Protocol

G6667 **Panorama® Human Cancer OligoArray®**

Set of two 24 x 8 cm gene arrays

Technical Protocol

Transparency orientation template – gene location guide

Microsoft® Excel spreadsheet including gene layout with hyperlinks to GenBank®

SIGMA
GENOSYS

Panorama ä

Human Cancer OligoArray™

Protocol Booklet

For use with Catalog Number Sigma G6667

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Notice to the Customer

Panorama™ Gene Arrays are to be used for research purposes only. The responsibility of all patent considerations in the use of this kit rests solely with the user. Suggestions and recommendations for the use of this kit are not to be taken as license to operate under or infringe upon any patent.

Trademarks & Patents

Limitations of the Procedure

- FOR RESEARCH PURPOSES ONLY.
- Any variation in operator, pipetting technique, washing technique, incubation time or temperature, and kit age can alter the performance of the kit.

Components

- **Panorama™ Gene Arrays.** Two charged, nylon membranes containing multiple-oligonucleotides representing cancer-related genes. Each array contains 2,886 different human cancer-related genes, 9 housekeeping genes, 3 *E. coli* genes, 1x TE Buffer and human genomic DNA.
- **Protocol booklet.**
- **Transparency.** An array template to facilitate spot location within a field on autoradiographs.
- **Floppy diskette.** Containing a spreadsheet of gene-specific information and showing the layout of genes on the arrays. The spreadsheet is in Microsoft® Excel (for Office 2000) format.

Storage of Components

Storage at room temperature: Panorama™ Gene Arrays, keep dry and protected from light.

Optional components:
Panorama™ Human Cancer cDNA Labeling and Hybridization Kit***** (Catalog No: CDLBL-HCN):

- Hybridization Solution, 100 mL, store at 4°C.
- cDNA Spin columns, 10 columns, store at 4°C.
- 10 mM dGTP, 15 µL, store at -20°C.
- 10 mM dATP, 15 µL, store at -20°C.
- 10 mM dTTP, 15 µL, store at -20°C.
- 10 mM dCTP, 15 µL, store at -20°C.
- 500 units AMV Reverse Transcriptase, 20 µL, store at -20°C.
- 5x Reverse Transcriptase Buffer, 100 µL, store at -20°C.
- Human Cancer cDNA Labeling Primers, lyophilized. Upon receipt, store desiccated at 4°C. After resuspension with 40 µL sterile distilled water, store in the short-term (several days) at 4°C. For longer-term storage of several weeks, it is recommended that the primers are stored at -20°C.
- Panorama™ Armored RNA® *E. coli*-B1444 RNA for Normalizing Arrays

***** The above reagents are sufficient to perform 10 labeling reactions and hybridizations.

Additional reorder items:

- Panorama™ Hybridization Solution (Sigma, P5485)
- AMV Reverse Transcriptase (Sigma, A8970)
- Human Cancer cDNA Labeling Primers (Sigma, C9861)
- Panorama™ Armored RNA® *E. coli*-B1444 RNA for Normalizing Arrays (Sigma, R1901)

Protocol Ver. 1.2

Materials and Equipment Required but Not Provided (for Radioactive detection)

Materials

- Total RNA Isolation Reagent, TRI Reagent® (Sigma, T9424) and Poly(A)+ RNA Isolation Kit (optional). Phenol, pH 4.3, optional (Sigma, P4682).
- cDNA Labeling and Hybridization Kit (Sigma, CDLBL-HCN).
- [α-^{33}P]-deoxycytidine 5-triphosphate (dCTP) (i.e. NEN Life Science Products, Inc., NEG 613H, 2,000-3,000 Ci/mmol). [α-^{32}P]-deoxycytidine 5-triphosphate (dCTP) may also be used (i.e. NEN Life Science Products, Inc., NEG 513H, 2000-3000 Ci/mmol).
- Human Cancer cDNA Labeling Primers (Sigma, C9861)
- Ribonuclease inhibitor (from human placenta) (Sigma, R2520).
- RNase-free DNase I (optional; Sigma, AMP-D1).
- Microcentrifuge Tubes (0.5 mL, 1.5 mL), sterile, nuclease-free.
- Conical Tube (50 mL screwcap).
- Scintillation vials and scintillation fluid (optional).
- 20x SSPE (Sigma, S2015).
- Panorama™ Hybridization Solution (Sigma, CDLBL-HCN (kit); Sigma, P5485 (component)).
- X-ray film (Kodak BioMax MR, 20 x 25 cm - Catalog number 870-1302 or 35 X 43 cm - Catalog number 870-5187). Use with Kodak BioMax intensifying screens; standard autoradiography cassette.
- Kodak Low Energy Storage Phosphor Screens HD measuring 20.3 cm x 25.4 cm or 35 cm x 43 cm (Molecular Dynamics LE177-956) or Fujifilm screens (Fuji Medical Systems YBIP2025MS, 20 cm x 25 cm or YBIP3543MS, 35 cm x 43 cm); standard autoradiography cassette.
- Micropipettors and nuclease-free disposable tips: 1.0 µl to 1000 µl capacity.

Equipment

- Spectrophotometer.
- Geiger-Mueller Counter.
- Heating block (90-95°C, 70°C and 42°C) or thermal cycler.
- Centrifuge (capable of 1100 x *g*) for spin column purification of cDNA labeling reaction.
- Microcentrifuge.
- Scintillation counter.
- Tweezers for handling the gene array membrane.
- Hybridization oven and roller bottles.
- An alternative system to roller bottles for the hybridization/wash steps is to use the following: sealable storage bags; water bath; container with cover (large enough to accommodate an 8 x 24 cm membrane and 200 mL minimum capacity); and shaking platform to accommodate the gene array hybridization and wash steps.
- Film developer and/or Phosphorimager for obtaining the gene array image.
- Computer (PC capable of running Microsoft® Excel, Office 2000) for viewing the cDNA array spreadsheet and analyzing phosphorimages.
- Radioisotope solid and liquid waste containers.

Additional Materials and Equipment Required but Not Provided (for Non-Isotopic, Chemiluminescent Detection)

Materials

- Biotin-16-dUTP, 1 mM Biotin-16-2'-deoxyuridine-5'-triphosphate (Enzo Biochem, Inc., Catalog number 42811).
- Western Blocking Reagent (Roche, Catalog number 1921673).
- Streptavidin-Horseradish Peroxidase (Ultra sensitive, Sigma, S2438).
- Maleic acid (Sigma, M0375).
- ECL Western blotting detection reagents (Amersham Pharmacia Biotech, Cat. No. RPN2209).
- Sodium Chloride (Sigma, S7653).
- X-ray film (Kodak BioMax ML, 8 in x 10 in - Catalog number 1788207 or 35 X 43 cm - Catalog number 870-5187). Standard autoradiography cassette.
- Tween 20 (Sigma, P7949).
- Filter unit, 500 mL, 0.2 µm pore size (Sigma, Z37,063-0).
- Sodium dodecylsulphate (Sigma, L4390).
- Sodium Hydroxide (Sigma, S8045).

Equipment

- CCD imaging system for generating a digital image from chemiluminescence (e.g. MiraiBio Inc., CCDBIO 16C).

Overview

The Panorama™ Human Cancer OligoArray™ provides researchers with a rapid, semi-quantitative tool to identify differentially-expressed Cancer-related genes. The development of gene array technology allows researchers to study the relative mRNA levels of hundreds to thousands of genes simultaneously, in a single experiment. This method can be used to determine which genes are “turned on” or “turned off” in response to developmental cues, a particular physiological or pathological condition, external stimuli, or a variety of stresses and cell treatments. In the past, an equivalent amount of information could only be derived using differential screening methods, such as, differential display RT-PCR, differential cDNA library screening or by performing gene-specific methods such as, hundreds of Northern blot experiments or RT-PCR reactions. Figure 1 shows the steps that are involved in an expression profiling experiment using gene arrays.

The Panorama™ Human Cancer OligoArray™ represents a comprehensive collection of 2,886 genes relevant to cancer research and cancer-related pathways. The gene set includes genes important in cellular processes such as angiogenesis, apoptosis, mitosis, intracellular signaling, and metastasis. Also present are oncogenes and tumor suppressor genes as well as clinically-important NF-κB target genes. The majority of genes on the Panorama™ Human Cancer OligoArray™ are represented by up to 3 long oligonucleotides to more accurately represent the expression of the mRNAs. The use of multiple oligonucleotides per gene maximizes the detection potential of the labeled target that may be compromised by variables such as anomalies resulting from target secondary structure and differential degradation along the length of the mRNA. In order to enhance specificity of signal detection, probes were chosen that target the 3’ end of each gene to maximize splice variant detection and increase the potential for specificity. All oligonucleotide probes are 70 bases long and have been designed to be as unique as possible with optimal hybridization characteristics. Probe design at Sigma-Genosys includes provisions to ensure that the melting temperatures (Tm) of probes are matched for equivalent performance under a given set of hybridization conditions. Secondary structures within probes, such as hairpins and dimer formation, are kept to a minimum. The oligonucleotides are printed on nylon filters, allowing for radioactive or chemiluminescent non-isotopic detection of gene expression. Each array is comprised of two fields where each gene is represented by duplicate spots of long oligo probes. Also included on the gene arrays are a number of positive control "housekeeping" genes, human genomic

DNA and negative controls. The signal from one or more of the positive control housekeeping genes may be used to normalize signals of all genes between two comparative samples. A recommended option for normalizing signals would be to include *Panorama™ Armored RNA® E. coli-B1444 RNA for Normalizing Arrays* (Sigma, R1901) in the labeling reaction (described later in this protocol). Human genomic DNA is spotted at the four corners of each field on the array and during any expression profiling experiment, the genomic DNA spots will act as a positive control, showing some degree of hybridization signal. The genomic DNA spots can also be used to orient the corners of the array (Figure 2).

Key to the sensitivity of these arrays is the use of the Human Cancer cDNA Labeling primers prepared with an optimized mix of gene-specific cDNA labeling primers. Use of these primers dramatically increases the signal sensitivity compared to probes generated using oligo(dT) labeling primers (Figure 3).

The layout of genes on the array is described in Appendix C and detailed information on each gene and the array layout is included in the accompanying Microsoft® Excel spreadsheet. High-quality expression data is obtained provided that the protocols detailed in the methods section are closely followed.

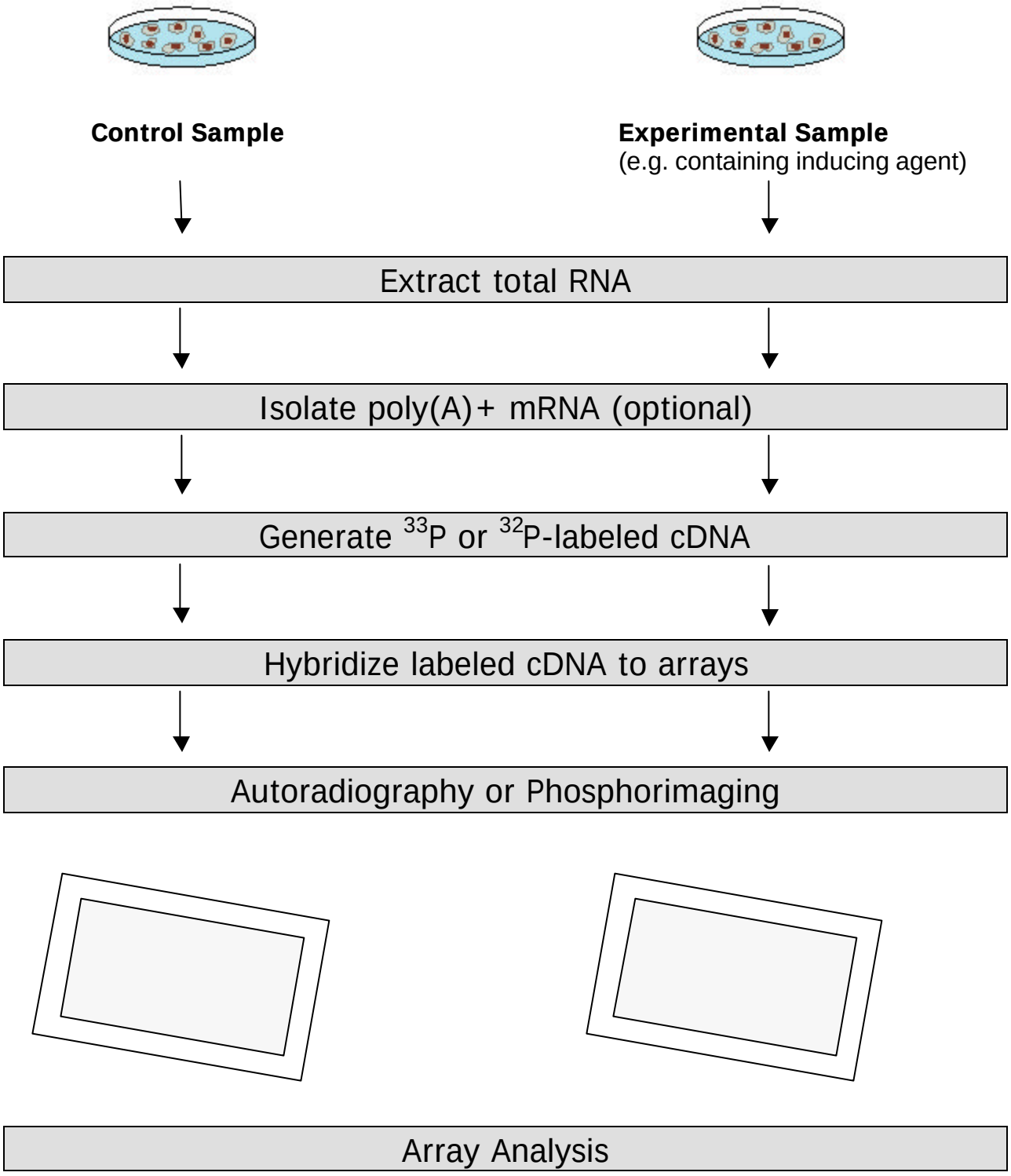


Figure 1. ***Diagrammatic representation of the expression profiling process using Panorama™ gene arrays.***

An example of differential gene expression in unstimulated versus stimulated THP-1 cells is shown in Figure 2. RNA was prepared from unstimulated THP-1 cells and THP-1 cells that were stimulated for 4 hours with phorbol myristate acetate (PMA) and bacterial lipopolysaccharide (LPS). Equal amounts of total RNA from each sample were then reverse transcribed using the Human Cancer cDNA labeling primers and the resulting radiolabeled cDNA samples were hybridized to the Panorama™ Human Cancer Gene Arrays. The membranes were then exposed to phoshorimaging plates and the signals quantified using the ArrayVision™ software.

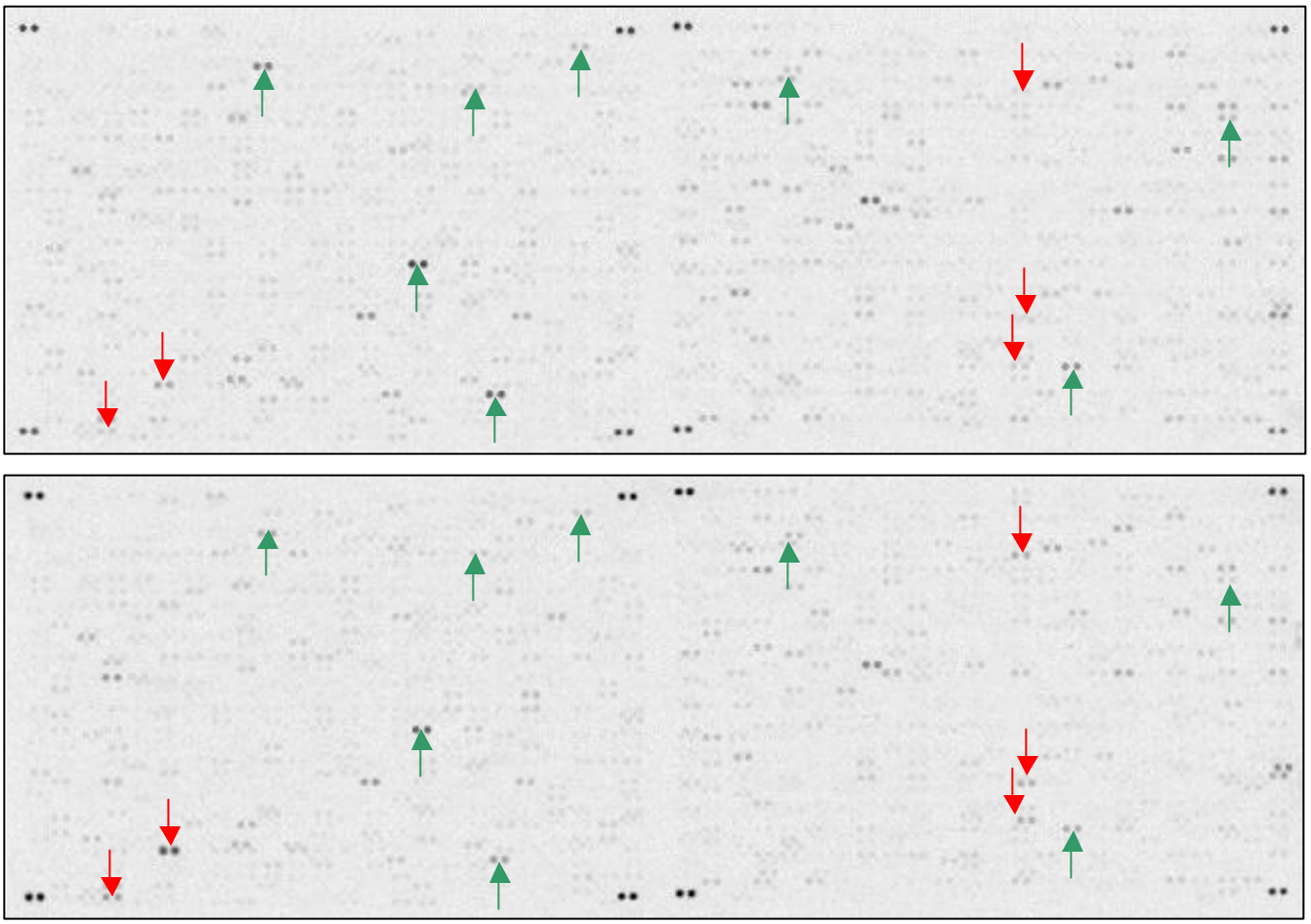


Figure. 2 Differential gene expression in stimulated THP-1 cells. Two Panorama™ Human Cancer OligoArrays were probed with labeled cDNA from unstimulated THP-1 cells (bottom panel) or THP-1 cells stimulated with PMA (1 pg/mL) and LPS (10 µg/mL) for 4 hr (top panel). Equal amounts of total RNA were reverse transcribed using the Human Cancer cDNA labeling primers and ^{33}P-dCTP. The labeled cDNAs were hybridized to the arrays overnight at 65^{o}C. The images were obtained following an overnight exposure to low-energy phosphorimaging screens.

Methods

Part One – RNA Extraction

A. Total RNA Isolation

The methodology for RNA extraction is critical for the success of any expression profiling experiment. It is essential to purify total RNA without any contaminating genomic DNA. Traces of genomic DNA in the RNA preparation may contribute to background hybridization. For high quality total RNA isolation with minimal genomic DNA contamination, we recommend the use of the *TRI Reagentâ* (Sigma, T-9424). An abbreviated protocol for using the *TRI Reagentâ* is shown below. An additional step is suggested to reduce genomic DNA contamination (see *):

Abbreviated Protocol:

1.	HOMOGENIZATION	Use 1 mL *TRI Reagentâ* per: 50-100 mg tissue, 5-10 x 10^6 cells in suspension or 10 cm^2 of cell culture plate.
		* Prior to the phase separation step, pellet insoluble material (extracellular membranes, genomic DNA, polysaccharides) by centrifugation at 12,000 x *g* for 10 minutes at 4°C. Transfer the clear supernatant to a fresh tube and proceed to phase separation step.
2.	PHASE SEPARATION	Homogenate + 0.2 mL chloroform.
3.	RNA PRECIPITATION	Aqueous phase + 0.5 mL isopropanol. Pellet by centrifugation.
4.	RNA WASH	Wash pellet with 1 mL 75% ethanol. Air dry.
5.	RNA SOLUBILIZATION	Sterile distilled water.

After washing the precipitate with 75% ethanol (step 4), discard the supernatant, taking care NOT to discard the RNA pellet. Drain well. The pellet may easily dislodge from the bottom of the tube. Air-dry the pellet for 10-15 minutes. When the RNA pellet appears clear or translucent, dissolve the RNA in 100-200µl of sterile, RNase-free water or 1xTE Buffer. Place the tube at 37°C for 15-30 minutes and then pipet repeatedly to thoroughly dissolve the RNA. Keep on ice for immediate use or store at -20°C until ready to proceed with the poly(A)+ isolation step.

B. Quantitation of RNA

1. Quantitate the RNA sample by measuring the absorbance at 260nm of an appropriate dilution (5-10 µl of RNA in 1 mL water) in a spectrophotometer. For example, dilute 10 µl sample to 1 mL with water, which is equivalent to a 1:100 dilution (dilution factor = 100). Read the absorption in optical density units (OD) of this dilution at 260nm.
2. Calculate the concentration of your RNA sample:
   Concentration of RNA sample (µg/mL) = OD units x Dilution factor x 40 µg/mL

C. Check the Quality of the Total RNA Sample

1. A total RNA sample that is essentially free of proteins should have an $A260_{nm}/A280_{nm}$ absorbance ratio of 1.6 - 1.9. If the RNA is resuspended in water with a pH <7.0, then the $A260_{nm}/A280_{nm}$ ratio may be falsely decreased, giving the impression of protein contamination in the sample. It is important to check both the integrity of the RNA and the amount of genomic DNA contamination by agarose gel electrophoresis. A non-denaturing agarose gel is used so that any genomic DNA contamination can be easily observed. Mix 5-10 µg RNA sample with non-denaturing loading dyes. Load the sample on a 1.2% agarose gel and separate the nucleic acids by electrophoresis. The 28S (~4.8 kb) and 16S (~1.9 kb) ribosomal RNA bands should be clearly visible at a staining intensity of about 2:1 ratio (28S:16S). A significantly lower ratio of staining may be indicative of RNA degradation and hence, the RNA sample may not be suitable for use. If genomic DNA is present in the RNA sample, it will be seen as high molecular weight-staining material. Note that some RNA species may appear as discrete bands of sizes up to ~15 kb. Typically, if the *TRI Reagentâ* total RNA extraction procedure is strictly followed, then genomic DNA contamination will be minimal. If excessive amounts of genomic DNA are present, it may be necessary to consider treating the RNA sample with RNase-free DNase I (Sigma, AMP-D1). Note: For details on running agarose gel electrophoresis, refer to Sambrook, J. *et al.*, (1989) *Gel Electrophoresis of DNA.* In *Molecular Cloning: A Laboratory Manual. 2nd ed.* Cold Spring Harbor Laboratory Press, N.Y., p. 6.1.

D. Poly(A)+ mRNA Isolation

The *Human Cancer cDNA Labeling Primers* may be used to synthesize cDNA with total RNA as template. However, using poly(A)+ mRNA (rather than total RNA) with the cDNA labeling primers may help to achieve a slightly greater sensitivity of detection. The majority of total RNA

is comprised of ribosomal RNA (rRNA) and transfer RNA (tRNA). Messenger RNAs comprise a small percentage of the total RNA (<5%). Hence, any mRNA enrichment step will help to increase the specific activity of the cDNA probe needed for hybridization to the arrays (in *Parts Two* and *Three* of this protocol). Poly(A)+ mRNA isolation kits are available from a number of suppliers. An oligo(dT)-cellulose or oligo(dT)-latex bead method should work well for these purposes. We recommend the use of GenElute™ mRNA Miniprep Kit (Sigma, MRN-10) from Sigma for purification of mRNA from total RNA or isolation of mRNA directly (GenElute™ mRNA Miniprep Kit; Sigma, DNM-10). Ensure that all RNA samples to be used with the Panorama™ gene arrays are processed in the same manner. Do not compare two samples where the poly(A)+ mRNAs have been isolated by two different approaches.

Part Two – Generating Labeled cDNA

Panorama™ Human Cancer OligoArrays™ can be used with either ^{33}P or ^{32}P or non-isotopically labeled probes (refer to Part Four of this protocol) for expression profiling. The signals from cDNAs probes synthesized using ^{33}P generate well-defined spots on the arrays that allow easier quantitation. Spot signals from images generated with ^{32}P may tend to bleed into one another, necessitating the analysis of several exposures from the same array. Whenever possible, Sigma-Genosys recommends the use of ^{33}P. Also, Sigma-Genosys recommends the use of the "Human Cancer cDNA Labeling Primers" rather than oligo(dT) primers in the cDNA labeling reactions. The poly(A) tract of an mRNA is downstream of the position of all the oligonucleotide probes representing each gene. It is possible that position of these oligonucleotide probes may be hundreds of base pairs removed from the poly(A) tail. Therefore, cDNA targets generated using oligo(dT) may not be long enough to include the region complementary to the oligonucleotide probes present in the array and this is likely to result in lower detection potential of the array. In contrast, cDNA targets generated with the optimized sequence-specific Human Cancer cDNA Labeling Primers are more likely to include the regions complementary to the oligonucleotide probes present in the array and therefore increase the detection potential of the array (Figure 3).

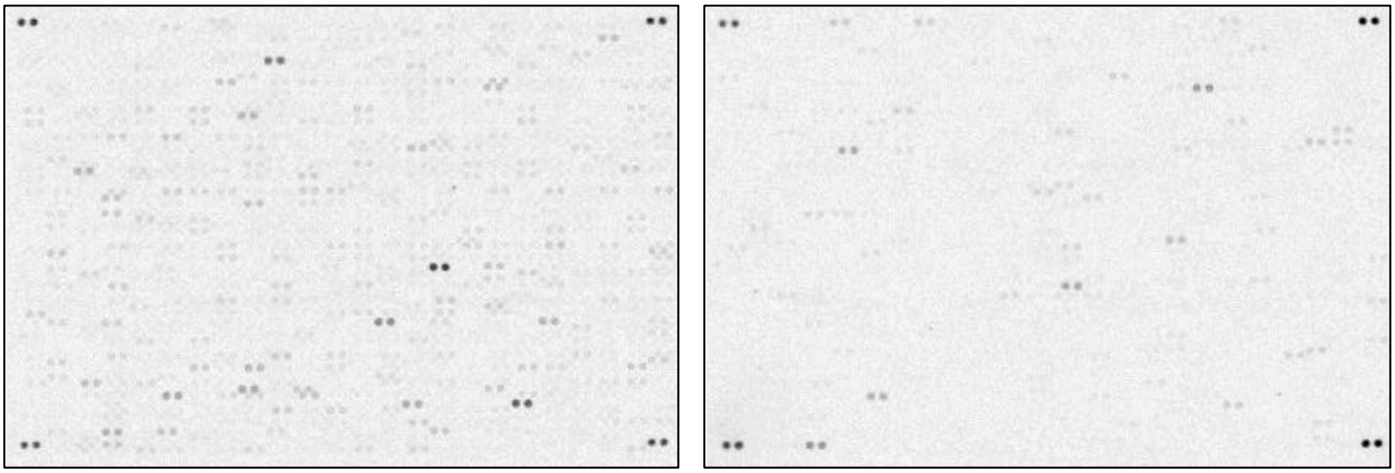


Figure 3. Hybridization of cDNA generated from unstimulated THP-1 cells using cDNA specific primers or oligo(dT) primers. Two Panorama™ Human Cancer OligoArrays™ were hybridized with labeled cDNA generated from unstimulated THP-1 using the sequence-specific Human Cancer cDNA Labeling Primers (left panel) or oligo(dT) primers (right panel). Equal amounts of total RNA were reverse transcribed using the appropriate cDNA labeling primers and ^{33}P-dCTP. The labeled cDNA targets were hybridized to the OligoArrays overnight at 65^{o}C. The images were obtained following an overnight exposure to low-energy phosphorimaging screens.

Preparation of Labeled cDNA Using *Human Cancer cDNA Labeling Primers*

The cDNA labeling reactions are performed in two steps. In the first step, the Human Cancer Labeling Primers are annealed to the RNA template. During the second step, radiolabeled nucleotide and reverse transcriptase are added to initiate the cDNA synthesis reaction. Components for the cDNA labeling reaction can be obtained from Sigma-Genosys (Panorama™ Human Cancer cDNA Labeling and Hybridization Kit; CDLBL-HCN).

To enable normalization of hybridization signal between two array samples that have been labeled independently, Sigma-Genosys strongly recommends the use of Panorama™ Armored RNA®* E. coli-*B1444 RNA for Normalizing Arrays (Catalog number: R1901). Spiking each of the cDNA synthesis reactions with equal amounts of* E. coli-*B1444 RNA results in the synthesis of labeled B1444 cDNA that binds to one of the negative controls, B1444 target gene present in the array. The hybridization signal obtained from the B1444 gene can then be used to normalize the signals between different arrays.

Table 1.

Final Concentration	Stock Reagent	Volume for one reaction
2 µg total RNA or ~0.5 µg poly(A)+ RNA		X µl
Human Cancer cDNA Labeling Primers, resuspended (Sigma, C9861)		4 µl
Panorama™ Armored RNA® *E. coli*-B1444 RNA for Normalizing Arrays (Sigma, R1901)		2 µl
	Sterile distilled water to	**14.5 µl**

Table 2.

Final Concentration	Stock Reagent	Volume for one reaction
Components from Table 1		14.5 µl
1x Reverse Transcriptase Buffer	5x	6 µl
333 µM dATP	10 mM	1 µl
333 µM dGTP	10 mM	1 µl
333 µM dTTP	10 mM	1 µl
40 µCi [α-^{33}P] dCTP or 40 µCi [α-^{32}P] dCTP (2,000-3,000 Ci/mmol)	10 µCi/µl	4 µl
20 U Ribonuclease Inhibitor (from human placenta)	40 U/µL	0.5 µl
50 U AMV Reverse Transcriptase	25 U/µl	2 µl
	Sterile, distilled water to	**30 µl**

1. For the annealing step, assemble the reaction in a 0.5 mL tube using the components listed in Table 1.
2. Anneal the *Human Cancer cDNA Labeling Primers* to the RNA template by placing the tube in a thermal cycler. Program the cycler to heat to 90°C for 2 minutes and then ramp to 42°C over a period of 20 minutes. Alternatively, place the tube in a heat block at 90°C. After 2 minutes, remove the block from the heating device and place on the work bench. Allow to cool to 42°C.
3. Once the thermal cycler (or heat block) has reached 42°C, add the components for the cDNA labeling step as listed in Table 2. **The final reaction volume = 30 µl**.
   Mix carefully by pipetting and return to the thermal cycler or heat block. Incubate at 42°C for 2-3 hours.

   ** Use appropriate personal protective equipment and adopt your institutions handling and waste disposal procedures for use of radioactive materials.*

4. The unincorporated radiolabeled nucleotide must be removed from labeled cDNA by purification over a Sephadex® G-25 gel-filtration spin column. (*Part Two* of this protocol).
1. After removal of unincorporated radiolabeled nucleotides, the labeled cDNA probe is ready to be used in a hybridization with the Panorama™ gene array (*Part Three* of this protocol).

B. Purification of Labeled cDNA Using Spin Columns

It is important to remove the excess, unincorporated-radioactive nucleotides from the labeling reaction. Removing the unincorporated nucleotides will help prevent background during the hybridization to the gene arrays. In addition, removing the excess nucleotides allows a general determination of the efficiency of incorporation into the cDNA. Use a Sephadex® G-25 spin column (component of the kit CDLBL-HCN).

1. Gently invert the gel-filtration spin column several times to resuspend the column matrix.

2. Carefully remove the top cap from the column, then remove the bottom cap and allow the buffer to drain by gravity into a 1.5 mL microcentrifuge tube. Discard the tube with eluted buffer.
3. Place the column in a collection tube (1.5 mL microcentrifuge tube) and then place the whole device in a 50 mL screw-top conical tube.
4. Centrifuge at 1100 x *g* for 2 minutes to remove all of the buffer from the column.
5. Add the sample to the center of the column bed (make sure that the column is in an upright position) and place the column in a fresh 1.5 mL collection tube within the 50 mL screw-top conical tube.
6. Spin the column at 1100 x *g* for 4 minutes and save the eluate. This is the purified cDNA sample.
7. A hand-held Geiger-Mueller counter can be used to determine a rough estimate of the percentage incorporation of radioactive nucleotide into the cDNA. Measure the radioactivity left in the column (unincorporated radioactive nucleotide, "U") and the amount of radioactivity in the collection tube (radioactive nucleotide incorporated into the cDNA, "I"). To determine the approximate percentage incorporation, calculate (I/(I+U)) x 100%. Typically, an incorporation of 20%-50% will yield acceptable results. Alternatively, an aliquot of the sample before (T) and after the column purification (I) can be counted in a scintillation counter to determine percent incorporation (I/T x 100).

Part Three – Hybridization and Analysis of Arrays

A. Hybridization and Washing of the Arrays

After preparing the radioactively-labeled cDNA in *Part Two*, the next step is to perform a hybridization to the Panorama™ gene array. The hybridizations are best performed in roller bottles in a hybridization oven, where minimal volumes of hybridization solutions are employed. Hybridizations can also be performed in sealed plastic bags in a heated air incubator or immersed in a water bath, with agitation. The following protocol presumes the use of roller bottles in a hybridization oven (volumes may have to be increased for use with sealed bag hybridizations). See Appendix A for buffer compositions.

1. Rinse the arrays in 50 mL 2x SSPE at room temperature for 5 minutes. Drain and discard the solution.

2. Pre-warm the hybridization oven to 65°C. Warm the *Hybridization Solution* (including salmon testes DNA; a component of the Sigma-Genosys kit CDLBL-HCN, or can be purchased separately: Sigma P5485) to 65°C prior to use. Pre-hybridize the Panorama™ gene array in 5 mL *Hybridization Solution* for at least 1 hour at 65°C, using roller bottles at 6 r.p.m. (or continuously agitate if using sealed bags).
3. Add the entire labeled cDNA generated from *Part Two* of this protocol to 2-3 mL *Hybridization Solution,* in a 15 mL conical screw-top tube. Denature the cDNA at 90-95°C for 10 minutes in a water bath or heat block.
4. Decant and discard the *Hybridization Solution* from the pre-hybridized array. Add the denatured labeled cDNA in *Hybridization Solution* to the array in the roller bottle.
5. Hybridize overnight (12-18 hours) at 65°C.
6. Decant the *Hybridization Solution* and save for future use or discard appropriately.
7. Add 40-50 mL of *Wash Solution I* to the roller bottle. Wash the array by inverting the roller bottle by-hand, at room temperature for 2-3 minutes. Decant and discard the *Wash Solution I* in an appropriate manner for radioactive waste solutions.

   Note: An alternative wash method may be adopted for the arrays. Arrays may be washed in a suitably-sized plastic food container (dedicated for use with radioactive materials). Agitate the container on a rocking table or use a shaking water bath.
8. Repeat step 7 two more times.
9. Pre-warm the *Wash Solution I* to 65°C. Add 80-100 mL *Wash Solution I* to the roller bottle. Wash the arrays in the hybridization oven at 65°C for 20 minutes (6 r.p.m.). Decant and discard the *Wash Solution I* to an appropriate radioactive waste container.
10. Repeat step 9 one more time.
11. Pre-warm the *Wash Solution II* to 65°C. Add 80-100 mL *Wash Solution II* to the roller bottle. Wash the arrays in the hybridization oven at 65°C for 20 minutes (6 r.p.m.).
12. Remove the array from the roller bottle (or the alternative washing container). Lay the array on a sheet of blotting paper.
13. Air-dry the array for 1-2 minutes.

    Warning: do NOT let the array dry completely. If allowed to dry completely, then stripping of the array for re-probing will be less efficient. A protocol for stripping the gene arrays can be found in Appendix B.

14. Wrap the array in clear plastic food wrap and subject it to autoradiography using Kodak BioMax MR (for ^{33}P-labeled cDNA), BioMax MS (for ^{32}P-labeled cDNA) X-ray film or expose phosphor screens for analysis by phosphorimagery. When performing phosphorimagery with ^{33}P, we strongly recommended the use of Kodak Low Energy Storage Phosphor Screens HD (Molecular Dynamics LE177-956, 20.3 cm x 25.4 cm or 35 cm x 43 cm) or Fujifilm screens (YBIP2025MS, 20 cm x 25 cm or YBIP3543MS, 35 cm x 43 cm). Images with these screens have a significantly higher resolution than with other general purpose phosphor screens. Imaging screens should be scanned at a 50 µm rather than a 100 µm or 200 µm pixel size, for greater resolution of spots and more accurate quantitation of pixel values. The file sizes of 50 µm scans may be extremely large. It is advisable to crop the images after scanning to one per image file. If using phosphorimaging screens, care must be taken to ensure that there is no wrinkles in the clear plastic wrap separating the screen from the array. Also, make sure that the entire array is pressed firmly against the imaging screen. Typically, a 2-4 day exposure to X-ray film or an overnight exposure to a phosphorimager screen will yield quantifiable results. It may be necessary to perform several exposures for different time periods to distinguish between extremes of expression levels.

NOTE: For all experiments it is recommended that an autoradiograph be generated for each array. Depending upon the phosphor screen being used, spots from phosphorimaging may appear diffuse. Autoradiographs typically show spots with sharper edge boundaries and provide a qualitative "hard copy" of the array image.

15. If the arrays are to be reused, it is essential that they be stripped before the membrane dries out. It is advisable to strip the arrays as soon as possible following imaging (see Appendix B).

B. Analysis of the Gene Arrays

Quantitation of gene expression signals is best determined from phosphorimager-generated image files. The image files may be analyzed using the phosphorimager manufacturer's software, e.g. ImageQuant from Molecular Dynamics, Quantity One from Bio-Rad Laboratories, OptiQuant from Packard Instrument Company or MacBas from Fuji. Sigma-Genosys has created an image analysis “template” for use with the ArrayVision™ software from Imaging Research. Pixel data that has been obtained using the template can be exported

to a Microsoft® Excel spreadsheet. This data can then be copied and pasted into another spreadsheet that contains the gene names in relation to their position in the template. Both the ArrayVision™ template and the appropriate Microsoft® Excel spreadsheet may be downloaded from the Sigma-Genosys website (http://www.genosys.com/expression).

Refer to Appendices C and D to determine the layout of genes on the Panorama™ Human Cancer Gene Arrays. Refer to the accompanying computer disk for a spreadsheet that details the array layout and for more information on each gene represented on the array.

The process of image analysis involves the following steps:

1. Set up a quantitation template to analyze the pixel intensity in each spot of the array.
2. Subtract background signal from each spot: Use signal from a clear area of the array or from the 1x TE Buffer negative control spot as a background value.
3. Export signal values to a spreadsheet file for manipulation in a program such as Microsoft® Excel.
4. Expression signals from different arrays must be "normalized" to allow cross-comparison. Normalizing signal values helps to correct for different experimental variables such as subtle differences in amounts of RNA used in the cDNA labeling steps or differences in labeled nucleotide incorporation efficiencies between samples, etc. Sigma-Genosys recommends normalizing relative signals from different arrays by representing the spot signal of each gene as a percentage of the signal from an internal control gene. For this purpose, Sigma-Genosys provides an internal control RNA (Panorama™ Armored RNA® *E. coli*-B1444 RNA for Normalizing Arrays; Catalog number R1901) that may be added to the labeling reaction. Signal from the B1444 gene spot can then be used to normalize the spot signals of the sample genes in the arrays that are being compared.
5. An alternate method for normalizing relative signals from different arrays is to represent the spot signal of sample genes as a percentage of the signal from a housekeeping gene known not to change between the samples. The signals from certain housekeeping genes may change following different cell treatments. In these cases, it may be necessary to normalize all gene signals to the averaged signal from several or all housekeeping genes.

6. Compare normalized signals of corresponding spots from different samples on different arrays. Divide the normalized values from the "experimental" array by the normalized values from the "control" array. This will determine fold-induction or fold-reduction in expression of gene-specific mRNAs between samples.

Part Four – Non-Isotopic Detection

Panorama™ gene arrays can be used with non-isotopically labeled cDNA probes for expression profiling. The following protocol uses a biotin-streptavidin system for chemiluminescent detection. The process involves the incorporation of biotin-16-dUTP into the cDNA synthesis reaction and the hybridization of biotin-labeled-cDNA to the array. Following high stringency washes, the array is incubated with a blocking agent and then with a streptavidin-horseradish peroxidase conjugate. The array is then washed to remove the unbound conjugate and the peroxidase activity is assayed by using a chemiluminescent substrate. The light emitted in the reaction is detected by either exposure to X-ray film or a digital image can be captured with a CCD camera detection system. For maximum sensitivity, Sigma-Genosys recommends the use of an ultra-sensitive streptavidin-HRP polymer from Sigma (Catalog number S2438).

A. Preparation of Biotin-Labeled cDNA

The cDNA labeling reactions are performed in two steps. In the first step, the Human Cancer cDNA Labeling Primers are annealed to the RNA template. During the second step, biotin-labeled nucleotide and reverse transcriptase are added to initiate the cDNA synthesis reaction. Most components for the cDNA labeling reaction can be obtained from Sigma-Genosys (CDLBL-HCN, Panorama™ cDNA Labeling and Hybridization Kit and Ultra sensitive Streptavidin-HRP polymer (Sigma S2438)).

Table 1.

Final Concentration	Stock Reagent	Volume for one reaction
10 µg total RNA		X µl
Human Cancer cDNA Labeling Primers, resuspended (Sigma, C9861)		4 µl
Panorama™ Armored RNA® *E. coli*-B1444 RNA for Normalizing Arrays-**Non-isotopic** (Sigma, R3027)		2 µl
	Sterile distilled water to	**14.5 µl**

Table 2.

Final Concentration	Stock Reagent	Volume for one reaction
Components from Table 1		15 µl
1x Reverse Transcriptase Buffer	5x	6 µl
333 µM dATP	10 mM	1 µl
333 µM dGTP	10 mM	1 µl
333 µM dCTP	10 mM	1 µl
110 µM dTTP	**3.3 mM***	1 µl
50 µM Biotin-16-dUTP	1 mM	1.5 µl
20 U Ribonuclease Inhibitor (from human placenta)	40 U/µL	0.5 µl
50 U AMV Reverse Transcriptase	25 U/µl	2 µl
	Sterile, distilled water to a *final* volume of	**30 µl**

***** 1:3 dilution of 10 mM stock solution

1. For the annealing step, assemble the reaction in a 0.5 mL tube using the components listed in Table 1.
2. Anneal the cDNA Labeling Primers to the RNA template by placing the tube in a thermal cycler. Program the cycler to heat to 90°C for 2 minutes and then ramp to 42°C over a period of 20 minutes. Alternatively, place the tube in a heat block at 90°C. After 2 minutes, remove the block from the heating device and place on the work bench. Allow to cool to 42°C.
3. Once the thermal cycler (or heat block) has reached 42°C, add the components from the cDNA labeling step as listed in Table 2. **The final reaction volume = 30µl.**
4. Mix carefully by pipetting and return to the thermal cycler or heat block. Incubate at 42°C for 2-3 hours.

B. Purification of Biotin-Labeled cDNA

It is important to remove the excess, unincorporated biotinylated nucleotides from the labeling reaction. Removing the unincorporated nucleotides will help prevent high background signals following hybridization to the gene arrays. Use a Sephadex® G-25 spin column (component of the Sigma-Genosys kit, CDLBL-HCN).

1. Gently invert the gel-filtration spin column several times to resuspend the column matrix.
2. Carefully remove the top cap from the column, then remove the bottom cap and allow the buffer to drain by gravity into a 1.5 mL microcentrifuge tube. Discard the tube with buffer.
3. Place the column in a collection tube (1.5 mL microcentrifuge tube) and then place the whole device in a 50 mL screw-top conical tube.
4. Centrifuge at 1100 x *g* for 2 minutes to remove all of the buffer from the column.
5. Add the sample to the center of the column bed (make sure that the column is in an upright position) and place the column in a fresh 1.5 mL collection tube within the 50 mL screw-top conical tube.
6. Spin the column at 1100 x *g* for 4 minutes and save the eluate. This is the purified cDNA sample.
7. The cDNA sample is now ready for hybridization.

C. Hybridization and Washing of Arrays

After preparing the biotin-labeled cDNA (step B, above), the next step is to perform a hybridization to the Panorama™ gene array. The hybridizations may be performed in roller bottles in a hybridization oven, where minimal volumes of hybridization solutions are employed or in sealed plastic bags in a heated air incubator or immersed in a water bath, with agitation. To reduce background signals in the array, the following precautions should be adopted:

a) Handle arrays carefully by their edges using blunt-ended forceps.
b) Never touch the arrays with bare or gloved hands.
c) Do not fold or crease the arrays.

d) Do not allow the array to become dry, even slightly.

e) Transfer of array from one Wash solution to another should be rapid and ensure that the array does not stick to the sides of the container as this would cause uneven washing and therefore increase the background signal.

The following protocol presumes the use of roller bottles in a hybridization oven (volumes may have to be increased for use with sealed bag hybridizations). See Appendix A for buffer compositions.

1. Rinse the arrays in 50 mL 2x SSPE at room temperature for 5 minutes. Drain and discard the solution.
2. Pre-warm the hybridization oven to 65°C. Warm the *Hybridization Solution* (including salmon testes DNA; a component of the Sigma-Genosys kit, CDLBL-HCN, or stand-alone item, P5485) to 65°C prior to use. Pre-hybridize the Panorama™ gene array in 5 mL *Hybridization Solution* for at least 1 hour at 65°C, using roller bottles at 6 r.p.m. (or continuously agitate if using sealed bags).
3. Add the entire biotin-labeled cDNA to 2-3 mL *Hybridization Solution,* in a 15 mL conical screw-top tube. Denature the cDNA at 90-95°C for 10 minutes in a water bath or heat block.
4. Decant and discard the *Hybridization Solution* from the pre-hybridized array. Add the denatured labeled cDNA in *Hybridization Solution* to the array in the roller bottle.
5. Hybridize overnight (12-18 hours) at 65°C.
6. Decant the *Hybridization Solution* and save for future use. The biotin-labeled probe can be stored at -20°C, or -70°C for longer storage times.
7. Add 40-50 mL of *Wash Solution I* to the roller bottle. Wash the array by inverting the roller bottle by-hand, at room temperature for 2-3 minutes. Decant and discard the *Wash Solution I*.

   Note: An alternative wash method may be adopted for the arrays. Arrays may be washed in a suitably-sized plastic food container. Agitate the container on a rocking table or use a shaking water bath.
8. Repeat step 7 two more times.

9. Pre-warm the *Wash Solution I* to 65°C. Add 80-100 mL *Wash Solution I* to the roller bottle. Wash the arrays in the hybridization oven at 65°C for 20 minutes (6 r.p.m.). Decant and discard the *Wash Solution I*.
10. Repeat step 9 one more time.
11. Pre-warm the *Wash Solution II* to 65°C. Add 80-100 mL *Wash Solution II* to the roller bottle. Wash the arrays in the hybridization oven at 65°C for 20 minutes (6 r.p.m.). Decant and discard the *Wash Solution II*. Immediately proceed to the next section (development of signal).

D. Development of chemiluminescent signal.

After the post-hybridization washes, we recommend continuing the detection of biotin by using Roche Molecular Biochemicals' Western Blocking Reagent (Catalog number 1 921 673) and Amersham's ECL Western blotting detection reagents (Kit Catalog number RPN 2209). All incubations are at room temperature, unless specified otherwise. For each step below, prepare enough of the reagents to cover the arrays in your container. The reagent volumes stated are merely suggested volumes. Actual volumes required will vary depending of container size.

1. Using forceps, transfer the membrane to a clean dish or a plastic container.
2. Block the membrane by gentle agitation in 50mL of *Blocking Buffer* (Western blocking reagent diluted 1:5 with Maleic Acid Buffer, for 2 hours. Longer blocking times are acceptable.
3. Prepare 30 mL *Conjugate Buffer* by making a 1:10 dilution of Western blocking reagent with Maleic Acid Buffer containing a final concentration of 1µg/mL Streptavidin-HRP (Sigma -S2438)
4. Decant the Blocking Buffer and incubate the membrane for 20 min in the Conjugate Buffer.
5. Discard the Conjugate Buffer. Gently wash the membrane in 50mL *Wash Buffer III* for 10 minutes.
6. Repeat the wash step (step 5) three more times.
7. Decant the Wash Buffer and equilibrate the membrane in 50 mL Maleic acid buffer for 5 minutes. *It is important that the filter is kept wet before the chemiluminescent substrate is applied. If the membrane is even slightly dry, high background signals may occur.*
8. Prepare 24 mL of ECL detection reagent according to manufacturer's protocol.

9. Remove the membrane from the bottle or bag using tweezers, and place between two sheets of acetate (plastic page protectors). Gently lift the top sheet of plastic and, with a sterile pipet, add (dropwise) 12 mL of the chemiluminescent substrate on top of the membrane, scattering the drops over the surface of the membrane. Incubate for 1 minute at room temperature. Lower the top sheet of plastic and cover the membrane. With a damp tissue, gently squeeze excess liquid out and wipe the top sheet to create a liquid seal around the membrane.
10. For detection of the chemiluminescent signal, the membrane should be exposed to standard BioMax-MR film. As a starting point for the exposure time, we recommend 5 minutes. Adjust the exposure time to the signal strength. Note that several exposures may be required in order to generate the appropriate signal strength.
11. The autoradiograph image may be scanned using a flatbed scanner for semiquantitative analysis. Alternatively, the chemiluminescent signals may be captured with a CCD camera imaging system for more quantitative analysis (e.g. MiraiBio Inc., CCDBIO 16C). The spot signals should be normalized as described in Part Three of the protocol.

Appendices

Appendix A - Solution Compositions

1x TE BUFFER
10 mM Tris-HCl, pH 8.0
1 mM EDTA

5x REVERSE TRANSCRIPTASE BUFFER
250 mM Tris-HCl, pH 8.5
40 mM $MgCl_2$
150 mM KCl
5 mM dithiothreitol (DTT)

HYBRIDIZATION SOLUTION
5x SSPE
2% SDS
5x Denhardt's Reagent
100 µg/mL sonicated, denatured salmon testes DNA

WASH SOLUTION I
0.5x SSPE
1% SDS

WASH SOLUTION II
0.1x SSPE
1% SDS

1x SSPE
0.18 M NaCl
10 mM sodium phosphate, pH 7.7
1 mM EDTA
NOTE: Na_2HPO_4 is added to NaH_2PO_4 to bring sodium phosphate to pH 7.7.
Typically, different concentrations of SSPE are prepared by dilution of a 20x stock solution.

1x DENHARDT'S REAGENT
0.02% Ficoll (MW 400,000)
0.02% polyvinylpyrrolidone (PVP; MW 40,000)
0.02% bovine serum albumin (BSA)

STRIPPING SOLUTION
10 mM Tris-HCl, pH 8.0
1 mM EDTA
1% (w/v) SDS

Solutions for Non-Isotopic Detection:

MALEIC ACID BUFFER
0.1 M Maleic acid, pH 7.5 @ 22°C
0.1 M NaCl
0.2 µm filtered

BLOCKING BUFFER
1:5 dilution of Western Blocking Reagent in Maleic Acid Buffer

CONJUGATE BUFFER
1:10 dilution of Western Blocking Reagent in Maleic Acid Buffer,
Streptavidin-HRP conjugate to a final concentration of 1µg/mL

WASH BUFFER III
0.1 M Maleic acid, pH 7.5 @ 22°C
0.1 M NaCl
0.3% (v/v) Tween 20
0.2 µm filtered

NON-ISOTOPIC STRIPPING SOLUTION
0.2M NaOH
0.1% SDS

Appendix B - Stripping the Arrays

A. Stripping of Radioactive Targets

It is important to make sure that the arrays do not dry out before performing the stripping procedure. If arrays are allowed to dry, prior to stripping, then the labeled probe may be irreversibly bound to the membrane.

1) Prepare 1000 mL *Stripping Solution* (Sigma S3312, Appendix A). Pour 500 mL of the stripping solution into a "Tupperware" container or a Pyrex dish and cover with clear plastic food wrap. Fold back one corner of the plastic wrap for venting. Bring the *Stripping Solution* to a boil using a microwave oven (about 5 minutes at full power). NOTE: If a microwave oven is not available, then add the *Stripping Solution* to a Pyrex dish and place the dish on a variable temperature hotplate. Bring the solution to a boil on the hotplate.

2) Add the array to the heated solution and continue a low boil using the "defrost" setting (about 33% power) for 15 minutes. If using a hotplate, lower the temperature to a simmer rather than a “hard” boil.

3) Place the array on a clean surface and decant the *Stripping Solution*. Using fresh *Stripping Solution*, repeat steps (1) and (2) one more time.

4) Drain the excess *Stripping Solution* and re-wrap the array in clear plastic wrap. Expose the array to phosphorimaging plates or subject it to autoradiography. The duration of the exposure should be same as for a typical experiment, i.e. overnight exposure.

5) Analyze the image by phosphorimagery (or develop the autoradiograph). Compare the signals from the stripped array with the experimental signals obtained prior to stripping. Typically, >95% of the signal should have been removed from the array. If significant signals persist, then repeat the stripping procedure one-three more times depending upon the signal intensity, using fresh *Stripping Solution* each time.

6) If not proceeding directly to a new hybridization experiment, then store the array in plastic wrap at -20°C until ready to use. . Alternatively, the array can be stored at room temperature in a sealed plastic bag with 20mL of 2X SSPE solution. If additional stripping are to be performed, ensure that the gene arrays do not dry completely.

Each time an array is stripped, there will be a slight loss of bound DNA from the filter. In addition, depending on the signal strength of the probe used, some labeled spots are likely to remain after stripping. For new experiments, it is advisable to compare signals only from similarly stripped arrays. For example, do not use a fresh array for the control sample and a stripped array for the test sample.

B. **Stripping of Non-isotopic Targets**

1) Transfer the array to a "Tupperware" container or a roller bottle. Wash the array with gentle agitation in distilled water for 2 minutes at room temperature.

2) Prepare 100 mL of *Non-isotopic Stripping Solution* (Appendix A).

3) Decant the water form the "Tupperware" containing the array and add 50 mL of the stripping solution.

4) Incubate the array for 20 minutes at 37°C.

5) Repeat steps 3 and 4 one more time.

6) Rinse the array in 50 ml of 2X SSPE for 2 minutes at room temperature.

7) Re-wrap the array in clear plastic wrap or seal in a bag with 2X SSPE. Expose the array to autoradiography film. The duration of the exposure should be the same as for a typical experiment, i.e. 5 minutes.

8) Analyze the image by autoradiography. Compare the signals from the stripped array with the experimental signals obtained prior to stripping. Typically, >95% of the signal should have been removed from the array. If significant signals persist, then repeat the stripping procedure one-two more times depending upon the signal intensity, using fresh *Non-isotopic Stripping Solution* each time.

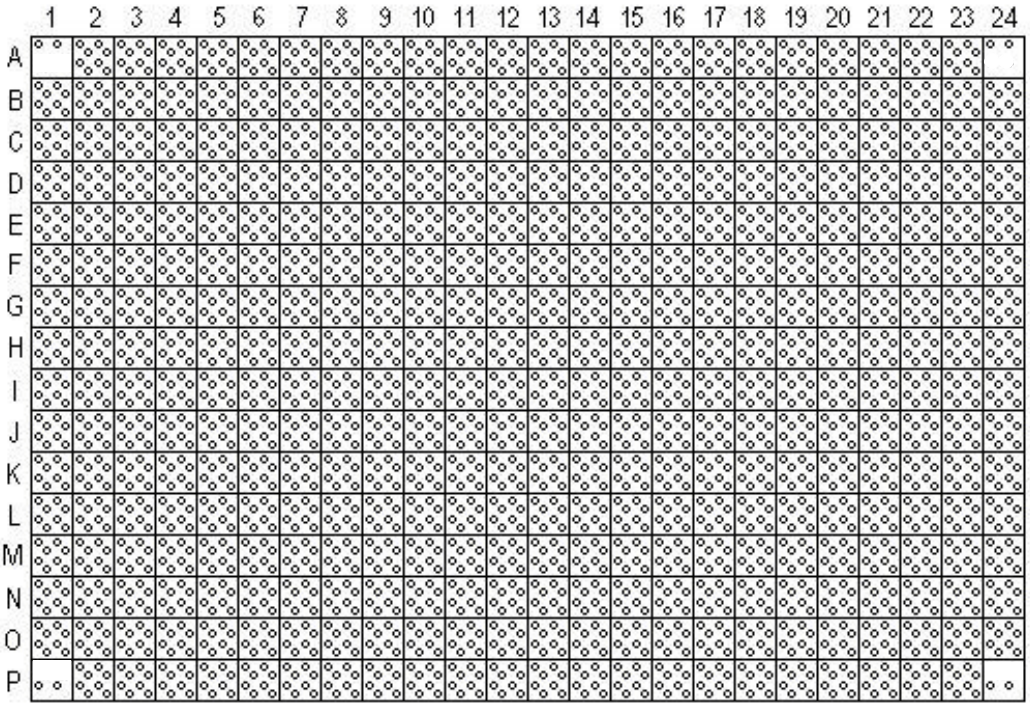
9) If not proceeding directly to a new hybridization experiment, store the array in a sealed bag with 2X SSPE at 4°C. If additional stripping are to be performed, ensure that the gene arrays do not dry completely.

Each time an array is stripped, there will be a slight loss of bound DNA from the filter. In addition, depending on the signal strength of the probe used, some labeled spots are likely to remain after stripping. For new experiments, it is advisable to compare signals only from similarly stripped arrays. For example, do not use a fresh array for the control sample and a stripped array for the test sample.

Appendix C - Layout of Genes on the Array

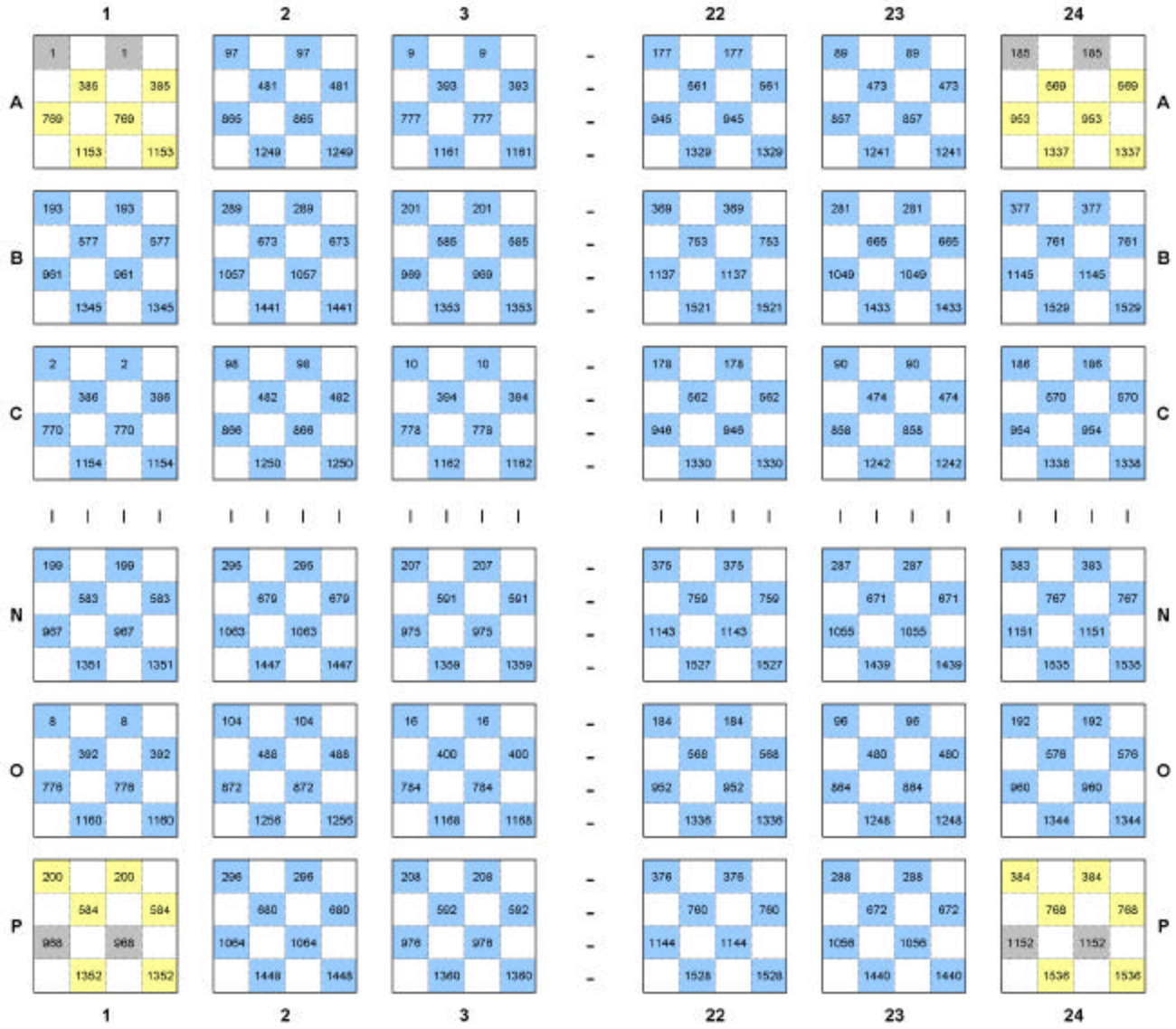
A. Layout of Primary Grid:

Field-1:



Each array consists of two fields and each field is divided into a 24 x 16 Primary Grid.

B. Layout of Secondary Grids:



Each primary grid contains 384 secondary grids. Most of the secondary grids contain 4 gene spots arrayed in duplicate (8 spots per secondary grid). For each of the two fields, the four corner grids contain human genomic DNA as a positive control/orientation marker. Note that in the second field, some of the grids do not contain a full complement of spotted genes.

Appendix D - Gene List and Locating Spots of Interest

A. Gene List:

For all genes listed in the spreadsheet on the accompanying disk, a Genbank® DNA sequence accession number has been assigned. Wherever possible, a RefSeq accession number has been used. Use the hyperlinks in the spreadsheet file on the accompanying disk to access the Genbank® sequence and other useful links with an internet browser.

B. Locating Spots of Interest:

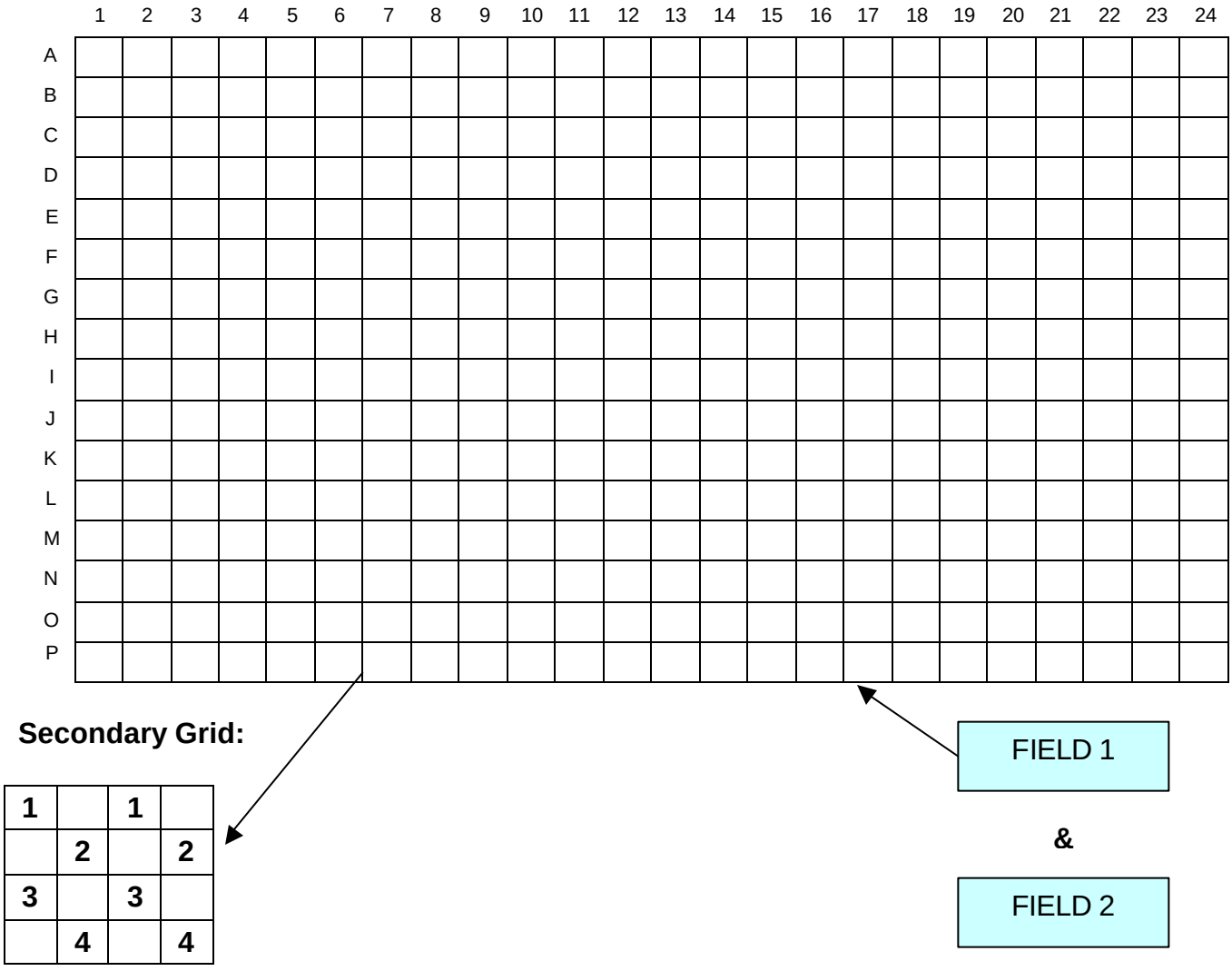
Analysis of the arrays may appear to be intimidating. The following is a guide to help determine gene "spot" numbers and the gene information associated with that spot.

1. Locate the position of the spot on the autoradiogram or the phosphorimager image.
2. Each array is divided into **2 fields** with genomic spots/anchors for each field.

   Each field is divided into **24 columns (1 – 24)** and **16 rows (A – P)**, in other words **384 primary grid squares.**

   Each of the **384 primary grid squares** contains **4 genes** spotted in duplicate (secondary grid, see below). Within the secondary grid, gene spot positions **1** and **3** appear left justified and gene spot positions **2** and **4** appear right justified.
3. Look up the spot coordinates in the Excel Worksheet ***(Array Layout).*** This will give you the spot number.
4. For example, look up the gene for Field **1**, Row **H**, Column **1** and spot position **4** (right justified). On the worksheet ***(Array Layout)*** you can see that this corresponds to spot number **1348**.

Primary grid:



Determining the Name of the Gene Corresponding to the Spot Number:

After determining the spot number from the coordinates of the field of the array (above), use the spreadsheet file to determine the identity of the gene at that spot:

1. Select the "***Gene Information"*** button located at the top of the ***Array Layout*** spreadsheet. Once selected, the button will appear highlighted. Double-click on a spot number in the ***Array Layout*** worksheet. The active cell should "jump" or link to the appropriate row with the spot number in the ***Gene Information*** worksheet. If the ***"Look-up Spot Info"*** button is selected in the ***Array Layout*** worksheet, then the active cell will "jump" to the ***Look-up Spot Info*** worksheet.

2. Alternatively, go to the worksheet labeled ***"Look-up Spot Info"*** of the Excel spreadsheet. Note: it is possible to switch between worksheets within a file by pressing *Ctrl + Page Up* or *Ctrl + Page Down*, simultaneously. Type in the spot number in the space that says ***"Enter spot number here"*** and hit the return key. For example, type **602.** All the information concerning the gene should now be displayed. Example, **IL8**.

Determining the Spot Number for a Known Gene Name:

1. Go to the worksheet labeled ***"Gene Information"*** of the Excel spreadsheet.
2. Select the **Gene Name** column by clicking on the column header (D). Note that gene description may be found under the **Gene Description** column (column Q).
3. Under the Edit pull down menu, select *FIND*. Alternatively type Ctrl + F.
4. Enter the name of the gene in the *Find* window and hit return. For example *IL8*.
5. The row with the gene name will now be deselected.
6. Note down the spot number corresponding to the gene name. For example **602** for *IL8*. Proceed to the next section to find the location of this gene in the array.

To Locate the Position of a Known Spot Number in the Array:

1. Go to worksheet labeled ***"Array Layout"*** of the Excel spreadsheet.
2. Under the Edit pull down menu, select *FIND*. Alternatively type Ctrl + F.
3. Enter the spot number in the *Find* window and hit return. For example, enter **602**.
4. Make sure that the *"Find Entire cells only"* box is **checked**.
5. The position of the spot will now be highlighted. For example **602** would be Field **1**, Row **D**, Column **7**, Spot Position **2**.
6. Use the ***Zoom*** function to zoom-in or -out on the array as desired.

Appendix E - DNase I treatment of total RNA

1. Pipette 50-100 µg of RNA sample into a fresh 1.5 mL microfuge tube add 5 µL of DNase I buffer (10X) and 5 units of DNase I (1 unit/µL) (*Sigma-AMP-D1*). Make up to a total volume of 50 µL with sterile, distilled water.
2. Incubate at room temperature for 15 minutes.
3. Add 150 µL of water to bring up the volume to 200 µL.
4. Add 200 µL of hot acidic phenol (pH 4.3, Sigma P4682), mix thoroughly with a vortex and incubate for 3 minutes at 65°C.
5. Cool tubes on ice for 3 minutes.
6. Separate the phases by centrifuging the sample in a microcentrifuge at maximum speed (~12,000 x *g*) for 2-3 minutes. Transfer the upper aqueous phase to a fresh tube.
7. Repeat the phenol extraction two more times.
8. Add 200 µL of acidic phenol:chloroform:isoamyl alcohol (25:24:1), vortex, and separate the phases by centrifuging the sample in a microcentrifuge at ~12,000 x *g* for 2-3 minutes. Transfer the upper aqueous phase to a fresh tube.
9. Add 200 µL of chloroform:isoamyl alcohol (24:1), vortex, and separate the phases by centrifuging the sample in a microcentrifuge at ~12,000 x *g* for 2-3 minutes. Transfer the upper aqueous phase to a fresh tube.
10. Precipitate the RNA by adding 0.1x volumes of 3M sodium acetate (pH 5.2) and vortex to mix. Add 3 volumes of absolute ethanol and mix by inverting the tube several times. Samples may be stored at -20°C indefinitely or proceed directly to the next step.
11. Pellet the RNA by centrifugation at 12,000 x *g* for 30 minutes. Carefully discard the supernatant either by decanting or by pipetting.
12. Wash the RNA pellet by adding 1 mL 70% ethanol. Gently invert the tube several times and centrifuge at maximum speed for 15 – 20 minutes.
13. Air dry the pellet for 10-15 minutes.
14. Dissolve the RNA in 50 µL of sterile water. Place tube at 37°C for 15 – 30 minutes to thoroughly dissolve the RNA. Keep on ice for immediate use or store at -20°C for long term storage.

It is advisable to check equivalent amounts of RNA before and after the DNase I digestion by non-denaturing agarose gel electrophoresis to ensure the quality of the RNA and to observe the effect of the DNase I treatment.

DISCLAIMER and WARRANTY

Sigma-Genosys, L.P. ("Seller") warrants to a purchaser ("Buyer") only that the Panorama™ Gene Arrays ("Arrays") sold by Seller shall substantially conform to Seller's specifications as in effect on the date of order by Buyer. Seller's sole liability under this warranty is limited to the replacement, at no charge, of any nonconforming Arrays. Seller shall not be responsible for failure of the Arrays to perform specific functions or any non-conformance caused by or attributable to misuse, improper storage, outdating, neglect or abuse of, or accident to, the Arrays. Buyer assumes all risks and liability arising from unloading, discharge, shipping, storage, handling and use of the Arrays, including risk of damages resulting from use of such Arrays either alone or in combination or conjunction with other substances. The Arrays are intended for research purposes only. They are not to be used for drug or diagnostic purposes and are not intended for human use.

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Claims relating to quantity, shipping damages, condition or losses must be made within 3 days of receipt of the ARRAYS. All shipping containers and packaging materials must be retained for inspection by carrier.

Claims relating to quality and conformance to specifications of the Arrays must be made within ten days of receipt of Arrays.

Title to the Arrays passes to buyer upon delivery of items to the carrier by Sigma-Genosys, L.P.