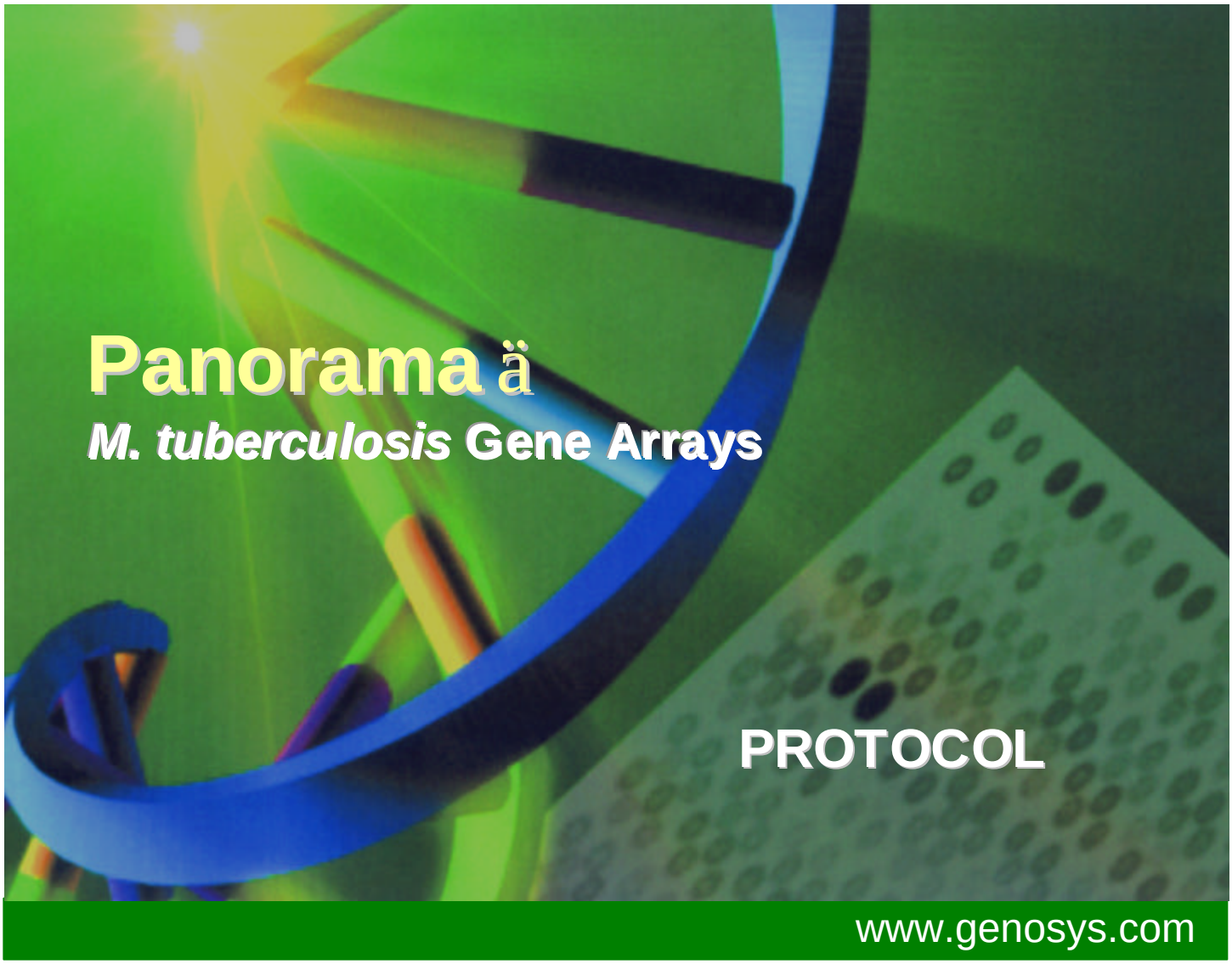


www.genosys.com

G4666 **Panorama™ *M. tuberculosis* Gene Arrays**

Set of two 8 x 24 cm arrays

Transparency orientation grid – spot/gene location guide

Microsoft® Excel spreadsheet including
gene layout and hyperlinks to Genbank®

Protocol

Optional Components:

CDLBL-TB Panorama™ *M. tuberculosis* cDNA Labeling and Hybridization Kit

C2731 Panorama™ *M. tuberculosis* cDNA Labeling Primers

SIGMA GENOSYS

Contents

Notice to the Customer

The use of the Panorama™ *M. tuberculosis* Gene Arrays is 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.

The Panorama™ Gene Arrays have been manufactured by Sigma-Genosys, Inc. using PCR technology owned by Hoffmann-La Roche, Inc. under a license granted by Hoffmann-La Roche, Inc. to Sigma-Genosys, Inc. The sale and purchase of this product does not convey any license to the purchaser to practice PCR or any other technology owned by Hoffmann-La Roche, Inc.

Trademarks and Patents

Panorama™ is a trademark of Sigma-Genosys, Inc.
Genosys® is a registered trademark of Sigma-Genosys, Inc.
Sephadex® is a registered trademark of Pharmacia Biotech AB.
ArrayVision™ is a trademark of Imaging Research, Inc
Microsoft® is a registered trademark of the Microsoft Corporation.
The PCR process is covered by patents owned by Hoffmann-La Roche, Inc.

Components

- **Panorama™ *M. tuberculosis* Gene arrays (G4666) .** Two charged, nylon membranes containing spotted PCR products representing 3,875 ORFs of *Mycobacterium tuberculosis*, strain H37Rv.
- **Protocol booklet.**
- **Transparency.** An array template to facilitate spot location on autoradiographs.
- **Floppy diskette.** Containing a spreadsheet showing the layout of the arrays along with ORF information. and the spreadsheet is in Microsoft® Excel (for Office 2000) format.

Storage of Components

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

Optional Components

Panorama™ *M. tuberculosis* cDNA Labeling and Hybridization Kit***** (Catalog No. CDLBL-TB):

- 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.
- Panorama™ *M. tuberculosis* 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.

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

Additional reorder items:

- Panorama™ Hybridization Solution (Catalog No. P5485/PRHY0001)
- AMV Reverse Transcriptase (Catalog No. A8970/PRRT0001)
- Panorama™ *M. tuberculosis* cDNA Labeling Primers - optimized for *M. tuberculosis* (Cat. No. C2731)

Materials and Equipment Required but Not Provided

Materials

- *Resuspension Buffer* (sucrose, Sigma S0389; Sodium acetate (pH 4.2), S7899); *Lysis Buffer* (SDS, L4390; EDTA, E7889); phenol (pH 4.3) (P4682); chloroform:isoamylalcohol (24:1); Ethanol; Array Wash solution (M1560).
- *M. tuberculosis* cDNA Labeling and Hybridization Kit (Cat. No. CDLBL-TB).
- Panorama™ *M. tuberculosis* cDNA Labeling Primers (Cat. No. C2731) - not required if using *M. tuberculosis* cDNA Labeling and Hybridization Kit (CDLBL-TB).
- [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).
- RNase-free DNase I (optional; Sigma AMP-D1).
- Panorama™ Microbial Array Wash Solution (Cat. No. M1560)
- Panorama™ Array Stripping Solution (optional; Cat. No. S3312)
- Microcentrifuge Tubes (0.5 mL, 1.5 mL), sterile, nuclease-free.
- Conical Tube (50 mL screwcap).
- Scintillation vials and scintillation fluid (optional).
- 20x SSPE.
- Panorama™ Hybridization Solution (Cat. No. P5485/PRHY0001).
- X-ray film (Kodak BioMax MR, 20 in x 25 cm; Cat. No. 870-1302 or 35 X 43 cm; Cat. No. 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 LE177-940) 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 for analyzing phosphorimages.
- Radioisotope solid and liquid waste containers.

Overview

Tuberculosis is a chronic infectious disease caused by the tubercle bacillus and is the leading cause of death due to infection worldwide. The causative agent of tuberculosis is the gram positive, non-motile, rod-shaped *Mycobacterium tuberculosis*. Due to its importance as a widely spread human pathogen, two strains of *Mycobacterium*, an infectious laboratory strain (H37Rv) and a recent clinical isolate (CDC-1551) from the Kentucky/Tennessee region have been sequenced by the Sanger Center[1] and TIGR[2], respectively. The completion of the genomic sequencing has made possible a comprehensive genomic approach to the biology of this important pathogen and to the drug discovery process.

Sigma-Genosys, Inc. offers a macroarray for the study of global gene expression in this important gram-positive human pathogen. It is now possible for investigators to study expression at the transcriptional level of nearly all open reading frames (ORFs) simultaneously in one experiment. The Panorama™ *M. tuberculosis* Gene Arrays contain 3,875 PCR-amplified open reading frames. The entire genome contains 3,918 putative ORFs. However, extensive homology in the remaining ORFs renders analysis of these ORFs redundant. PCR products have been designed to be less than 500b.p. in length and in most part have been designed to represent a region of the ORF that has as little homology to other ORFs as possible. The gene array now makes expression profiling accessible to the most molecular biology laboratories and opens new horizons for basic and applied microbial genome research.

The development of gene array technology allows researchers to study the relative mRNA levels of thousands of genes simultaneously, in a single experiment. This method can be used to determine which genes are "turned on" or "turned off" between two or more samples. These studies are often referred to as "expression profiling" experiments. In the past, the equivalent amount of information could only be derived using differential screening methods, such as differential display RT-PCR or differential cDNA library screening; 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. A typical expression profiling experiment involves the following steps: 1) growth of cultures under various conditions for comparing gene expression, 2) extraction of total RNA from each culture, 3) generation of radioactively-labeled cDNA from all RNA samples, 4) hybridization of labeled cDNAs to duplicate arrays (one sample per array), 5) autoradiography or phosphorimaging of the arrays, and 6) analysis of the expression patterns.

Phosphorimager files allow quantification of the expression signals from the genes spotted on the arrays. Gene expression signals can be compared between a control sample and a number of experimental samples. A number of factors affect the expression signal strength, such as length of a particular PCR fragment spotted on the array (signal may increase with longer fragments). In general, macroarray based analysis is considered semi-quantitative, where trends in the changes of expression between samples is reproducible. Figure 2 shows results from an expression profiling experiment where bacteria were either in log-phase or stationary-phase of growth. A set of two membranes allows for the analysis of two bacterial samples. Arrays may be stripped and re-probed to analyze additional samples.

The Panorama™ *M. tuberculosis* Gene Arrays contain 3,875 PCR-amplified ORFs, from *M. tuberculosis,* strain H37Rv. These arrays provide a rapid means of determining global gene expression during cellular responses to developmental cues, external stimuli, or stresses.

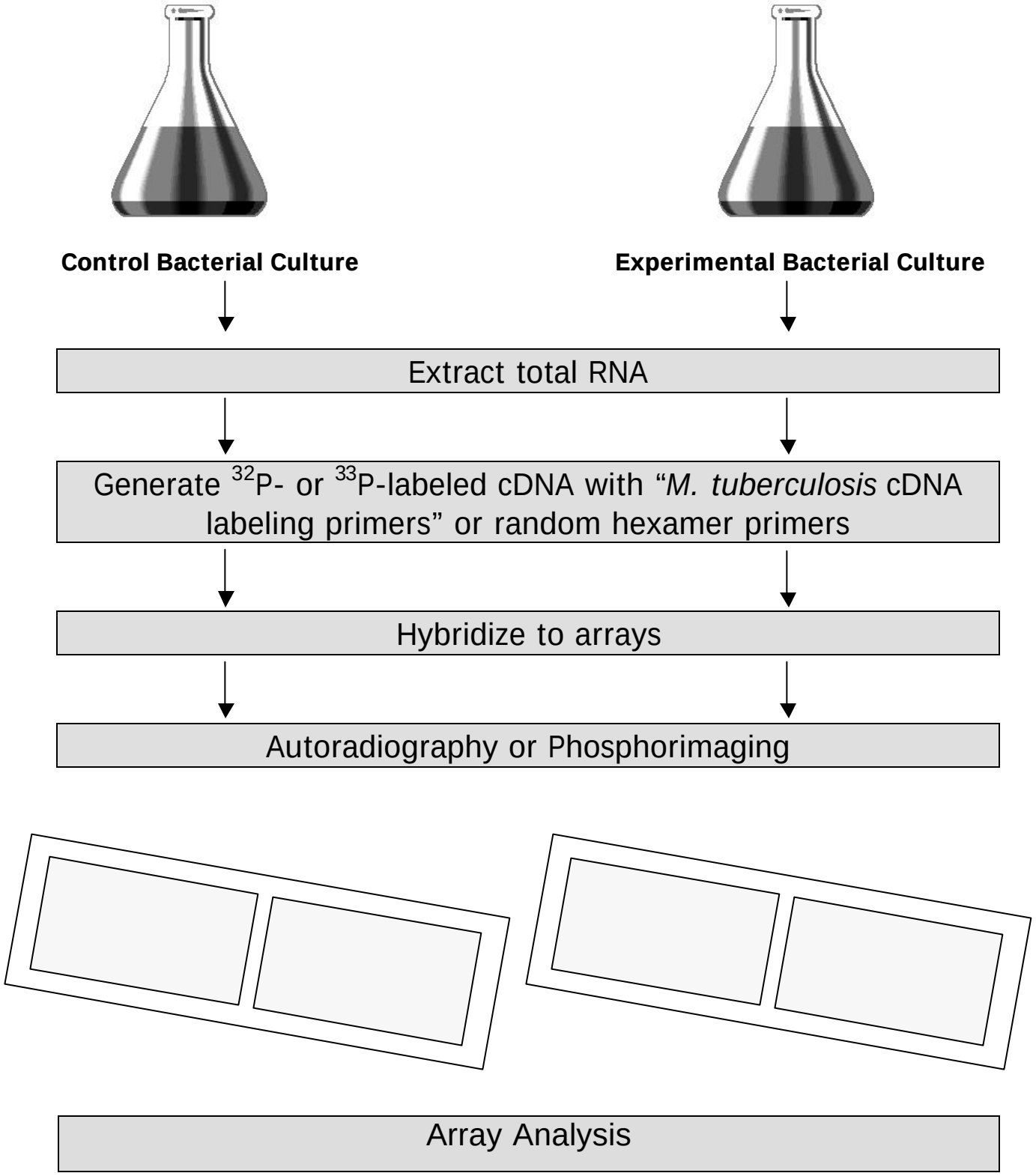


Figure 1. ***Diagrammatic representation of the expression profiling process using macroarrays.***

Expression Profiling Figures

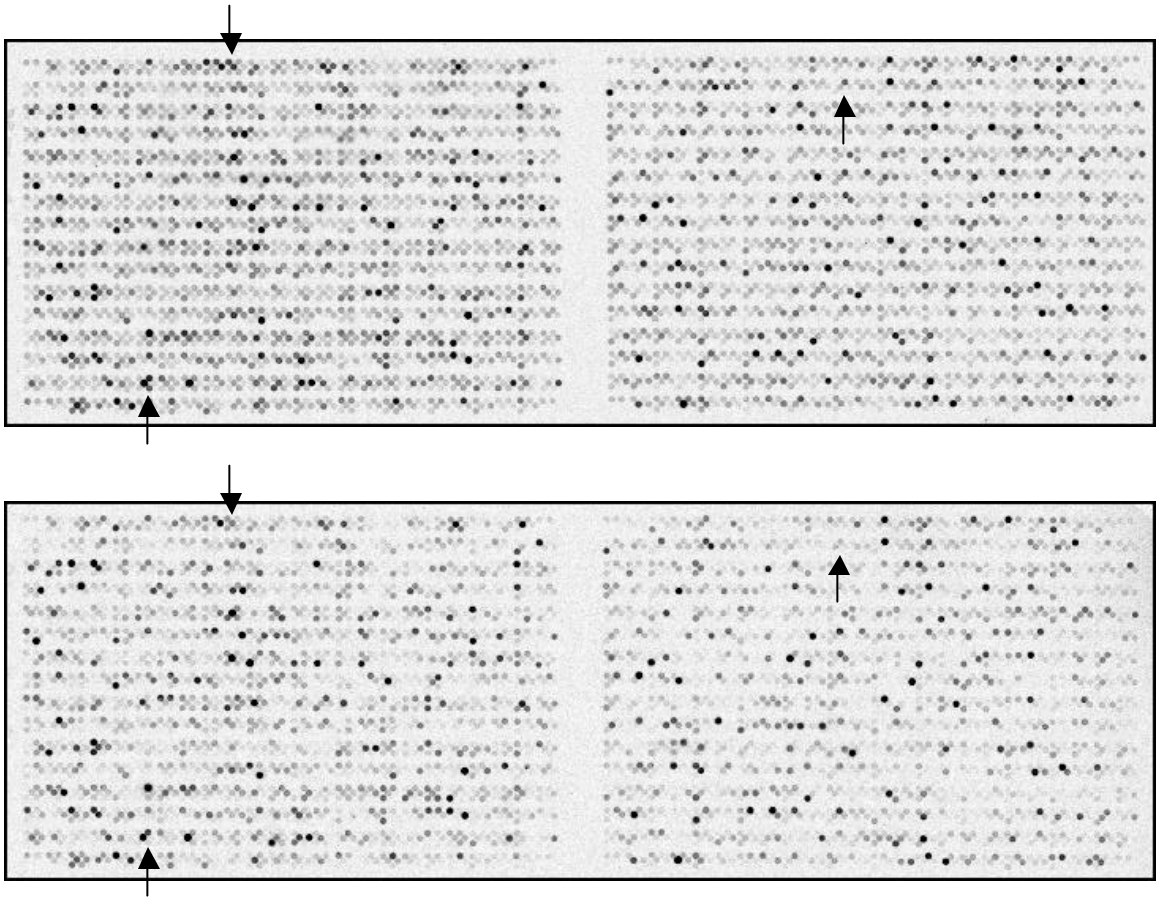


Figure 2. *Differential gene expression between log-phase and stationary-phase growth in* M. tuberculosis. Two Panorama™ *M. tuberculosis* Gene Arrays were probed with ^{33}P-labeled cDNA from cells grown to log phase (upper panel) or cells grown to stationary phase (lower panel). The labeled cDNA was prepared using the *M. tuberculosis* cDNA labeling primers (Cat. No. C2731). The above images were obtained by phosphorimaging following an overnight exposure to imaging plates.

Product Description

Each Panorama™ *M. tuberculosis* gene array contains PCR-amplified open reading frames (ORFs) from the *Mycobacterium tuberculosis* genome (strain H37Rv). For the majority of ORFs, the PCR products have been designed to represent a region of the ORF that has as little homology to other ORFs as possible. Information regarding the genome sequence and the annotations may be found on the World-Wide Web:

For the strain sequenced by the Sanger Centre (H37Rv)

http://www.ncbi.nlm.nih.gov/cgi-bin/Entrez/framik?db=Genome&gi=135

For the Strain sequenced by TIGR (CDC1551)

http://www.tigr.org/tigr-scripts/CMR2/GenomePage3.spl?database=gmt

For the Panorama™ *M. tuberculosis* Gene Array, all of the PCR products from 3,875 ORFs of strain H37Rv have been printed onto positively-charged nylon hybridization membranes. Following printing, each array was cross linked with UV light. For orientation purposes, the top right-hand corner of each array has been clipped.

The arrays are 8 cm x 24 cm in size. Each *Mycobacterium tuberculosis* array comprises of two fields and each field consists of a primary grid containing secondary grid of spots. The primary grid is composed of 16 rows (A to P) by 24 columns (1 to 24) of secondary grids. Each secondary grid contains six genes spotted as 3 rows and 2 columns (see the Appendices B and C for representations of the arrays). Note that all of the secondary grids do not contain a full complement of 6 genes. The four corners of the primary grid, A1, A24, P1 and P24, contain *M. tuberculosis* genomic DNA spotted in duplicate. These secondary grids contain 2 genomic DNA spots only. During any expression profiling experiment where labeled cDNA is used as probe, the genomic DNA spots will act as a positive control and should always show signal. The genomic DNA spots can be used to (a) orient the corners of the fields of the array and (b) anchor image analysis templates after phosphorimaging.

A Microsoft® Excel spreadsheet accompanies the arrays. The spreadsheet shows the spot numbers within each of the two fields of the array. The spreadsheet can be used to determine the ORF associated with each spot (or *vice-versa*). The file also includes spreadsheets with detailed information on each ORF along with web-based hyperlinks to pertinent gene information.

NOTE: Please read and familiarize yourself with the entire Panorama Gene Array protocol before use.

Methods

Part One – RNA Extraction

The methodology for RNA extraction is critical for a successful expression profiling experiment. It is essential to purify RNA free of any contaminating genomic DNA from the bacterial chromosome. Traces of genomic DNA in the RNA preparation may contribute to background hybridization to all spots on the array. Since *Mycobacterium* cell walls are hard to break open, a cell disruptor may be helpful in obtaining high yields of RNA. The protocol described by Cheung et al. (1994) uses a reciprocating high-speed shaking device together with 0.1 mm zirconia/silica beads[3]. The bacteria may be resuspended in extraction reagent containing phenol and cetyltrimethyl-ammonium bromide (CTAB) and then, homogenized with the beads. Alternatively, bacteria can be resuspended in heated (90°C) guanidinium thiocyanate solution prior to mechanical disruption[4]. The protocol described here does not make use of any type of cell disruptor and therefore, the yield of RNA may be lower. Nevertheless, 3-5 mL of bacterial culture should yield enough high quality RNA with minimal DNA contamination for several cDNA labeling reactions. Refer to Appendix A for buffer compositions. The reagent volumes cited below are for use with cell pellets from up to 5 mL bacterial culture. The yield of RNA should be sufficient for several cDNA labeling reactions. Scale-up reagents for use with larger culture volumes. **All of the following steps should be performed on ice, unless otherwise stated.**

1. After performing the desired treatments, growth conditions, etc., transfer each culture to one or more 1.5 mL microcentrifuge tubes on ice. Pellet the bacterial cells from 1.5 – 5mL cultures in a microcentrifuge at maximum speed, (~12,000 x *g*) for 1 minute at 4°C. The yield of RNA should be sufficient for several cDNA labeling reactions.
2. Decant and discard the growth medium. At this point, an optional rapid-freeze step may be included. A rapid-freeze of the cell pellet at -70°C or in liquid nitrogen may help to arrest cellular function.
3. If using mechanical disruption, resuspend the bacterial cells in 1 mL of extraction reagent and transfer to a 2 mL microcentrifuge tube containing 0.5 mL of zirconia/silica beads (0.1 mm size) and shake the tubes using a reciprocating high-speed shaking device at 6,000 rpm for 20 seconds (Savant Instruments). Then, skip steps 4-8 and go directly to step 9 for the rest of the protocol.
4. If the bacterial cells are not being disrupted by mechanical means, then add 250 µL *Resuspension Buffer* containing 1mg/mL lysozyme (Sigma L7651) to the cell pellet. Pipette thoroughly to resuspend the bacterial cell pellet. If resuspending the cell pellets from several

tubes, then resuspend one tube first and transfer the resuspension to the next tube. Continue pipetting and transferring until all pellets are resuspended and pooled.

5. Incubate the cells on ice for 10 minutes.
6. Add 375 µL *Lysis Buffer*, mix thoroughly using a vortex.
7. The lysate must be subjected to three "freeze-thaw" cycles. To freeze the cells, either immerse the tube in an insulated receptacle containing liquid nitrogen for 1-2 minutes or incubate on dry ice for 3 minutes. To thaw the tubes, incubate in a water bath at room temperature for 2-3 minutes, until thawed.
8. Transfer the tube to a heat-block at 65 °C and incubate for 3 minutes.
9. Add 700 µL of acidic phenol (pre-warmed to 65°C), vortex vigorously to mix and incubate for 3 minutes, at 65°C. Note that the use of acidic phenol is essential to minimize genomic DNA contamination of the RNA sample. The acidic phenol is prepared and buffered with 0.1 M citrate acetate, pH 4.3 (Sigma P4682).
10. Cool the sample on ice for 3 minutes.
11. Separate the phases by centrifuging the sample in a microcentrifuge at ~12,000 x *g* (maximum speed) for 5 minutes. Transfer the upper aqueous phase to a fresh tube. Avoid any solid matter near the interphase.
12. Repeat the phenol extraction two more times (steps 58 to 710). Note that if the optional rapid-freeze step was included (see step 2), then an additional phenol extraction is recommended. The rapid-freeze step may result in the release of more chromosomal DNA from the cells and an extra acidic phenol extraction is useful in minimizing DNA contamination of the RNA sample.
13. After transferring the aqueous phase to a fresh tube, add 700 µL of a mixture of acidic phenol:chloroform:isoamyl alcohol (25:24:1), at room temperature. Vortex to mix and separate phases again in a microcentrifuge for 52 minutes.
14. Transfer the aqueous phase to a fresh tube and perform one final extraction with an equal volume of chloroform:isoamyl alcohol (24:1). Vortex to mix and separate phases in a microcentrifuge at maximum speed for 52 minutes.
15. Transfer the aqueous phase to a fresh tube. **NOTE: Care must be taken to avoid transferring any of the organic-phase along with the aqueous-phase.** Organic-phase carry-over may severely affect the efficiency of cDNA synthesis in Step Two of this protocol.
16. Divide the aqueous phase equally between two fresh tubes and add 0.1x volumes (~35 µL) of 3M sodium acetate (pH 5.2). Vortex to mix. Precipitate the RNA by adding 2.5 volumes of absolute ethanol. Invert the tube several times to mix. Samples may be stored at this stage at -20°C, indefinitely.

17. To proceed, pellet the RNA by centrifugation at 12,000 x g (or maximum speed in a microcentrifuge) for 30 – 60 minutes. Carefully discard the supernatant either by decanting or by pipetting. Take care NOT to discard the RNA pellet. Note that the pellet may be barely visible.
18. Wash the RNA pellet by adding 1 mL 70% ethanol. Gently invert the tube several times and centrifuge at maximum speed for 10 – 15 minutes.
19. Discard the supernatant, again 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.
20. When the RNA pellet appears clear or translucent, dissolve the RNA in 100 – 200 µL of sterile, RNase-free water or 1xTE Buffer. Place 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 cDNA labeling step (Part Two of the protocol).
21. Quantify the RNA sample by measuring the absorbance at 260 nm of an appropriate dilution (5 – 10 µL of RNA in 1 mL water) in a spectrophotometer.
22. It is important to check both the integrity of the RNA and the degree 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 2 – 5 µg RNA sample with a non-denaturing gel loading dye and run the sample on a 1.2% agarose gel. If genomic DNA is present in the RNA sample, it will be seen as high molecular weight-staining material. Typically, if the above procedure is strictly followed, genomic DNA contamination will be negligible. If excessive amounts of genomic DNA are present, it may be necessary to treat the RNA sample with RNase-free DNase I (**Appendix F**).

Part Two – Generating Labeled cDNA

PREPARATION OF LABELED cDNA

Expression profiling in bacteria is impacted by the fact that there is no effective method for purifying mRNAs from total RNA. Current methods for generating cDNA from microbial RNA employ random hexamer-primed synthesis using reverse transcriptase. When generating labeled cDNA using random hexamer primers with total RNA, the majority of the label is incorporated into rRNA as opposed to mRNA. Hence, the sensitivity of message detection is low for bacterial expression profiling applications. Sigma-Genosys has circumvented this problem by designing cDNA primers that preferentially prime mRNAs in *M. tuberculosis*. These cDNA labeling primers may be purchased alone (Cat. No. C2731) or in the labeling and hybridization kit (Cat. No. CDLBL-TB). The optimized primers result in labeled cDNAs that more truly represent complements of mRNAs, with a significantly higher specific activity compared to random hexamer-primed cDNA. The following protocols give options for generating labeled cDNA with either random hexamers or with the Sigma-Genosys *M. tuberculosis* cDNA labeling primers. Note that Sigma-Genosys recommends the use of ^{33}P rather than ^{32}P for expression profiling with the Panorama™ Gene Arrays. Signal detection with ^{33}P gives a sharper image with well-defined spots on the arrays that allow easier quantitation. Signals generated using ^{32}P are more diffuse making quantitation of individual spots less reliable and spot borders appear less discrete.

Using *M. tuberculosis* cDNA Labeling Primers

The cDNA labeling reactions are performed in two steps. In the first step, the *M. tuberculosis* cDNA 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™ cDNA Labeling and Hybridization Kit, Cat. No. CDLBL-TB).

Table 1.

Final Concentration	Stock Reagent	Volume for one reaction
1 µg total RNA from *M. tuberculosis*		X µL
M. tuberculosis cDNA Labeling Primers, reconstituted with 40µL sterile, distilled water (Sigma-Genosys, C2731)		4 µL
	Sterile distilled water to	**15 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 dTTP	10 mM	1 µL
20 µCi [α-^{33}P] dCTP (2,000-3,000 Ci/mmol)*	10 µCi/µL	2 µL
50 U AMV Reverse Transcriptase	25 U/µL	2 µL
	Sterile, distilled water to a *final* volume of	**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 *M. tuberculosis* 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 and 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**.
4. 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 institution's handling and waste disposal procedures for use of radioactive materials.*
5. The unincorporated-radiolabeled nucleotide must be removed from labeled cDNA by purification over a Sephadex® G-25 gel-filtration spin column. Use the spin column protocol detailed below.
6. After removal of unincorporated-radiolabeled nucleotides, the labeled cDNA probe is ready for hybridization with the Panorama™ gene array (*Part Three* of this protocol).

Using Random Hexamer Primers

The protocol for generating cDNA with random hexamers is similar to that described above with the following exceptions:

Use 42 pmol (~83 ng) of random hexamers instead of the "*M. tuberculosis* cDNA Labeling Primers" and use 10-20 µg of total RNA. Before assembling the reaction, heat the RNA to 70°C for 3 minutes, then immediately chill on ice. Assemble the entire reaction on ice, adding the components listed in Tables 1 and 2, above. After assembling the reaction, mix carefully by pipetting and incubate at 42°C for 2 – 3 hours. After the incubation period, proceed to the next section to remove unincorporated radiolabeled nucleotides.

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 the generation of background during the hybridization to the 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.

1. Gently invert the gel-filtration spin column 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 Falcon tube.
4. Centrifuge at 1,100 x *g* for 3 – 4 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.
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 counter can be used to determine a rough estimate of the percentage incorporation of labeled nucleotides into the cDNA. Measure the radioactivity left in the column (unincorporated labeled nucleotide, "U") and the amount of radioactivity in the collection tube (labeled nucleotide incorporated into the cDNA,

"I"). To determine the approximate percentage incorporation, calculate (I/(I+U)) x 100. Typically, the incorporation should be >30%. 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

HYBRIDIZATION AND WASHING OF THE ARRAY

The next step is to hybridize the radioactively-labeled cDNA (prepared in Part Two) to the Panorama™ gene array. The hybridizations are best performed in roller bottles in a hybridization oven where minimal volumes of hybridization solutions can be employed. Nevertheless, hybridizations can also be performed in sealed plastic bags in a heated air incubator or immersed in a water bath. 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). Please see Appendix A for buffer compositions

1. Rinse the blots in 50 mL 2x SSPE for 5 minutes. Drain and discard the solution.
2. Pre-warm the hybridization oven to 65°C. Warm the Hybridization Solution (Cat. No. P5485/PRHY0001 or Kit Cat. No. CDLBL-TB) to 65°C for about 10 minutes prior to use. Note that the contents of the Hybridization Solution may come out of solution during storage at 4°C. Pre-warming to 65°C will help ensure that components go into solution before use. Swirl solution to mix. Then, add the appropriate quantity of salmon testes DNA to achieve a final concentration of 100 µg/mL, immediately prior to using the Hybridization Solution.
3. 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).
4. Add the entire volume of labeled cDNA generated from Part Two of this protocol to 3 mL Hybridization Solution. Heat at 90 – 95°C for 10 minutes in a water bath to denature the cDNA.

5. 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.
6. Hybridize overnight (12 – 18 hours) at 65°C, using roller bottles at 6 r.p.m. (or continuously agitate if using sealed bags).
7. Decant the hybridization solution and save for future use or discard appropriately.
8. Add 40 – 50 mL of Wash Solution to the roller bottle (see appendix A or use Microbial Array Wash Solution, Cat. No. M1560). Wash the array by inverting the roller bottle by-hand, at room temperature for 2 – 3 minutes. Decant and discard the Wash Solution 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.
9. Repeat step 8 two more times.
10. Pre-warm the Wash Solution to 65°C. Add 80 – 100 mL Wash Solution to the roller bottle. Wash filters in the hybridization oven at 65°C for 20 minutes (6 r.p.m.). Decant and discard the Wash Solution to an appropriate radioactive waste container.
11. Repeat step 10 two more times.
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 significantly less efficient. An array stripping protocol can be found in Appendix E.
14. Wrap the array in clear food wrap and subject to autoradiography using Kodak Bio-Max MR (for ^{33}P-labeled cDNA), Bio-Max MS (for ^{32}P-labeled cDNA) X-ray film or expose phosphorimager screens for analysis by phosphorimagery. When performing phosphorimagery with ^{33}P, Sigma-Genosys strongly recommends the use of Kodak Low Energy Storage Phosphor Screens HD (Molecular Dynamics, LE177-956, 20.3 cm x 25.4 cm) or Fujifilm screens (YBIP2025MS, 20 cm x 25 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 field per image file. If using phosphorimaging screens, care must be taken to ensure that there are 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 blots are to be used again, it is essential that they be stripped before the membrane dries out. It is advisable to strip the arrays as soon as possible following image exposure (see Appendix E).

ANALYSIS OF THE ARRAYS

Quantification 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. Many software packages may not be able to analyze simultaneously the large number of spots represented on the Panorama *M. tuberculosis* Gene Arrays. Sigma-Genosys uses ArrayVision™ software (developed by Imaging Research, Inc.) which can analyze all the spots on the *M. tuberculosis* array. The user may wish to analyze the arrays in smaller subsets, depending on the software being used. The process of analysis involves the following steps:

1. Set up a quantitation template to analyze pixel intensity in each spot of the array.
2. Subtract background signal from each spot.
3. Export signal values to a spreadsheet file for manipulation in a program such as Microsoft® Excel.

4. Normalize relative signals from different arrays by representing the spot signal as a percentage of the total signal from all spots on the array (preferred method). Alternatively, represent each gene's signal as a percentage of the averaged signal from all of the positive control genomic DNA spots.
5. Compare normalized signals of corresponding spots from different samples on different arrays to determine fold-induction or fold-reduction in expression between samples.

For images that are scanned at 50 micron resolution and are being analyzed using the ArrayVision™ software, the ArrayVision™ spot quantitation template and the corresponding Microsoft® Excel Spreadsheet (containing the appropriate gene names corresponding to each spot) may be obtained from Sigma-Genosys or can be downloaded from the Sigma-Genosys web pages:
(http://www.genosys.com/expression/frameset.html).

REFERENCES:

1. Cole, S.T. et al. (1998). Deciphering the biology of Mycobacterium tuberculosis from the complete genome sequence. *Nature* **393:**537-544.

2. Valway, S.E. et al. (1998). An outbreak involving extensive transmission of a virulent strain of Mycobacterium tuberculosis. *N. Engl. J. Med.* **338:**633-639.

3. Cheung, A. L., Eberhardt, K. J. and Fischetti, V. A. (1994). A method to isolate RNA from Gram-positive bacteria and Mycobacteria. *Anal. Biochem.* **222:**511-514.

4. Graham, J. E. and Clark-Curtiss J. E. (1999). Identification of Mycobacterium tuberculosis RNAs synthesized in response to phagocytosis by human macrophages by selective capture of transcribed sequences (SCOTS). *Proc. Natl. Acad. Sci. USA.* **96:**11554-11559.

Appendices

Appendix A - Composition of Solutions

RESUSPENSION BUFFER

0.3 M sucrose

10 mM sodium acetate, pH 4.2

EXTRACTION BUFFER

40 % phenol

0.6 % Cetyltrimethylammonium bromide

50 mM sodium acetate, pH 4.5

1 mM dithiothreitol

LYSIS BUFFER

2% SDS

10 mM sodium acetate, pH 4.2

1x TE BUFFER

10 mM Tris, 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

0.5x SSPE

0.2% 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.

1x DENHARDT'S REAGENT

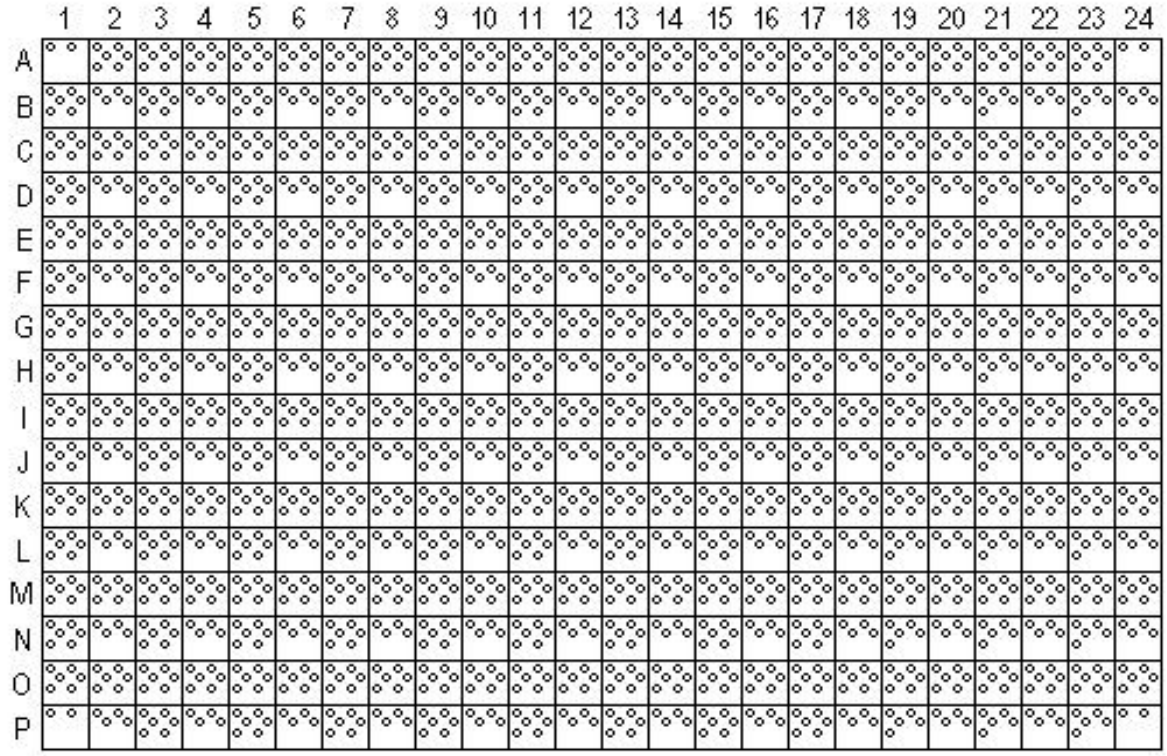
0.02% Ficoll (MW 400,000)

0.02% polyvinylpyrrolidone (PVP; MW 40,000)

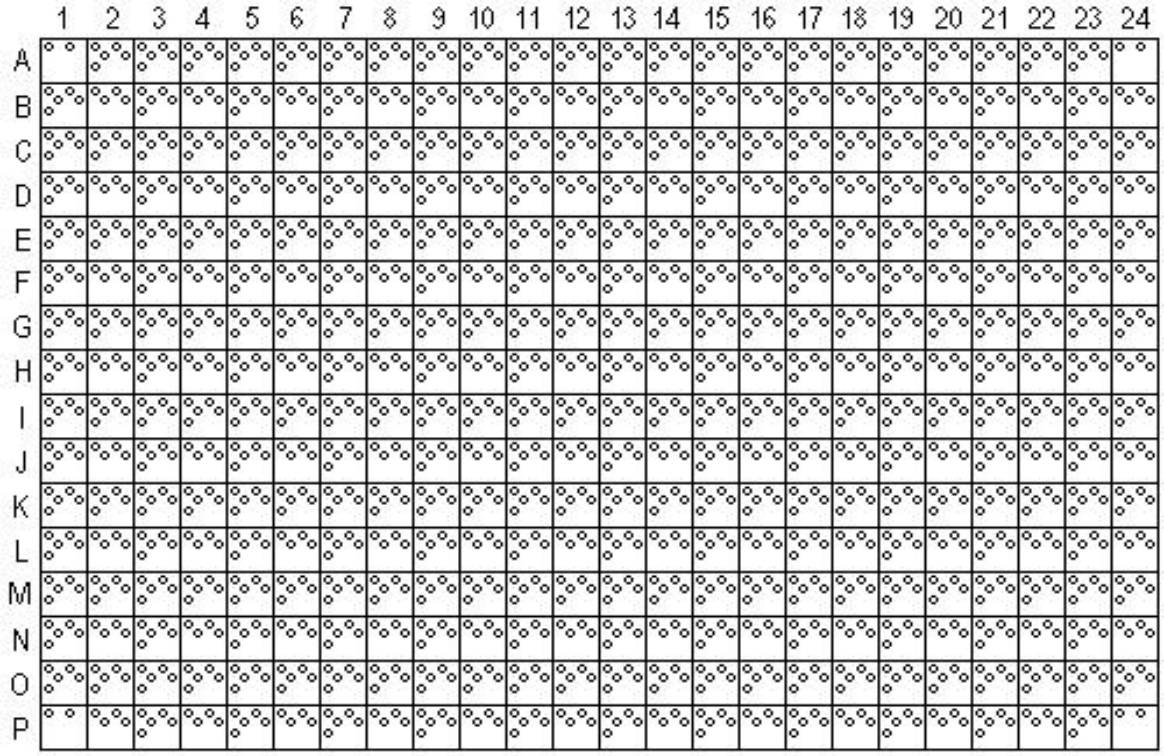
0.02% bovine serum albumin (BSA)

Appendix B - Overall Array Layout

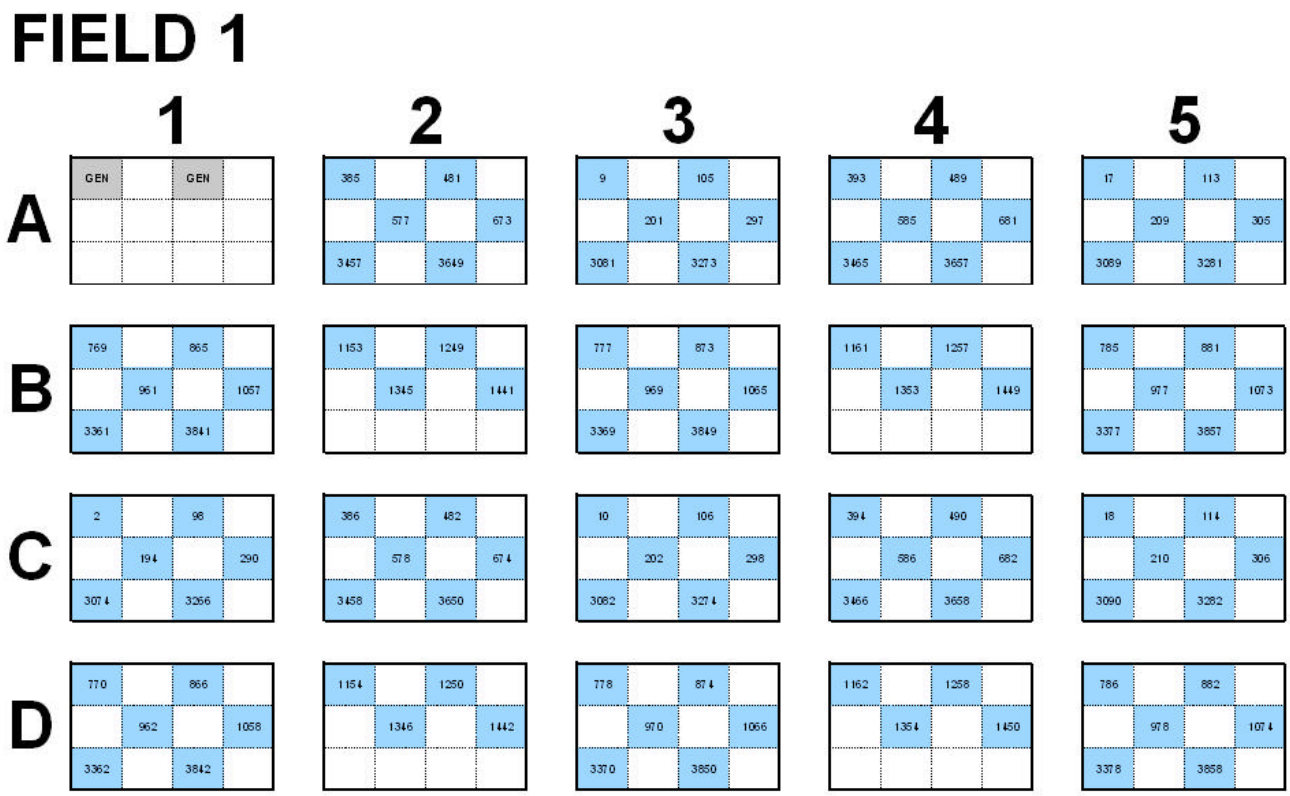
FIELD 1



FIELD 2



Appendix C - Layout of Secondary Grids



The primary grid contains 384 secondary grids. Many of the secondary grids contain 6 ORFs (6 spots per secondary grid). The four corner grids contain *M. tuberculosis* genomic DNA as a positive control (see spots labeled as “GEN” above). Note that some of the grids do not contain a full complement of spotted genes (refer to Appendix B). Each ORF has been assigned a spot number. Please refer to the accompanying spreadsheet for full details of gene information and the layout of ORFs on the array.

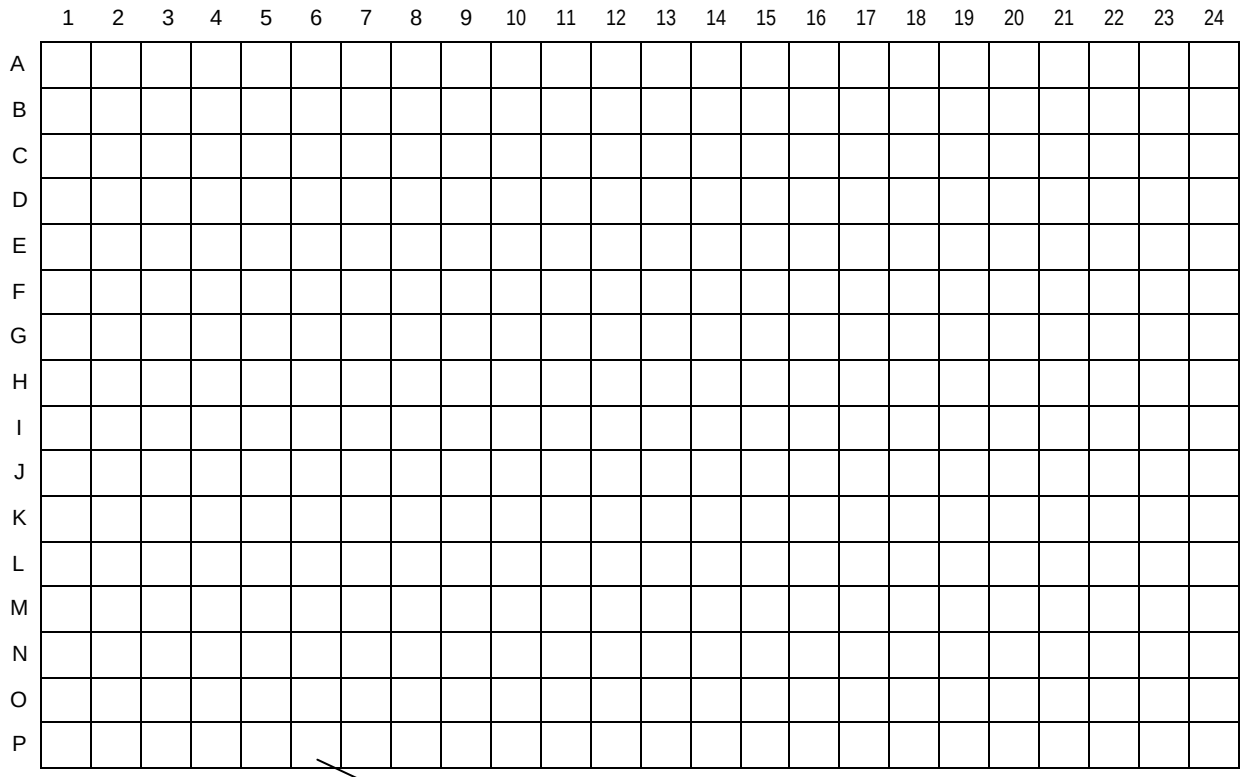
Appendix D - Determining Spot Numbers and Gene Identification

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. The analysis is facilitated by use of the accompanying spreadsheet "*M. tuberculosis* Array Information version 1.0.xls*"* (and transparency grid, if analyzing autoradiographs). Note that the spreadsheet file contains three worksheets (*Array Layout*, *Look-up Spot Info* and *Gene Information*).

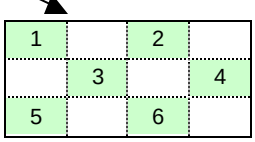
1. Locate the position of the spot on the autoradiogram or the phosphorimager image.
2. Each array contains genomic DNA spots/anchors in the four corners of the array. Each array is divided into **24 columns (1 – 24)** and **16 rows (A – P)**, in other words **384 primary grid squares.** Each of the **384 primary grid squares** contains up to **6 genes** (secondary grid, see below). Within the secondary grid, gene spot positions **1, 2** and **5, 6** appear left justified and gene spot positions **3** and **4** appear right-justified.

   NOTE: All of the Secondary grids do not have **6 gene** spots.

Primary grid:



Secondary Grid:



3. Look up the spot coordinates in the Excel spreadsheet file (see ***Array Layout*** worksheet)***.*** This will give you the spot number.
4. For example, look up the gene for Row **H**, Column **1** and spot position **5** (left-justified). On the worksheet, ***Array Layout,*** this corresponds to spot number **3364**.

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 "*M. tuberculosis* Array Information version 1.0.xls" spreadsheet 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 **3364.** All the information concerning the gene should now be displayed. Example, **Rv3467***.*

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 (E). Note that alternate names or synoyms may be found under the **Synonym** column (column F).
3. Under the *Edit* pull down menu, select *Find*. Alternatively, press Ctrl and F, simultaneously.

4. Enter the gene name in the *Find* window and hit return. For example ***Rv3467***
5. The row with the gene name will now be deselected.
6. Note down the spot number corresponding to the gene name. For example, **3364** for ***Rv3467***.

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 and F, simultaneously.
3. Enter the spot number in the *Find* window and press return. For example, enter **205**.
4. Make sure that the *"Find entire cells only"* box is checked in the "*Find*" window.
5. The position of the spot will now be highlighted. For example, **205** would be Field-1, Row **I**, Column **3**, Spot Position **3**.
6. **IMPORTANT: Use the *Zoom* function to zoom in or out on the array layout as desired.**

Appendix E - Stripping the arrays

Stripping Solution (Cat. No. S3312):

	Stock	Volume
10 mM Tris, pH 7.5/8.0	1 M	5 mL
1 mM EDTA	0.5 M	1 mL
1% SDS	20%	25 mL

Add 469 mL of H_2O to make a total volume of 500 mL.

1) In a "Tupperware" container or in a Pyrex dish covered with clear plastic food wrap, bring the stripping solution to a boil using a microwave oven (about 5 minutes at full power).

2) Add the blot to the heated solution and continue a low boil using the "defrost" setting (about 33% power) for 20 minutes.

3) Drain the excess solution and re-wrap the array in clear plastic food wrap. Expose the array to phosphorimaging plates or subject to autoradiography. The duration of the exposure should be same as for a typical experiment, e.g. overnight exposure.

4) Analyze the image by phosphorimagery (or develop the autoradiograph). Compare the signals from the stripped blot with the experimental signals obtained prior to stripping. Typically, >95% of the signal should have been stripped from the blot. If significant signals persist, then repeat the stripping procedure one more time, using fresh stripping solution.

5) If user is not proceeding directly to a new hybridization experiment, then store the array in plastic wrap at -20°C until ready to use.

Each time a blot is subjected to a stripping protocol, there will be a slight loss of bound DNA from the filter. Also, some labeled signal is likely to remain after stripping. For new experiments, it is advisable to compare signals only from similarly-treated blots. For example, do not use a fresh array for the control sample and a stripped array for the test sample.

Appendix F - 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 (as described in step 7, page 8), 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.

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