Preface

Dramatic technological advances have marked every quantum leap in our understanding of biological systems. Advances in manipulating and sequencing DNA triggered the last such leap. More than 20 years ago this technology began finding its way into biology laboratories, and multiple landmark discoveries have followed, including the sequencing of the genomes of several prokaryotic and eukaryotic species. This, in turn, spawned a new interest in bioinformatics, structural biology, and high-throughput methods that would allow scientists to look at the response of the entire genome to physiological, pharmacological, and pathological changes. Whether this transition was driven by exhaustion of the existing hypothesis pool, a subconscious adoption of the old "new paradigm" of biological complexity, or an instinctual urge among biologists to look for new and interesting phenomena is not really important. What is important is that high-throughput methods are becoming more and more routine and available, and experimentalists and theoreticians must be prepared to take advantage of them. Spotted DNA microarrays fall into this category. They power functional genomics, a nascent research field dealing with the structure and activity of genomes and global relationships between genotype and phenotype. The birth of the field can be traced back to three seminal papers ***(2,3,12)***. These three works grew from the realization that by placing individual sequence elements on a solid surface one can probe by hybridization a nearly unlimited number of targets simultaneously. Since then, similar *ad infinitum* approaches have been used to monitor relative protein levels ***(6)***, protein functions ***(15)***, cellular activities ***(16)***, and molecular interactions ***(10)***. These breakthrough studies will set the stage for the development of the fields of functional proteomics, metabolomics, etc. However, at the moment only functional genomic techniques on solid surfaces enjoy relatively wide acceptance because they are based on sounder physical-chemical principles.

The long-term impact of functional genomics will depend on multiple factors— standardization and simplification of the protocols used, robust error assessment, streamlining of the analytical techniques, the sustainability of cost. The goal of the volume is to familiarize its readers with available, reproducible protocols in the field, and to attempt to introduce an audience of biologists to data processing techniques that will become critically important as we start dealing with increasing quantities of information. The "Methods" are divided in two sections: (i) Methods in Data Generation and (ii) Methods in Data Analysis. The first section focuses on bench techniques that have been

developed and are being routinely used in several hard-core genomics laboratories. Besides general applicability of the techniques, the articles represented in the first section were selected on the basis of one major criterion: that they give sufficiently robust protocols to be adopted without modification by workers who have just begun their journeys in the field of genomics. The section opens with articles describing ways to manufacture and use spotted microarrays on three different solid surfaces: glass, plastic, and nylon membranes. Arrays manufactured on glass surfaces are usually interrogated with fluorescent nucleic acid probes, and Chapter 4 describes an optimized RNA labeling procedure that is applicable to the known spectrum of RNA sources. This chapter is followed by articles dealing with issues and protocols that are common to the field of bacterial functional genomics. The last two years' work in the field of functional genomics was marked by development of specialized applications that have added to its depth. In this period there were techniques introduced that allow one to monitor subcellular RNA localization *in masse* ***(4,9,14)***, to map chromosomes at the resolution of a single gene ***(8,13)***, and to survey the steady-state genome-wide distribution of DNA binding proteins in vivo ***(5,7,11)***. Chapters 6–8 deal primarily with the methodologies behind these advances; Chapter 7 also provides a link between expression profiling and determination of gene copy number using whole-genome DNA microarrays.

The issues of inference, experimental design, and reproducibility are of the paramount importance to researchers who deal with massive data sets. The second section of the "Methods" volume focuses on experimental design, data analysis, data display techniques, and bioinformatics. The section opens with a comprehensive overview of the inferential issues in microarray data analysis. The next four articles (Chapters 10–13) address in sequential manner some of the issues outlined in the overview article ***(9)***: design of microarray experiments ***(10)***; choice of the test statistic and assessment of the significance of observations ***(11)***; data reduction ***(12)***, and clustering, the most popular technique for microarray data classification ***(13)***. Accelerating and making the most of functional genomics studies are impossible without visualization and data storage, both of which—like the remaining methods in the field—are still in their infancy. These problems cannot be overlooked, however, because they allow genomics researchers and their colleagues in the scientific community to examine and mine experimental results. Two articles (***14*** and ***15***) describe some available approaches to the data visualization and database related issues. There are several things that were not reflected in any of the featured articles but are nevertheless worth noting. Spotted DNA microarrays are currently being used mainly for two purposes: 1. screening and 2. modeling. Truly successful application of microarrays in these two areas depends, albeit to different degrees, on technology standardization as well as the free, unimpeded flow

of experimental data. Although we believe that methods will become fairly standard in the near future, a good deal of useful and valuable information will be lost in the short run owing to the lack of enforceable standards and controlled vocabularies for experimental annotation. The Minimum Information About a Microarray Experiment (MIAME) specifically addresses this issue ***(1)***, and should be read by anyone who wants to engage in expression profiling.

The "methods" we have compiled do not provide advice about or comparisons of the available spotting platforms, image extraction and analysis algorithms, data storage and retrieval devices, and data analysis compendia. Although the available options range from simple, relatively affordable solutions to high-end, sometimes extremely expensive, commercial ones, we believe that information accumulated in the field is not yet sufficient and/or systematic enough to provide comprehensive comparisons of individual solutions and/or specific recommendations.

In the course of preparing this volume we surveyed available microarray web resources. We encourage readers interested in developments in the field to keep an eye on the following sites:

http://www.microarrays.org
http://derisilab.ucsf.edu/
http://www.mged.org/Workgroups/MIAME/miame.html
http://www.bioconductor.org/
http://ihome.cuhk.edu.hk/~b400559

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2

Nylon cDNA Expression Arrays

George Jokhadze, Stephen Chen, Claire Granger, and Alex Chenchik

1. Introduction

Nucleic acid arrays provide a powerful methodology for studying biological systems on a genomic scale. BD Atlas™ Arrays, developed by BD Biosciences Clontech, are expression profiling products specifically designed to be accessible to all laboratories performing isotopic blot hybridization experiments. We have developed two types of readily accessible BD Atlas Arrays: nylon macroarrays, well suited for high-sensitivity expression profiling using a limited gene set, and broad-coverage plastic microarrays, for a more extensive analysis of a comprehensive set of genes. In this chapter, we describe protocols for printing and performing gene expression analysis using nylon membrane–based arrays. For a more in-depth description and protocols related to plastic film–based arrays, please refer to Chapter 3.

Nylon membrane–based arrays offer several advantages for researchers. Compared with glass arrays, nylon arrays are usually less expensive to produce and require less complicated equipment. Nylon arrays are generally considered more user friendly, since analysis involves only familiar hybridization techniques. Detection of results is also straightforward—probes are radioactively labeled, so one can simply use a standard phosphorimager.

1.1. Sensitivity of Nylon Arrays

Nylon membranes are typically used to print low- (10–1000) to medium- (1000–4000) density cDNA arrays. Unlike high-density arrays, which are usually printed on glass or plastic supports, probes for nylon arrays can be labeled with ^{32}P, resulting in a much higher (>fourfold) level of sensitivity

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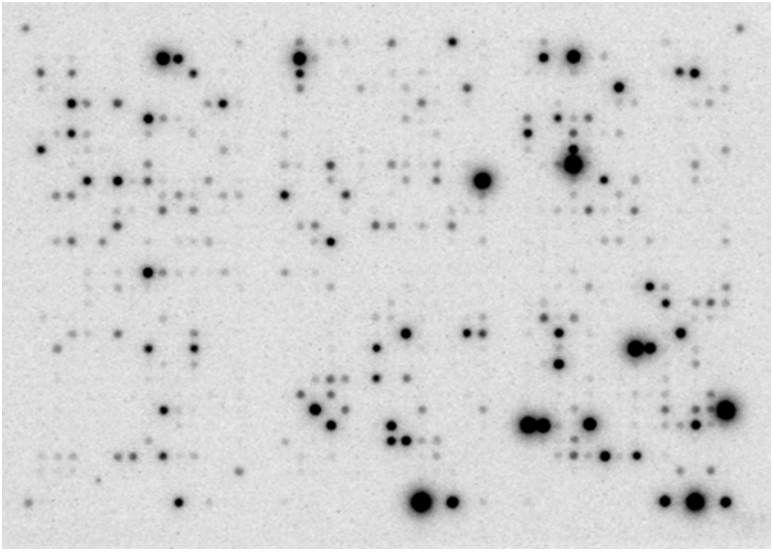

Fig. 1. Nylon array hybridized with a ^{32}P-labeled probe.

(**Fig. 1**). This means that the presence of even low-abundance transcripts can be detected.

Nylon arrays are printed with fragments of cDNA clones (200–600 bp) representing individual genes. Each cDNA fragment is amplified from the original clone using gene-specific or universal primers, denatured, and printed onto the membranes. cDNA fragments have a significantly higher hybridization efficiency than oligos yet generally do not allow discrimination between highly homologous genes, such as multigene family members. For this reason, cDNA fragments are ideal for nylon arrays that represent a limited number of genes. In an array experiment, the cDNA fragments on the array are designated as the "targets." The "probe" used to screen the array is a radioactively labeled pool of cDNAs, reverse transcribed from total or polyA^+ RNA extracted from a particular tissue or cell type. Duplicate arrays are screened with cDNA probes prepared from two or more tissues, cell lines, or differentially treated samples.

The single most important factor determining the success or failure of array experiments is the quality of the RNA used to make the probes. Poor-quality RNA preparation leads to high background on the membrane and/or a misleading hybridization pattern. The present protocol allows purification of total RNA and labeling of probes for array hybridizations in one straightforward procedure—no separate poly A^+ RNA purification step is needed. An acceptable

amount (10 µg) of high-quality total RNA can be isolated from as little as 10 mg of tissue or 10^5 cells.

With nylon membrane arrays, there is a choice of using ^{32}P or ^{33}P in the labeling reaction. The more appropriate method depends on the printing density of the array (*see* **Subheading 3.1.4.**) and the nature of the experiment. For general purposes, we recommend using ^{32}P because this isotope provides greater sensitivity. High sensitivity will be especially important if one is interested in any low-abundance transcripts. On the other hand, ^{33}P offers the advantage of higher-resolution signal, meaning that the signal produced by a spot on the array will be more closely confined to the spot's center, preventing signal "bleed" to neighboring spots. High signal bleed can complicate the interpretation of results for nearby genes. The ^{33}P method is particularly useful if highly abundant transcripts are of interest or one plans to quantitatively analyze the results by phosphorimaging. However, ^{33}P detection is generally only one-fourth as sensitive as ^{32}P detection ***(1)***. When labeling array probes, choose the method that best suits your needs.

2. Materials

Unless otherwise noted, all catalog numbers provided are for BD Biosciences Clontech products.

2.1. Nylon Membrane Array Printing

2.1.1. Nylon Membrane Printing Reagents

1. Nytran Plus Membrane, cut into 82 × 120 mm rectangles (Schleicher & Schuell).
2. BD TITANIUM™ *Taq* PCR Kit (cat. no. K1915-1).
3. Gene-specific or universal primers for amplifying cDNA fragments (*see* **Subheading 3.1.**).
4. Sequence-verified cDNA templates (vectors carrying clones with sequence-verified cDNA insert).
5. Milli-Q-filtered H_2O.
6. Printing dye (30% Ficoll, 1% thymol blue).
7. 3 *M* NaOAc, pH 4.0.
8. Membrane neutralization solution (0.5 *M* Tris, pH 7.6).

2.1.2. Nylon Membrane Array Printing Equipment

1. Polymerase chain reaction (PCR) reaction tubes (0.5 mL). (We recommend Perkin-Elmer GeneAmp 0.5-mL reaction tubes (cat. no. N801-0737 or N801-0180).
2. PCR machine/thermal cycler. We use a hot-lid thermal cycler.

3. 384-well V-bottomed polystyrene plates (USA Scientific), for use as a source plate during printing.
4. SpeedVac.
5. Arrayer robot. We use a *Bio*Grid Robot (*Bio*Robotics).
6. UV Stratalinker crosslinker (Stratagene).
7. Pin tool (0.7 mm diameter, 384 pin).
8. Sarstedt Multiple Well Plate 96-Well (lids only), used to hold nylon membranes for printing.
9. Adhesive sealing film (THR100 Midwest Scientific).
10. NucleoSpin Multi-8 PCR Kit (cat. no. K3059-1) or NucleoSpin Multi-96 PCR Kit (cat. no. K3065-1).

2.2. Reagents for RNA Isolation and Probe Synthesis

2.2.1. Reagents Provided with BD Atlas Pure Total RNA Labeling System

The BD Atlas™ Pure Total RNA Labeling System (cat. no. K1038-1) is available exclusively from BD Biosciences Clontech. Do not use the protocol supplied with the BD Atlas Pure Kit. The procedures for RNA isolation and cDNA synthesis in the following protocol differ significantly from the procedures found in the BD Atlas Pure User Manual.

1. Denaturing solution.
2. Saturation buffer for phenol.
3. RNase-free H_2O.
4. 2 *M* NaOAc (pH 4.5).
5. 10X termination mix.
6. Streptavidin magnetic beads.
7. 1X binding buffer.
8. 2X binding buffer.
9. 1X reaction buffer.
10. 1X wash buffer
11. DNase I (1 U/μL).
12. DNase I buffer.
13. Biotinylated oligo(dT).
14. Moloney murine leukemia virus reverse transcriptase (MMLV RT).

2.2.2. Additional Reagents/Special Equipment

1. Saturated phenol (store at 4°C). For 160 mL: 100 g of phenol (Sigma cat. no. P1037 or Boehringer Mannheim cat. no. 100728). In a fume hood, heat a jar of phenol in a 70°C water bath for 30 min or until the phenol is completely melted. Add 95 mL of phenol directly to the saturation buffer (from the BD AtlasPure Kit), and mix well. Hydroxyquinoline may be added if desired. Aliquot and freeze at –20°C for long-term storage. This preparation of saturated phenol will only have one phase.

2. Tissue homogenizer (e.g., Polytron or equivalent). For <200 mg of tissue, use a 6-mm probe. For >200 mg of tissue, use a 10-mm probe.
3. [α-^{32}P]dATP (10 µCi/µL; 3000 Ci/mmol) (cat. no. PB10204; Amersham) or [α-^{33}P]dATP (10 µCi/µL; >2500 Ci/mmol) (cat. no. BF1001; Amersham). Do not use Amersham's Redivue or any other dye-containing isotope.
4. Deionized H_2O (Milli-Q filtered or equivalent; do not use diethylpyrocarbonate-treated H_2O).
5. Magnetic particle separator (cat. no. Z5331; Promega, Madison, WI). It is important that you use a separator designed for 0.5-mL tubes.
6. Polypropylene centrifuge tubes: 1.5-mL (cat. no. 72-690-051; Sarstedt), 2-mL (cat. no. 16-8105-75; PGC), 15-mL (tubes cat. no. 05-562-10D, caps cat. no. 05-562-11E; Fisher), and 50-mL (tubes with caps cat. no. 05-529-1D; Fisher). Fifteen- and 50-mL tubes should be sterilized with 1% sodium dodecyl sulfate (SDS) and ethanol before use.
7. 10X dNTP mix (for dATP label; 5 m*M* each of dCTP, dGTP, dTTP).
8. 10X Random primer mix (N-15) or gene-specific primer mix (*see* **Subheading 3.4.3.**).
9. BD PowerScript™ Reverse Transcriptase and 5X BD PowerScript™ Reaction Buffer (available exclusively from BD Biosciences Clontech; cat. no. 8460-1).
10. Dithiothreitol (DTT) (100 m*M*).
11. NucleoSpin® Extraction Kit: NucleoSpin extraction spin columns, 2-mL collection tubes, buffer NT2, buffer NT3 (add 95% ethanol before use as specified on the label), buffer NE.

2.3. Reagents for Hybridization, Washing, and Stripping of Nylon Arrays

1. BD ExpressHyb™ hybridization solution (cat. no. 8015-1).
2. Sheared salmon testes DNA (10 mg/mL) (cat. no. D7656; Sigma).
3. **Optional:** 10X Denaturing solution (1 *M* NaOH, 10 m*M* EDTA) (*see* **Subheading 3.5.**).
4. **Optional:** 2X Neutralizing solution (1 *M* NaH_2PO_4 [pH 7.0]): 27.6 g of NaH_2PO_4•H_2O). Add 190 mL of H_2O, adjust the pH to 7.0 with 10 *N* NaOH if necessary, and add H_2O to 200 mL. Store at room temperature (*see* **Subheading 3.5.**).
5. C_ot-1 DNA (1 mg/mL).
6. 20X saline sodium citrate (SSC), 175.3 g of NaCl, 88.2 g of Na_3citrate•$2H_2O$. Add 900 mL of H_2O, adjust the pH to 7.0 with 1 *M* HCl if necessary, and add H_2O to 1 L. Store at room temperature.
7. 20% SDS: 200 g of SDS. Add H_2O to 1 L. Heat to 65°C to dissolve. Store at room temperature.
8. Wash solution 1: 2X SSC, 1% SDS. Store at room temperature.
9. Wash solution 2: 0.1X SSC, 0.5% SDS. Store at room temperature.

3. Methods

3.1. Printing of Nylon Membrane Arrays

cDNA fragments to be used for printing can be amplified by using either gene-specific primers or a pair of "universal" primers (i.e., T3, T7, M13F, or M13R) complementary to sites in the cloning vector flanking the cDNA clone. One advantage of using gene-specific primers is that a specific region of the cDNA clone to be amplified can be chosen. For example, the amplification of cDNAs used to print BD Atlas Arrays is specially designed to minimize nonspecific hybridization. All cDNA fragments are 200–600 bp long and are amplified from a region of the mRNA that lacks the poly A tail, repetitive elements, or other highly homologous sequences. Another advantage of using gene-specific primers is that the antisense primers used in array preparation can be pooled and subsequently used as a gene-specific primer mix to synthesize cDNA probes from experimental samples. The use of gene-specific probes provides higher sensitivity and lower background than random primers (*see* **Subheading 3.4.3.** for details).

3.1.1. Preparative PCR for cDNA Fragments

1. Prepare a 100-µL PCR reaction in a 0.5-mL PCR tube for each cDNA to be represented on the array. Calculate the amount of each component required for the PCR reaction by referring to **Table 1**. Universal primers, appropriate for your cloning vector, may be used in place of gene-specific primers. Adjust the volumes accordingly.
2. Commence thermal cycling using the following parameters: 30–35 cycles of 94°C for 30 s and 68°C for 90 s, 68°C for 5 min, and 15°C soak. These conditions were developed for use with a hot-lid thermal cycler; the optimal parameters may vary with different thermal cyclers. (Note that these parameters were optimized for amplification of fragments approx 200–600 bp long.)
3. Run 5 µL of each pooled PCR product (plus loading dye) on a 2% TAE agarose gel, alongside a molecular weight marker, to screen the PCR products.
4. Check each PCR product size by comparison with the molecular weight markers. If the size of the PCR product is correct, add EDTA (final concentration of 0.1 *M* EDTA, pH 8.0) to the pooled PCR products to stop the reaction.

3.1.2. Purification of cDNA Fragments

To purify amplified cDNA fragments, we recommend that you use either the NucleoSpin Multi-8 PCR Kit (cat. no. K3059-1) or NucleoSpin Multi-96 PCR Kit (cat. no. K3065-1) and follow the enclosed protocol. NucleoSpin PCR kits are designed to purify PCR products from reaction mixtures with speed and efficiency. Primers, nucleotides, salts, and polymerases are effectively removed using these kits; up to 96 samples can be processed simultaneously in less than

Table 1
cDNA Fragment PCR Set-Up

PCR master mix	Final concentration	Per 100-µL reaction (µL)
10X BD TITANIUM *Taq* PCR buffer	1X	10
10 µ*M* dNTP mix	200 µ*M*	2
Specific or universal primer mix, 20 µ*M* each	0.4 µ*M* each	2
Template (0.5–1 ng/µL)	0.025–0.05 ng/µL	5
50X BD TITANIUM *Taq* Mix	1X	2
Milli-Q H_2O	Bring volume up	79

60 min. Up to 15 µg of high-quality DNA can be isolated per preparation. Recovery rates of 75–90% can be achieved for fragments from 100 bp to 10 kb.

3.1.3. Standardization of cDNAs

1. In a 1.5-mL microcentrifuge tube, dilute 5 µL of the purified cDNA fragment stock in 995 µL of H_2O (a 1:200 dilution) and read the optical density of the dilution at 260 nm. Calculate the cDNA concentration in cDNA stock. Each PCR reaction should yield a total of 2 to 3 µg of DNA.
2. If the concentration of cDNA in the stock solution is >500 ng/µL, go to **step 5**; if <500 ng/µL, continue with the next step.
3. Concentrate the cDNA stock solution by evaporation in a SpeedVac. Repeat **steps 1** and **2**.
4. Adjust the concentration to 500 ng/µL by adding Milli-Q-H_2O: $V_{H_2O} = (C_i \times V_i/C_f) - V_i$, in which C_i and V_i are the initial concentration and volume of the main solution (before adding H_2O), respectively; and C_f is the final, desired concentration.
5. Store the normalized cDNA at –20°C.

3.1.4. Printing of cDNA Arrays on Nylon Membranes

An 80 mm × 120 mm rectangle of nylon membrane can be printed with as many as 3000 cDNA fragments (using a 384-pin tool with 0.7-mm-diameter pins) without encountering significant difficulties with image analysis due to signal bleed. If ^{32}P-labeled probes are used, the maximum printing density on a membrane of the same size should be no more than 1500, to avoid loss of signal resolution.

Depending on your experimental needs and organism, you may wish to include negative controls, such as genomic DNA, phage lambda DNA, or yeast

DNA. Some researchers also choose to include cDNA fragments representing certain housekeeping genes, known to be highly expressed in their experimental samples, to serve as positive controls.

1. Prepare the individual cDNA printing mixes. The final cDNA concentration for printing should be approx 100 ng/µL. The final NaOH concentration for printing should be 0.15 *N*. The final printing dye concentration for printing should be 1X. The volume of solution deposited by a single, 0.7-mm-diameter pin is 90 nL, which is equivalent to 10 ng of cDNA printed per spot. For example, to prepare 25 µL of ready-to-print cDNA solution with a ~110 ng/µL final concentration, combine: 5.5 µL of cDNA (500 ng/µL), 0.4 µL of 10 *N* NaOH, 2.5 µL of 10X dye, and 16.6 µL of Milli-Q H_2O, for a total of 25.0 µL. This volume is sufficient for printing approx 200 arrays with single spots for each cDNA, or 100 arrays with duplicate spots. (Printing from volumes of <2 to 3 µL may result in irregular spot morphology.)
2. Aliquot 25 µL of each cDNA printing mix into individual wells of a 384-well plate.
3. Prepare the arrayer for printing following the manufacturer's user manual. (We use a *Bio*Robotics *Bio*Grid.)
4. Place each nylon membrane onto a lid from a Sarstedt 96-well plate. This will hold the membrane securely during printing. Place the Nytran Plus membranes and lids into the filter tray (the *Bio*grid tray holds 24 membranes at a time).
5. Begin the printing process according to the manufacturer's instructions.
6. Replace the water and ethanol in the arrayer's trays after every second round of printing.
7. After the completion of printing, allow the membranes to dry for 45 min at room temperature.
8. Using forceps, pick up the dried, printed membranes, grasping each membrane only by the edge, and drop into a tray containing membrane-neutralizing solution. Gently agitate the membrane arrays for approx 1 min. Change the solution after every 48 membranes.
9. Crosslink the membranes using an energy of 120 mJ/cm^2 (1200 × 100 µJ/cm^2) in a UV Stratalinker Crosslinker. When complete, remove the membranes from the Stratalinker and lay flat to dry for at least 4 h. Dried arrays should be stored at –20°C, sealed individually in plastic bags.

3.2. RNA Isolation

3.2.1. RNA Isolation from Tissues

Conical 50-mL tubes can break under forces >10,000*g*. We recommend using sterile 15- and 50-mL round-bottomed, polypropylene centrifuge tubes at all times.

1. Harvest the tissue; use immediately or flash freeze in liquid nitrogen and store at –70°C. *Important:* When working with frozen tissue, be sure to keep the

Table 2
Reagents for RNA Isolation from Tissues

	Weight of tissue			
	10–100 mg	100–300 mg	300–600 mg	0.6–1.0 g
Recommended tube size (mL)	2 × 2	15[a]	50[a]	50[a]
Denaturing solution (mL)	1.0	3.0	6.0	10.0
Saturated phenol (mL)	2.0	6.0	12.0	20.0
Chloroform (mL)	0.6	1.8	3.6	6.0
Saturated phenol (2nd round) (mL)	1.6	4.8	9.6	16.0
Chloroform (2nd round) (mL)	0.6	1.8	3.6	6.0
Isopropanol (mL)	2.0	6.0	12.0	20.0
80% EtOH wash (mL)	1.0	3.0	6.0	10.0

[a]Conical tubes can break under forces greater than 10,000*g*. Ensure that round-bottomed tubes are used.

tissue frozen until you add the denaturing solution. Even partial thawing can result in RNA degradation. Perform all necessary manipulations on dry ice or liquid nitrogen.

2. Cut or crush the tissue into small pieces (<1 cm^3). When working with frozen tissue, prechill a mortar and pestle with liquid nitrogen, fill the mortar with liquid nitrogen, and break frozen tissue into smaller pieces.
3. Weigh out the tissue in a prechilled, sterile tube. See **Table 2** for the appropriate tube size.
4. Add the appropriate volume (*see* **Table 2**) of denaturing solution. Always add at least 1 mL/100 mg of tissue.
5. Grind the sample at 0–4°C using a tissue homogenizer (e.g., Polytron or equivalent) at the maximum setting for 1 to 2 min or until completely homogenized.
6. Incubate on ice for 5–10 min.
7. Vortex the sample thoroughly. Centrifuge the homogenate at 15,000*g* for 5 min at 4°C to remove cellular debris.
8. Transfer the entire supernatant to new centrifuge tube(s). Avoid pipeting the insoluble upper layer, if present.
9. Add the appropriate volume (*see* **Table 2**) of saturated phenol.
10. Cap the tubes securely and vortex for 1 min. Incubate on ice for 5 min.
11. Add the appropriate volume (*see* **Table 2**) of chloroform.
12. Shake the sample and vortex vigorously for 1 to 2 min. Incubate on ice for 5 min.
13. Centrifuge the homogenate at 15,000*g* for 10 min at 4°C.
14. Transfer the upper aqueous phase containing the RNA to a new tube. Take care not to pipet any material from the white interface or lower organic phase.
15. Perform a second round of phenol:chloroform extraction, using the amounts shown in **Table 2** for "2nd round" (*see* **Note 1**). Repeat **steps 9–14**.

Table 3
Reagents for RNA Isolation from Cultured Cells

	Cell number			
	10^6–10^7	1–3 × 10^7	3–6 × 10^7	6–10 × 10^7
Tube size (mL)	2 × 2	15[a]	50[a]	50[a]
Denaturing solution (mL)	1.0	3.0	6.0	10.0
Saturated phenol (mL)	2.0	6.0	12.0	20.0
Chloroform (mL)	0.6	1.8	3.6	6.0
Saturated phenol (2nd round) (mL)	1.6	4.8	9.6	16.0
Chloroform (2nd round) (mL)	0.6	1.8	3.6	6.0
Isopropanol (mL)	2.0	6.0	12.0	20.0
80% EtOH wash (mL)	1.0	3.0	6.0	10.0

[a]Conical tubes can break under forces greater than 10,000*g*. Ensure that round-bottomed tubes are used.

16. Transfer the upper phase to a new tube. Avoid touching the interface.
17. Add the appropriate volume (*see* **Table 2**) of isopropanol. Add slowly, mixing occasionally as you add it.
18. Mix the solution well and incubate on ice for 10 min.
19. Centrifuge the samples at 15,000*g* for 15 min at 4°C.
20. Quickly remove the supernatant without disturbing the RNA pellet.
21. Add the appropriate volume (*see* **Table 2**) of 80% ethanol.
22. Centrifuge at 15,000*g* for 5 min at 4°C. Quickly and carefully discard the supernatant.
23. Air-dry the pellet.
24. Resuspend the pellet in enough RNase-free H_2O to ensure an RNA concentration of 1 to 2 µg/µL. Refer to **Table 4** for approximate yields.
25. Allow the pellet to soak, then resuspend thoroughly by tapping the tube and pipeting.
26. Set aside a 2-µL aliquot to compare with your RNA sample following DNase treatment. Store the RNA samples at –70°C until ready to proceed with DNase treatment.

3.2.2. RNA Isolation from Cultured Cells

1. Transfer the cultured cells to a sterile tube. See **Table 3** for the appropriate tube size.
2. Centrifuge at 500*g* for 5 min at 4°C. Discard the supernatant.
3. Use the cells immediately, or flash freeze in liquid nitrogen and store at –70°C. When working with frozen cells, be sure to keep the cells frozen until you add

Table 4
Representative Total RNA Yields

Tissue/cell source	Amount of starting material	Yield of total RNA (µg)	Yield after DNase (70% recovery) (µg)
Rat liver	100 mg	600	420
Rat skeletal muscle	100 mg	90	60
Mouse brain	100 mg	125	90
Mouse spleen	100 mg	245	170
Mouse testes	100 mg	240	170
Mouse thymus	100 mg	85	60
Human cerebellum	100 mg	85	60
Human prostate tumor	100 mg	100	70
MCF-7 cell line	1×10^7 cells	70	50
Mouse fibroblasts	1×10^7 cells	800	560
U251 cell line	1×10^7 cells	95	65

the denaturing solution. Even partial thawing can result in RNA degradation. Perform all necessary manipulations on dry ice or liquid nitrogen.

4. Add the appropriate volume (**Table 3**) of denaturing solution.
5. Pipet up and down vigorously and vortex well until the cell pellet is completely resuspended.
6. Incubate on ice for 5–10 min.
7. Vortex the sample thoroughly. Centrifuge the homogenate at 15,000*g* for 5 min at 4°C to remove cellular debris.
8. Transfer the entire supernatant to new centrifuge tube(s). Avoid pipeting the insoluble upper layer, if present.
9. Add the appropriate volume (*see* **Table 3**) of saturated phenol.
10. Cap the tubes securely and vortex for 1 min. Incubate on ice for 5 min.
11. Add the appropriate volume (*see* **Table 3**) of chloroform.
12. Shake the sample and vortex vigorously for 1 to 2 min. Incubate on ice for 5 min.
13. Centrifuge the homogenate at 15,000*g* for 10 min at 4°C.
14. Transfer the upper aqueous phase containing the RNA to a new tube. Take care not to pipet any material from the white interface or lower organic phase.
15. Perform a second round of phenol:chloroform extraction, using the amounts shown in **Table 3** for "2nd round" (*see* **Note 1**). Repeat **steps 9–14**.
16. Transfer the upper phase to a new tube. Avoid touching the interface.
17. Slowly add the appropriate volume (*see* **Table 3**) of isopropanol, mixing occasionally as you add it.
18. Mix the solution well and incubate on ice for 10 min.

19. Centrifuge the samples at 15,000*g* for 15 min at 4°C.
20. Quickly remove the supernatant without disturbing the RNA pellet.
21. Add the appropriate volume (*see* **Table 3**) of 80% ethanol.
22. Centrifuge at 15,000*g* for 5 min at 4°C. Quickly and carefully discard the supernatant.
23. Air-dry the pellet.
24. Resuspend the pellet in enough RNase-free H_2O to ensure an RNA concentration of 1 to 2 µg/µL. Refer to **Table 4** for approximate yields.
25. Allow the pellet to soak, and then resuspend thoroughly by tapping the tube and pipeting.
26. Set aside a 2-µL aliquot to compare with your RNA sample following DNase treatment. Store the RNA samples at –70°C until ready to proceed with DNase treatment.

3.2.3. DNase Treatment

The following protocol describes DNase I treatment of 0.5 mg of total RNA prior to purification of poly A^+ RNA. If you are starting with more or less than 0.5 mg, adjust all volumes proportionally.

1. Combine the following reagents in a 1.5-mL microcentrifuge tube for each sample (you may scale up or down accordingly): 500 µL of total RNA (1 mg/mL), 100 µL of 10X DNase I buffer, 50 µL of DNase I (1 U/µL), and 350 µL of deionized H_2O, for a total volume of 1.0 mL. Mix well by pipeting.
2. Incubate the reactions at 37°C for 30 min in an air incubator.
3. Add 100 µL of 10X termination mix. Mix well by pipeting.
4. Split each reaction into two 1.5-mL microcentrifuge tubes (550 µL per tube).
5. Add 500 µL of saturated phenol and 300 µL of chloroform to each tube and vortex thoroughly.
6. Centrifuge at 16,000*g* for 10 min at 4°C to separate the phases.
7. Carefully transfer the top aqueous layer to a fresh 1.5-mL microcentrifuge tube. Avoid pipeting any material from the interface or lower phase.
8. Add 550 µL of chloroform to the aqueous layer and vortex thoroughly.
9. Centrifuge at 16,000*g* for 10 min at 4°C to separate the phases.
10. Carefully remove the top aqueous layer and place in a 2-mL microcentrifuge tube.
11. Add 1/10 vol (50 µL) of 2 *M* NaOAc and 2.5 vol (1.5 mL) of 95% ethanol. If treating <20 µg of total RNA, add 20 µg of glycogen.
12. Vortex the mixture thoroughly; incubate on ice for 10 min.
13. Spin in a microcentrifuge at 16,000*g* for 15 min at 4°C.
14. Carefully remove the supernatant and any traces of ethanol.
15. Gently overlay the pellet with 500 µL of 80% ethanol.
16. Centrifuge at 16,000*g* for 5 min at 4°C.
17. Carefully remove the supernatant.

18. Air-dry the pellet for approx 10 min or until the pellet is dry.
19. Dissolve the precipitate in 250 µL of RNase-free H_2O, and assess the yield and purity of the RNA as described in **Subheading 3.3.** Alternatively, store the RNA at –70°C.

3.3. Assessment of RNA Yield and Quality (see Table 4)

3.3.1. Calculation of A_{260}/A_{280} Ratio

Pure RNA exhibits a ratio of 1.9–2.1.

3.3.2. Gel Electrophoresis

Electrophorese 1 to 2 µg of total RNA on a 1% denaturing agarose gel. Examine the gel when the dye has migrated 3 to 4 cm from the wells. Total RNA from mammalian sources should appear as two bright bands (28S and 18S RNA) at approx 4.5 and 1.9 kb (*see* **Note 2**). The ratio of intensities of the 28S and 18S rRNA bands should be 1.5–2.5:1. Lower ratios are indicative of degradation. You may also see additional bands or a smear lower than the 18S rRNA band, including very small bands corresponding to 5S rRNA and tRNA.

3.3.3. Testing for DNA Contamination by PCR

A simple test for genomic DNA contamination is to use the total RNA directly as a template in a PCR reaction with primers for any well-characterized gene (e.g., actin or G3PDH). Select primers that will amplify a genomic DNA fragment <1 kb. Be careful that the primers are not separated by a long intron. If this reaction produces bands that are visible on an agarose/ethidium bromide (EtBr) gel, the RNA almost certainly contains genomic DNA. As a positive control, use different concentrations of genomic DNA as a template for PCR. This control will allow you to determine the approximate percentage of DNA impurities in the RNA sample. For a successful nylon array experiment, the RNA should contain <0.001% genomic DNA or produce no visible PCR product after 35 cycles.

3.4. Poly A^+ Enrichment and Preparation of Probes (see Note 3)

3.4.1. Preparation of Streptavidin Magnetic Beads

1. Resuspend magnetic beads by inverting and gently tapping the tube.
2. Aliquot 15 µL of beads per probe synthesis reaction into one 0.5-mL tube.
3. Separate the beads on a magnetic particle separator.
4. Pipet off and discard the supernatant.
5. Wash the beads with 150 µL of 1X binding buffer; pipet up and down.
6. Separate the beads on a magnetic particle separator.
7. Pipet off and discard the supernatant.

8. Repeat **steps 5–7** three times.
9. Resuspend the beads in 15 µL of 1X binding buffer per reaction.

3.4.2. Enrichment of Poly A^+ RNA

Perform the following steps for each total RNA sample. It is extremely important that you do not pause between any of these steps.

1. Preheat a PCR thermal cycler to 70°C.
2. Aliquot 10–50 µg of total RNA into a 0.5-mL tube. For synthesizing probes with the highest sensitivity, we recommend using as much RNA as possible, up to the 50-µg limit.
3. Add deionized H_2O to 45 µL.
4. Add 1 µL of biotinylated oligo(dT), and thoroughly mix by pipeting.
5. Incubate at 70°C for 2 min in the preheated thermal cycler.
6. Remove from heat and cool at room temperature for 10 min.
7. Add 45 µL of 2X binding buffer, and mix well by pipeting.
8. Resuspend the washed beads by pipeting up and down, and add 15 µL to each RNA sample.
9. Mix on a vortexer or shaker at 1500 rpm for 25–30 min at room temperature. Ensure that the beads remain suspended. Do not exceed 30 min.
10. Separate the beads using the magnetic separator. Carefully pipet off and discard the supernatant.
11. Gently resuspend the beads in 50 µL of 1X wash buffer.
12. Being careful not to lose particles, separate the beads and then pipet off and discard the supernatant.
13. Repeat **steps 11** and **12** one time.
14. Gently resuspend the beads in 50 µL of 1X reaction buffer.
15. Separate the beads, and then pipet off and discard the supernatant.
16. Resuspend the beads in 3 µL of deionized H_2O.

3.4.3. cDNA Probe Synthesis

The generation of cDNA probes from total or poly A^+ RNA is accomplished through reverse transcription. The reverse transcription reaction can be primed with a random primer mix, or with a gene-specific mix of antisense primers that generates cDNA for only those genes represented on your array (if the array contains less than 3000–4000 genes). We have found that preparing a gene-specific primer mix for each different array results in an approx 10-fold increase in sensitivity, with a concomitant reduction in nonspecific background. To prepare a 10X gene-specific primer mix for your array, prepare a mixture of 25-bp antisense primers representing each gene of the array, with a final, combined DNA concentration for all primers of 30–50 µ*M*.

1. Prepare a master mix for all labeling reactions plus one extra reaction (to ensure that you have sufficient volume). Combine the following (per reaction) in a 0.5-mL microcentrifuge tube at room temperature (*see* **Note 4**): 4 µL of 5X reaction buffer (*see* **Note 5**), 2 µL of 10X dNTP mix (for dATP label), 5 µL of [α-^{32}P]dATP (3000 Ci/mmol, 10 µCi/µL) or [α-^{33}P]dATP (>2500 Ci/mmol, 10 µCi/µL), and 0.5 µL of DTT (100 m*M*), for a total volume of 11.5 µL.
2. Preheat a PCR thermal cycler to 65°C.
3. Add 4 µL of 10X gene-specific primer mix or 4 µL of random primer mix to the resuspended beads. Mix well by pipeting.
4. Incubate the beads and primer mix in the preheated thermal cycler at 65°C for 2 min.
5. Reduce the temperature of the thermal cycler to 50°C (or 48°C if using an unregulated heating block or water bath); incubate the tubes for 2 min. During this incubation, add 2 µL of PowerScript Reverse Transcriptase (or MMLV RT; *see* **Note 5**) per reaction to the master mix by pipeting, and keep the master mix at room temperature.
6. After completion of the 2-min incubation at 50°C, add 13.5 µL of master mix to each reaction tube. Mix the contents of the tubes thoroughly by pipeting, and immediately return them to the thermal cycler.
7. Incubate the tubes at 50°C (or 48°C) for 25 min.
8. Add 2 µL of 10X termination mix, and mix well.
9. Separate the beads and pipet the supernatant (~approx 20 µL) into 180 µL of Buffer NT2.
10. Place a NucleoSpin extraction spin column into a 2-mL collection tube, and pipet the sample into the column. Centrifuge at 16,000*g* for 1 min. Discard the collection tube and flowthrough into the appropriate container for radioactive waste.
11. Insert the NucleoSpin column into a fresh 2-mL collection tube. Add 400 µL of buffer NT3 to the column. Centrifuge at 16,000*g* for 1 min. Discard the collection tube and flowthrough.
12. Repeat **step 11** twice.
13. Transfer the NucleoSpin column to a clean 1.5-mL microcentrifuge tube. Add 100 µL of buffer NE, and allow the column to soak for 2 min.
14. Centrifuge at 14,000 rpm for 1 min to elute the purified probe.
15. Check the radioactivity of the probe by scintillation counting:
    a. Add 2 µL of each purified probe to 5 mL of scintillation fluid in separate scintillation-counter vials.
    b. Count ^{32}P- or ^{33}P-labeled samples on the ^{32}P channel, and calculate the total number of counts in each sample. (Multiply the counts by a dilution factor of 50.) Probes synthesized using this procedure should have a total of $1–10 \times 10^6$ cpm. Store the probes at –20°C.
16. Discard the flowthrough fractions, columns, and elution tubes in the appropriate container for radioactive waste.

3.5. Hybridization to Nylon Arrays

1. Prepare a solution of BD ExpressHyb hybridization solution and sheared salmon testes DNA:
   a. Prewarm 5 mL of hybridization solution at 68°C (*see* **Note 6**).
   b. Heat 0.5 mg of the sheared salmon testes DNA at 95–100°C for 5 min, and then chill quickly on ice.
   c. Mix the heat-denatured sheared salmon testes DNA with the prewarmed hybridization solution. Keep at 68°C until use.
2. Fill a hybridization bottle with deionized H_2O. Wet the nylon array by placing it in a dish of deionized H_2O, and then place the membrane in the bottle. Pour off all the water from the bottle; the membrane should adhere to the inside walls of the container without creating air pockets. Add 5 mL of the solution prepared in **step 1**. Ensure that the solution is evenly distributed over the membrane. Perform this step quickly to prevent the array membrane from drying.
3. Prehybridize for 30 min with continuous agitation at 68°C. Do not remove the nylon array from the container during the prehybridization, hybridization, or washing steps. If performing the hybridization in roller bottles, rotate at 5–7 rpm during the prehybridization and hybridization steps.
4. Prepare the probe for hybridization as follows (see **step 5** for optional method):
   a. Add 5 µL of C_ot-1 DNA to the entire pool of labeled probe.
   b. Incubate the probe in a boiling water bath for exactly 2 min.
   c. Incubate the probe on ice for exactly 2 min.
5. **Optional:** We find that boiling is adequate to denature probes; however, if you prefer an alkaline denaturing procedure, you may use the following steps instead:
   a. Mix approx 100 µL of labeled probe (entire sample)~ and approx 11 µL (or 1/10 total volume) of 10X denaturing solution (1 *M* NaOH, 10 m*M* EDTA), for a total volume of~ approx 111 µL.
   b. Incubate at 68°C for 20 min.
   c. Add the following to the denatured probe: approx 115 µL (or 1/2 total volume) of 2X neutralizing solution (1 *M* NaH_2PO_4, pH 7.0), for a total volume of approx 230 µL.
   d. Continue incubating at 68°C for 10 min.
6. Being careful to avoid pouring the concentrated probe directly on the surface of the membrane, add the mixture prepared in **step 4** directly to the array and prehybridization solution. Make sure that the two solutions are mixed.
7. Hybridize overnight with continuous agitation at 68°C. Be sure that all regions of the membrane are in contact with the hybridization solution at all times. If necessary, add an extra 2 to 3 mL of prewarmed BD ExpressHyb hybridization solution.
8. The next day, prewarm wash solution 1 (2X SSC, 1% SDS) and wash solution 2 (0.1X SSC, 0.5% SDS) at 68°C.
9. Carefully remove the hybridization solution and discard in an appropriate radioactive waste container. Replace with 200 mL of prewarmed wash solution

1. Wash the nylon array for 30 min with continuous agitation at 68°C. Repeat this step three more times. If using roller bottles, fill to 80% capacity and rotate at 12–15 rpm during all wash steps.
10. Perform one 30-min wash in 200 mL of prewarmed wash solution 2 with continuous agitation at 68°C.
11. Perform one final 5-min wash in 200 mL of 2X SSC with agitation at room temperature.
12. Using forceps, remove the nylon array from the container and shake off excess wash solution. Do not blot dry or allow the membrane to dry. If the membrane dries even partially, subsequent removal of the probe (stripping) from the nylon array will be difficult.
13. Immediately wrap the damp membrane in plastic wrap.
14. Mount the plastic-wrapped nylon array on Whatman paper (3 MM Chr). Expose the nylon array to X-ray film at –70°C with an intensifying screen. Try several exposures for varying lengths of time (e.g., 3–6 h, overnight, and 3 d). Alternatively, use a phosphorimager. When exposing the nylon array to a phosphorimaging screen at room temperature, be sure to seal the nylon array membrane in plastic to prevent drying.

3.6. Stripping of Nylon Arrays

To reuse the nylon array after exposure to X-ray film or phosphorimaging, you may remove the cDNA probe by stripping. Perform all steps in a fume hood with appropriate radiation protection.

1. In a 2-L beaker, heat 500 mL of 0.5% SDS solution to boiling.
2. Remove the plastic wrap from the nylon array and immediately place the membrane into the boiling solution. Avoid prolonged exposure of the membrane to air.
3. Continue to boil for 5–10 min.
4. Remove the solution from the heat and allow to cool for 10 min.
5. Rinse the nylon array in wash solution 1 (2X SSC, 1% SDS).
6. Remove the nylon array from the solution and immediately wrap the damp membrane in plastic wrap. Check the efficiency of stripping with a Geiger hand counter and by exposure to X-ray film (*see* **Note 7**). If radioactivity can still be detected, repeat the stripping procedure (**steps 1–5**).
7. Place the nylon array in a hybridization container and proceed with the next hybridization experiment. Alternatively, the nylon array can be sealed and stored in plastic wrap at –20°C until needed. Do not allow the membrane to dry, even partially.

3.7. Interpretation of Results (see Note 8)

3.7.1. Sensitivity of Detection and Background Level

After hybridization and washing, we recommend that you perform a "trial run" exposure (for 3 to 4 h) of the nylon array membranes to X-ray film or

a phosphorimaging screen. This will allow you to assess the sensitivity and quality of the hybridization pattern so that you can determine the optimal exposure time for the experiment. For X-ray film, expose the membranes to Kodak BioMax MS film (with the corresponding BioMax MS intensifying screen) at –70°C overnight. In our experience, other X-ray films are two- to fivefold less sensitive than BioMax MS film. If available, a phosphorimager affords approximately the same sensitivity as BioMax MS film and allows you to quantify hybridization signals.

3.7.2. Exposure Time

As long as the RNA is of high quality, the signals corresponding to medium- to high-abundance mRNAs (0.05–0.5% of poly A^+ RNA) can be easily detected after several hours or an overnight exposure. Usually, an overnight exposure is not sufficient to reveal hybridization signals from rare- to medium-abundance mRNAs, especially when using ^{33}P-labeled probes. The exact number of hybridization signals depends on the complexity of the experimental RNA sample and the set of printed cDNAs and may differ by severalfold. The practical limit for sensitivity is the level of background generated by nonspecific hybridization of the probe to the membrane. Longer exposure times (>7 d) are useful only if the background level is low. Overexposure is not an issue if using a phosphorimager.

Some samples may produce signals that are similar or even higher in intensity than the abundant housekeeping genes. After an overnight exposure with ^{32}P-labeled probes, you should observe signals for the most abundant housekeeping genes, including ubiquitin, phospholipase A_2, α-tubulin, β-actin, and G3PDH. These genes are expressed at about 0.1–0.5% abundance in mammalian tissues or cells and can be used as universal positive controls. Note that the ratio of intensities of signals for different housekeeping genes may differ as much as two- to fivefold for different tissues or cells.

Another important parameter is the level of nonspecific hybridization, or background. After overnight exposure, there generally will not be hybridization with blank regions of the membrane or with any negative DNA controls.

3.7.3. Normalization of Hybridization Signals

The best approach for comparing hybridization signals for different samples is to equalize the intensity of the hybridization signals by adjusting exposure times. If one array is uniformly darker than the other, adjust the exposure time of one array until the overall signal is approximately the same on both arrays. The most common reason for different overall hybridization intensities is the quality of RNA samples used to prepare the hybridization probes. In our

experience, it is most effective and convenient to normalize arrays based on the overall signal from all genes on the array.

As an alternative to normalization based on the overall level of signal, some researchers prefer to identify one or more housekeeping genes that generate equally intense hybridization signals for the samples being compared. This housekeeping gene (or genes) can then serve as a standard for normalization. In cells or tissues that are closely related—i.e., where only a few genes change their expression levels—the expression of housekeeping genes generally remains constant. However, the expression levels of individual housekeeping genes may be variable depending on your experimental system, especially if different tissues are being compared.

4. Notes

1. For very RNase-rich samples (e.g., pancreas, liver, spleen), we recommend that you perform a third or fourth round of phenol:chloroform extraction.
2. If, on a denaturing formaldehyde/agarose/EtBr gel, the total RNA appears as a smear that is no larger than 2 kb, the RNA may be degraded. If this is the case, we suggest you prepare fresh RNA after checking the purification reagents for RNase or other impurities. If problems persist, you may need to find another source of tissue/cells.
3. Be sure to work through the enrichment/probe synthesis steps quickly, without pausing. Additionally, to help reduce any chance of RNA degradation, you may add 100 U of Ambion's ANTI-RNase (cat. no. 2692) after adding magnetic beads to the sample.
4. As discussed in the **Subheading 1.**, both ^{32}P- and ^{33}P-labeling methods are compatible with nylon membrane arrays. Compared with ^{32}P, the spatial resolution and quality of images are improved with ^{33}P. These characteristics tend to facilitate image analysis and signal quantification. However, also note that ^{33}P signals are approximately four times less intense, decreasing assay sensitivity.
5. If desired, you may also use the wild-type MMLV RT provided with the BD Atlas Pure Kit; however, you should use the same enzyme to label all probes that will be directly compared. Ensure that you use the correct 5X reaction buffer. For MMLV use 5X MMLV reaction buffer: 250 m*M* Tris-HCl (pH 8.3), 375 m*M* KCl, 15 m*M* $MgCl_2$.
6. Hybridization volume should be increased to 15 mL for large bottles. As a general rule, ensure that there is adequate volume to keep the array thoroughly bathed during the incubation.
7. If you observe high background when reprobing a nylon array, the membrane may not have been stripped completely or may have been allowed to dry. If a membrane is allowed to dry even partially, subsequent removal of the probe will be very challenging. To prevent drying after the final wash, shake off excess solution with forceps (do not blot dry) and immediately wrap the membrane in

plastic wrap or seal it in a polyethylene bag. When reprobing, unwrap the array, immediately place it in stripping solution, and follow the rest of the protocol provided for removing probes.

8. Because of sequence-dependent hybridization characteristics and variations inherent in any hybridization reaction, array data should be considered semiquantitative. We strongly recommend that you corroborate the results of your experiment using RT-PCR.

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