				
24	EA-LMB-372	no significant match in GenBank				
etc...	etc...					



This data could be graphed in excel or
exported to other programs for
additional analysis.

Interesting genes that are identified as significant can be queried further using the Blast Quest information database program. BlastQuest is a searchable information database that is web accessible. This database contains pertinent information for each gene that is spotted onto the gene arrays. For each gene, information such as expectation (e) values, bit scores, accession numbers, and links to sequence and literature information at the National Center for Biotechnology Information (NCBI) is available.

- Website: www.ecoarray.com (The link to the program is on “Products” page.)
- Access codes: (will be emailed).

Troubleshooting

A. Low counts of radiation

There are several possible reasons for having low counts.

- The RNA is degraded. The Ambion kit contains some control RNA that can be used to see if the RNA you are using is degraded. One of the most common reasons for having degraded RNA is multiple freeze-thaws of the RNA. Make 2 μg aliquots of your RNA before the labeling procedure. Run a check gel again to see if the RNA is intact (two distinct bands observed).
- The concentration of the RNA was not accurately determined and 2 μg were not used in the labeling reaction. Re-spec your samples.
- The enzymes in the labeling kit are degraded. Try to minimize the amount of time the reagents for the labeling kit are out of the -20°C freezer. Don't use a frost-free freezer to store the kit.
- The radiation has expired. ^{33}P has a half-life of approximately 2 weeks. Try to order a radiation lot that is as fresh as possible prior to labeling the RNA. If ordering radiation from Perkin Elmer, fresh lots of radiation are made the first and third Thursday of every month.

B. High background on membranes

This is most likely due to one of two scenarios. Either:

- the radiation during the hybridization step was pipetted onto the membrane and not well mixed, or
- adequately rinsing of the membranes did not occur during the first wash step.

C. Spots on membranes are faint

- Try exposing the membranes to the phosphor cassette screen for a longer time period.

D. Very few spots are observed on the membranes

- The hybridization temperature is too high. The optimal hybridization temperature for the membranes is between $60-64^{\circ}\text{C}$. Below this temperature range, non-specific binding will occur. Above this temperature range, binding of the RNA to some of the PCR products will not occur because the melting temperature of some of the probes is close to this temperature.

Appendix

A. Solutions (Protocols for 1 liter final volume.)

Both protocols require 20X Sodium saline citrate (SSC) and 20% Sodium dodecyl sulfate (SDS).

- **Wash Solution #1**
(2X SSC, 0.5% SDS)

$$(X)(20X\text{ SSC}) = (2X\text{ SSC})(1000\text{mL total volume})$$
$$X = 100\text{ mL } 20X\text{ SSC}$$

$$(Y)(20\% \text{ SDS}) = (0.5\% \text{ SDS})(1000\text{mL total volume})$$
$$Y = 25\text{ mL } 20\% \text{ SDS}$$

- **Wash Solution #2**
(0.5X SSC, 0.5% SDS)

$$(X)(20X\text{ SSC}) = (0.5X\text{ SSC})(1000\text{mL total volume})$$
$$X = 25\text{ mL of } 20X\text{ SSC}$$

$$(Y)(20\% \text{ SDS}) = (0.5\% \text{ SDS})(1000\text{mL total volume})$$

B. References

The following peer reviewed papers have been published using this gene chip technology:

- Larkin, P., Knoebl, I., Denslow, N. (accepted) Differential Gene expression in fish exposed to endocrine disrupting compounds. *Comparative Biochemistry and Physiology*.
- Larkin, P., Sabo-Attwood, T., Kelso, J., and Denslow, N. (2003) Analysis of gene expression profiles in largemouth bass exposed to the endogenous hormone, estradiol, and the environmental contaminants, nonylphenol, and *p, p'*-DDE. *EcoToxicology*. In Press.
- Larkin, P., Folmar, L.C., Hemmer, M.J., Poston, A.J., and Denslow, N. (2003) Expression profiling of estrogenic compounds using a sheepshead minnow cDNA macroarray. *Environmental Health Perspectives, Toxicogenomics*. 111, 29-36.
- Larkin, P., Sabo-Attwood, T., Kelso, J., and Denslow, N. (2002) Gene expression analysis of largemouth bass to estradiol, nonylphenol, and *p,p'*-DDE. *Comp Biochem Physiol B Biochem Mol Biol*, 133, 543-557.
- Larkin, P., Folmar, L.C., Hemmer, M.J., Poston, A.J., Lee, H.S., and Denslow, N. (2002) Array technology as a tool to monitor endocrine disruption in wild fish populations, *Marine Environ Res*. 54(3-5):395-9.
- Sabo-Attwood, T., Larkin, P., Kelso, J., and Denslow, N.D. (July 2002) Gene expression profiles of largemouth bass exposed to nonylphenol and ICI 182,780. *International Congress on the Biology of Fish*, University of British Columbia, Vancouver Canada.

EcoArray Bass Chip Spot Image

