Panorama ä

B. subtilis Gene Arrays

Protocol Booklet

SIGMA®
GENOSYS

Contents

Notice to the Customer

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The PCR process is covered by patents owned by Hoffmann-La Roche, Inc.

Components

- Panorama *B. subtilis* Gene array(s) (PRBS0002)
- Protocol booklet.
- Document showing gene names associated with array spot numbers.
- Transparency to facilitate spot location within a field on autoradiographs.
- Floppy disk containing a spreadsheet as a self-extracting archive. The spreadsheet shows the layout of the arrays and is in Microsoft® Excel (for Office 97) format.

Storage of Components

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

Optional Components

Panorama *B. subtilis* cDNA Labeling and Hybridization Kit***** (Catalog No. PRLB0001):

- 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.
- *B. subtilis* 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. PRHY0001)
- AMV Reverse Transcriptase (Catalog No. PRRT0001)
- cDNA Labeling Primers - optimized for *B. subtilis* (Catalog No. GPBS0001)

Overview

Bacillus subtilis is a gram-positive bacterium, well studied from a biochemical, genetic and pathological point of view. It is an ideal model for studies of other pathological organisms and is particularly important as it has the ability to sporulate. *B. subtilis* has been used extensively for industrial scale protein production as it secretes expressed proteins at high levels. This microbe has also been used as a model for resistance to antibiotics and other drugs. There are many useful applications of *B. subtilis* whole genome gene arrays including the study of gene pathways involved in sporulation, secretion and drug resistance.

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” 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, 5) autoradiography or phosphorimaging of the arrays, and 6) analysis of the expression patterns.

Phosphorimager files allow quantification of the 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 *B. subtilis* Gene Arrays contain 4,107 PCR-amplified open reading frames (ORFs), representing all putative *B. subtilis* protein-coding genes. These arrays provide a rapid way of determining global gene expression during cellular responses to developmental cues, external stimuli, or stresses.

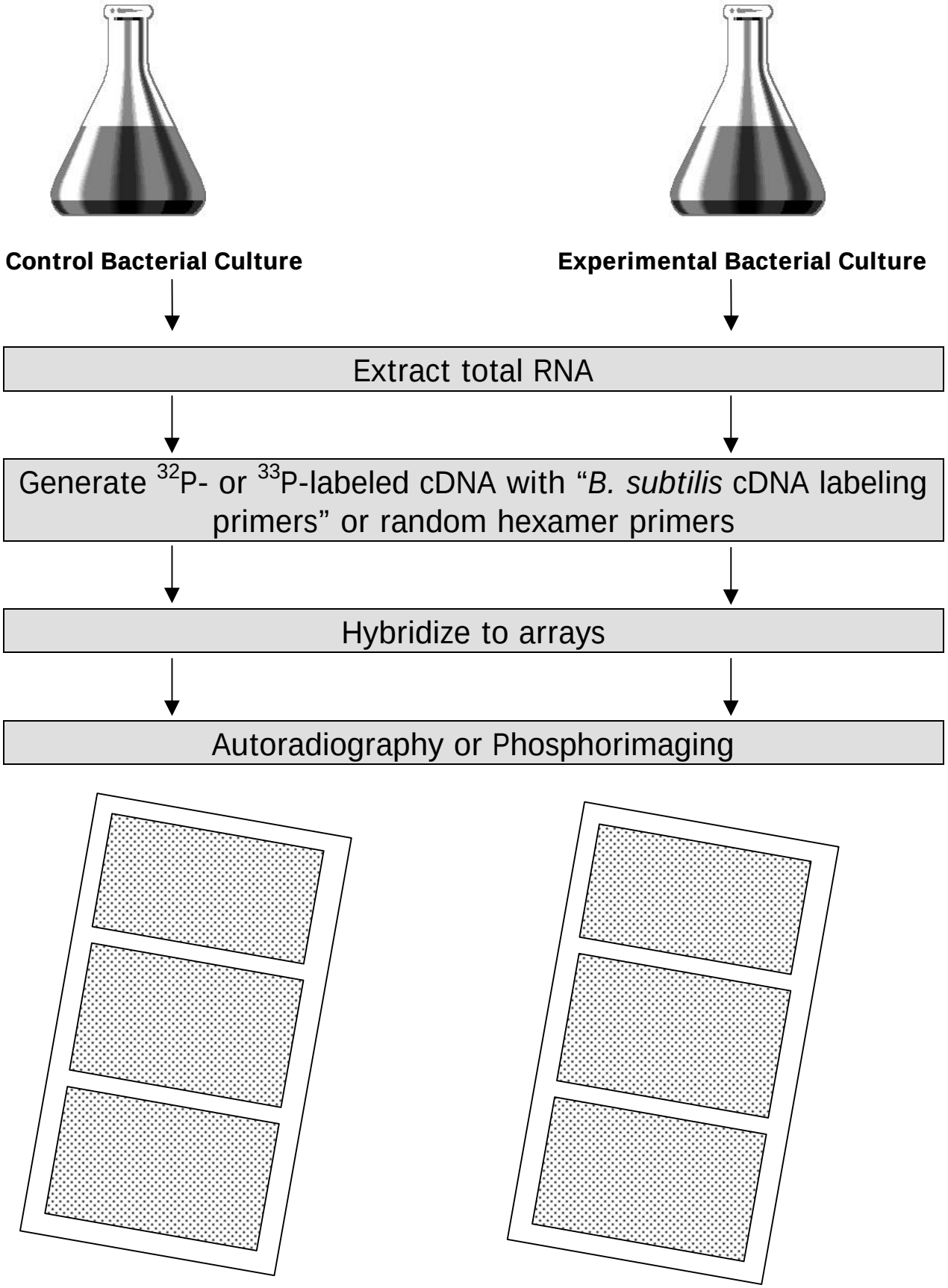


Figure 1. ***Diagrammatic representation of the expression profiling process using macroarrays.***

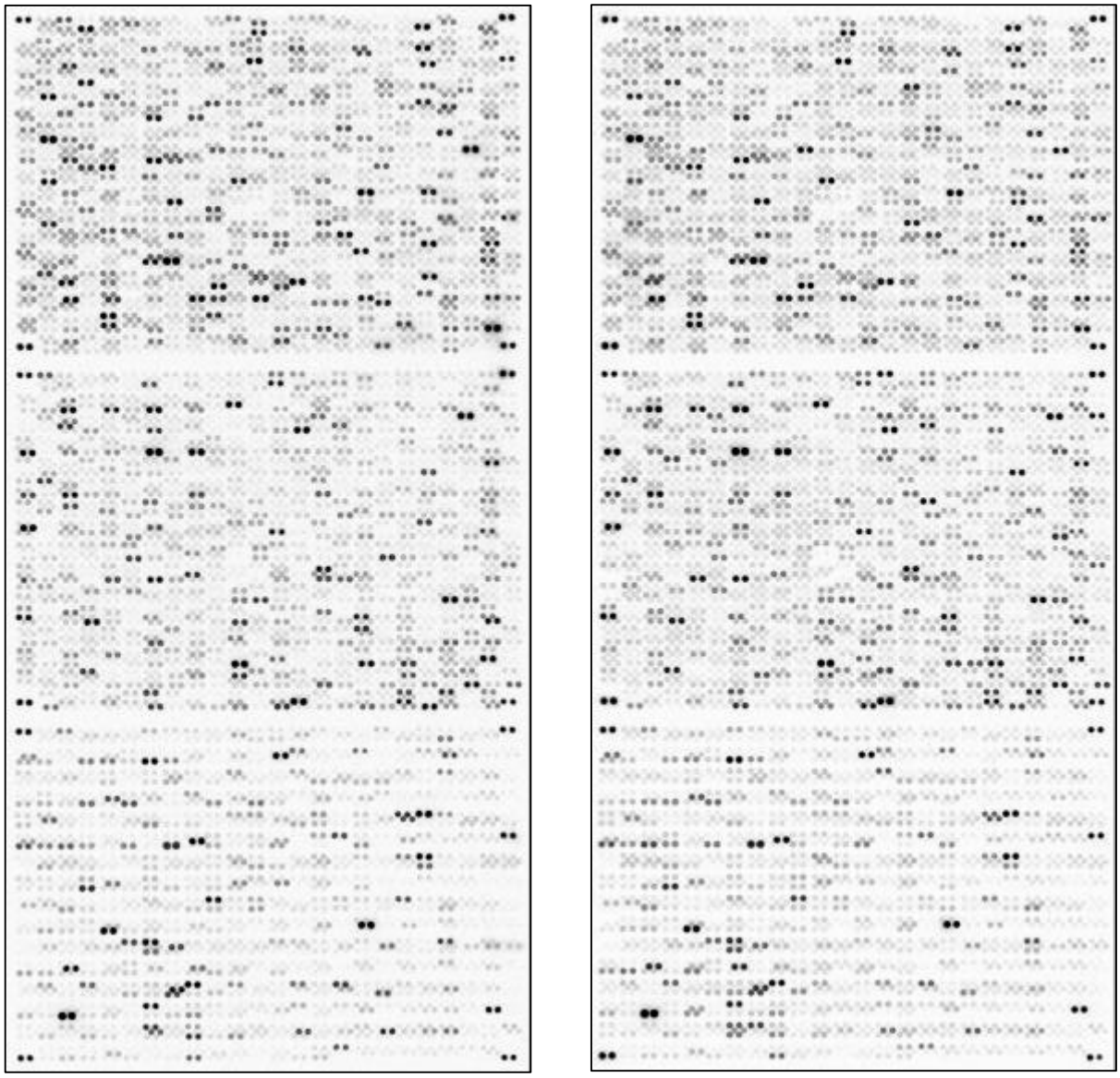


Figure 2. ***Differential gene expression during induction of the heat shock response in*** **B. subtilis.** Two Panorama *B. subtilis* Gene Arrays were probed with ^{33}P-labeled cDNA from cells grown continuously at 37^{o}C (left panel) or cells that have been exposed to heat shock at 48^{o}C, for 6 min. (right panel). The labeled cDNA was prepared using the *B. subtilis* cDNA labeling primers (Cat. No. GPBS0001). The above images were obtained by phosphorimaging following an overnight exposure to imaging plates.

Product Description

Each Panorama *B. subtilis* gene array contains PCR-amplified open reading frames (ORFs) from the *Bacillus subtilis* genome (strain 168). The majority of ORFs have been amplified in their entirety. Revisions to the original *B. subtilis* genomic DNA sequence and annotations have been made and are still ongoing. The latest updates in annotation revisions may be found on the "SubtiList World-Wide Web Server" (http://bioweb.pasteur.fr/GenoList/SubtiList/genome.cgi). The original annotation of the genome identified 4,100 ORFs. Recently, seven additional protein-coding genes have been identified. For the Panorama *B. subtilis* Gene Array, all of

the 4,107 ORFs have been printed in duplicate at 5 ng per spot 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 12 cm x 24 cm in size. Each array is divided into three fields. Each field is subdivided into smaller sections of spots. Each of the three fields consists of a primary grid containing secondary grids 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 four genes spotted in duplicate. Hence, there is a total of 8 spots per secondary grid (see the Appendices B and C for representations of the arrays). Note that the secondary grids in “Field 3” do not contain the full complement of genes spotted in duplicate. The four corners of the primary grid, A1, A24, P1 and P24 for each field, contain *B. subtilis* genomic DNA. 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 strong signals. The genomic DNA spots can be used to (a) orient the corners of the fields of the array, (b) anchor image analysis templates after phosphorimaging, and (c) normalize signals between replicate arrays.

A Microsoft® Excel spreadsheet accompanies the arrays. The spreadsheet shows the spot numbers within each of the three fields of the array. The spreadsheet can be used to determine the ORF associated with each spot (or *vice-versa*).

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 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 *Bacillus* cell walls are hard to break open, a cell disruptor may be helpful in obtaining high yields of RNA. The protocol described here does not make use of any type of cell disruptor and therefore, the yield of RNA will 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 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.
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. 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.
4. Incubate the cells on ice for 10 minutes.
5. Add 500 µl *Lysis Buffer*, mix thoroughly using a vortex.
6. 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.

7. Transfer the tube to a heat-block at 65°C and incubate for 3 minutes.
8. 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).
9. Cool the sample on ice for 3 minutes.
10. 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.
11. Repeat the phenol extraction two more times (steps 8 to 10). 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.
12. 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 2 minutes.
13. 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 2 minutes.
14. 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.
15. Divide the aqueous phase equally between two fresh tubes and add 0.1x volumes 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.
16. 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 which may be barely visible.

17. 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.
18. 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.
19. When the RNA pellet appears clear or translucent, dissolve the RNA in 50 – 100 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).
20. 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.
21. 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.

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 *B. subtilis* (Sigma-Genosys Cat. No. GPBS0001 or Kit Cat. No. PRLB0001). 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 *B. subtilis* 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 *B. subtilis* cDNA Labeling Primers

The cDNA labeling reactions are performed in two steps. In the first step, the *B. subtilis* 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 (PRLH0001, Panorama cDNA Labeling and Hybridization Kit).

Table 1.

Final Concentration	Stock Reagent	Volume for one reaction
1 µg total RNA		X µl
B. subtilis cDNA Labeling Primers, reconstituted with 40µl sterile, distilled water (Sigma-Genosys, GPBS0001)		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 *B. subtilis* 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 "*B. subtilis* 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 15 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 >50%. Alternatively, an aliquot of the sample before and after the column purification can be counted in a scintillation counter to determine percent incorporation.

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 (Sigma-Genosys Cat. No. PRHY0001 or Kit Cat. No. PRLB0001) to 65°C for about 10 minutes prior to use. Note that the contents of the Hybridization Solution (PRHY0001) 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 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.

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. 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 2 – 3 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. 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, 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 LE177-940, 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. 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 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 1 – 2 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. Autoradiographic images typically show spots with sharper edge boundaries and provide a qualitative "back-up" to phosphorimaging files.

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 *B. subtilis* Gene Arrays. Sigma-Genosys sells and uses ArrayVision software (developed by Imaging Research, Inc.) which can analyze all spots in one field on the *B. subtilis* array at one time. 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. Determine the average signal (pixel intensity) of the pair of duplicate spots representing each gene.
5. Normalize relative signals from different arrays by representing the "averaged" spot signal as a percentage of the total signal from all spots on the array. Alternatively,

represent each gene's "averaged" signal as a percentage of the averaged signal from all of the positive control genomic DNA spots.

6. Compare normalized signals of corresponding spots from different samples on different arrays to determine fold-induction or fold reduction in expression between samples.

Appendices

Appendix A - Composition of Solutions

RESUSPENSION BUFFER

0.3 M sucrose

10 mM sodium acetate, pH 4.2

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

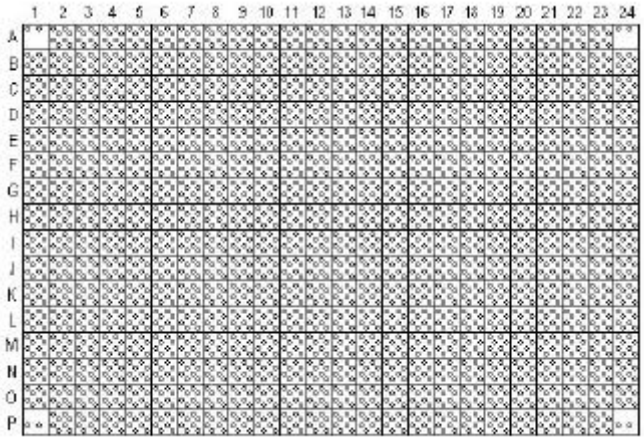
0.02% Ficoll (MW 400,000)

0.02% polyvinylpyrrolidone (PVP; MW 40,000)

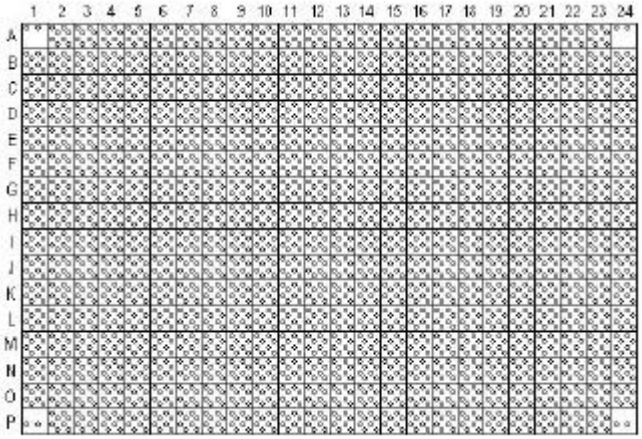
0.02% bovine serum albumin (BSA)

Appendix B - Overall Array Layout

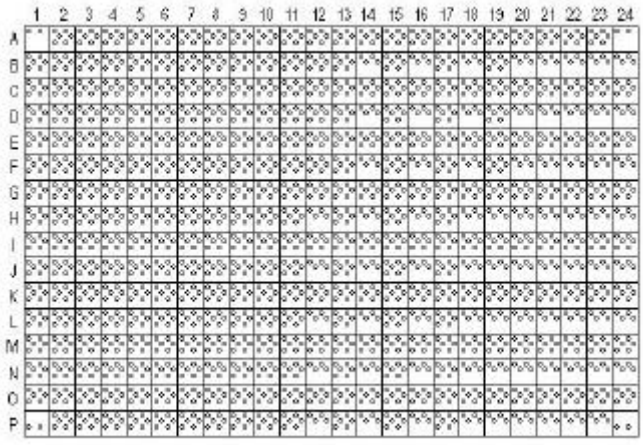
Field 1



Field 2

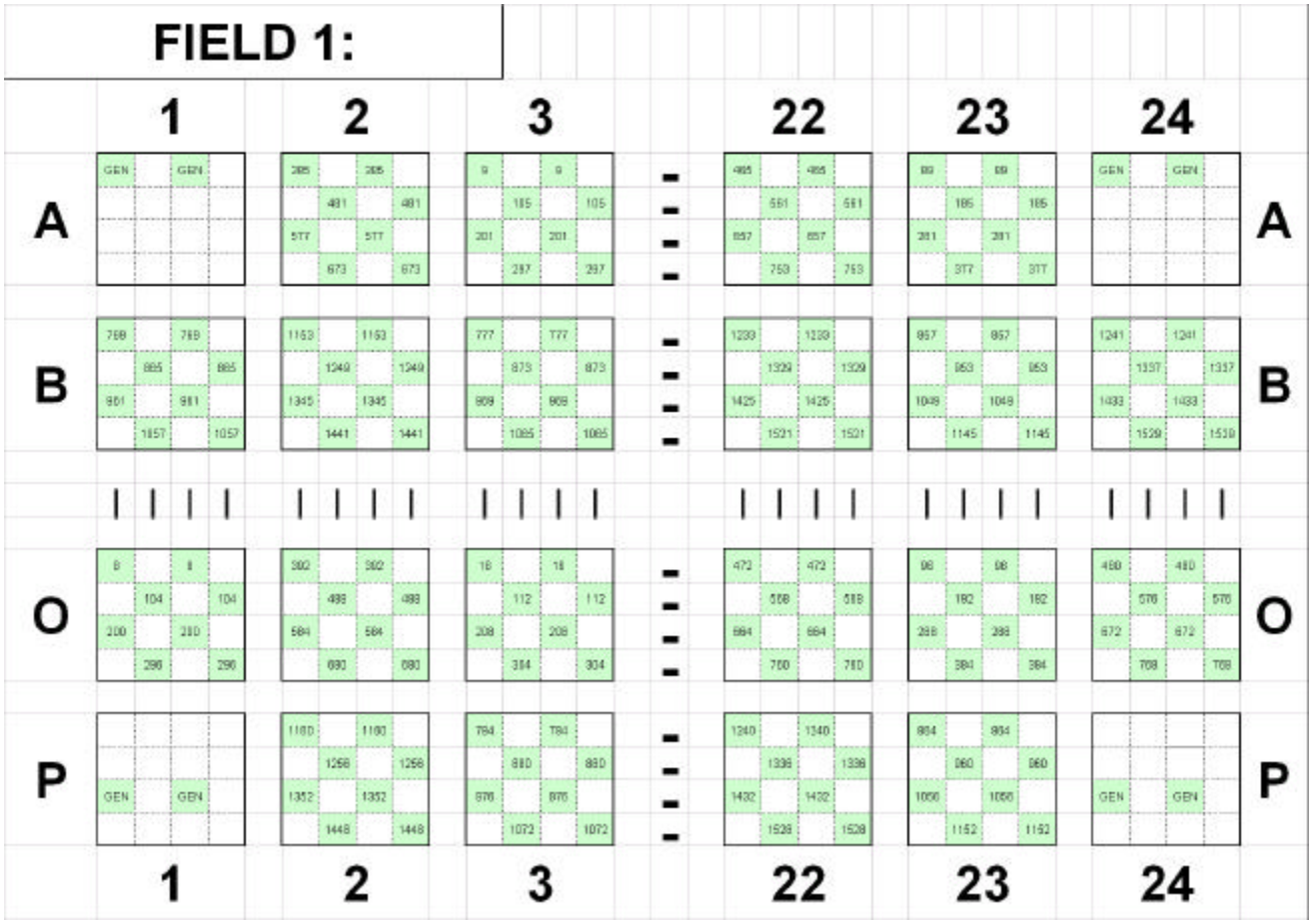


Field 3



Each *B. subtilis* array consists of three fields of ORFs. Each field is divided into a 24 x 16 Primary Grid.

Appendix C - Layout of Secondary Grids



Each primary grid contains 384 secondary grids. Most of the secondary grids contain 4 ORFs arrayed in duplicate (8 spots per secondary grid). For each of the three fields, the four corner grids contain *B. subtilis* genomic DNA as a positive control (see labeled G1 and G2 above). Note that in the third field, some of the grids do not contain a full complement of spotted genes (refer to Appendix B).

Appendix D - Determining Spot Numbers and Gene Identification

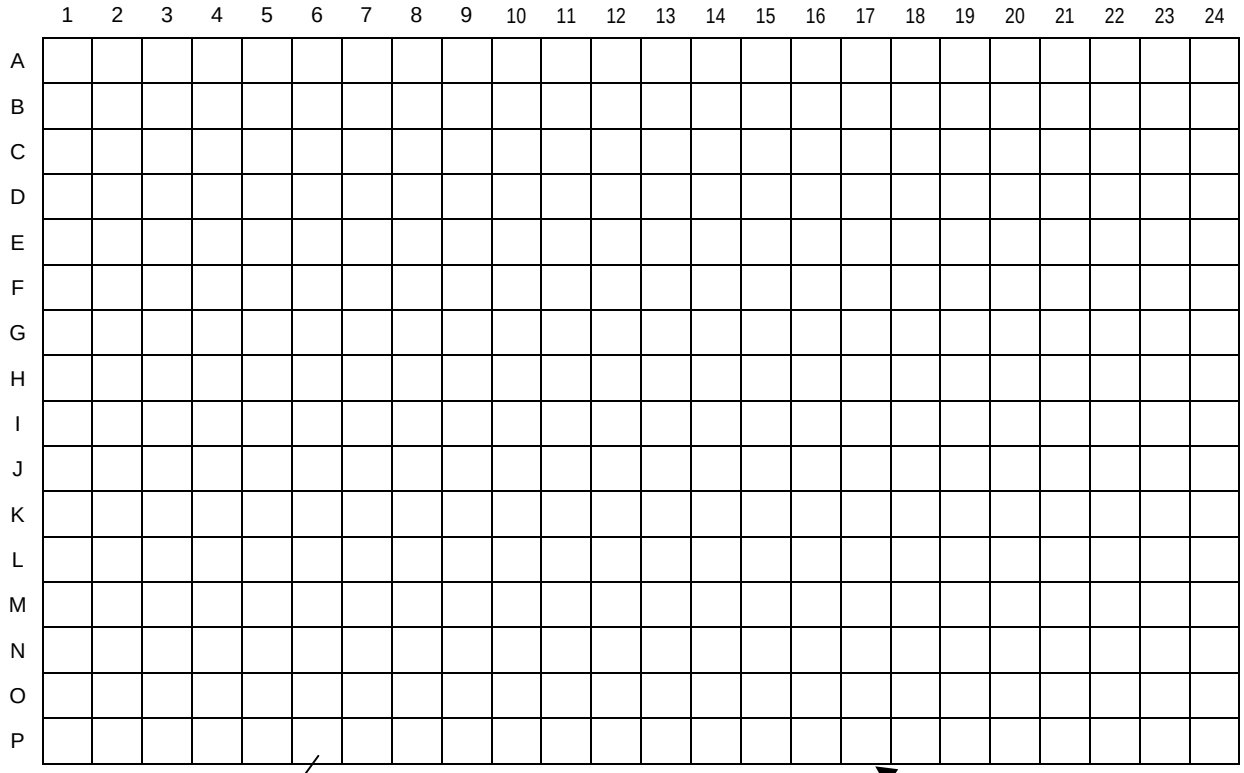
Analysis of the arrays may appear to be intimidating. The following is a guide to help determine gene "spot" numbers and the gene information associated with that spot. The analysis is facilitated by use of the accompanying spreadsheet "*B. subtilis* Array Information.xls" (and transparency grid, if analyzing autoradiographs).

1. Locate the position of the spot on the autoradiogram or the phosphorimager image.
2. Each array is divided into **3 fields** with genomic spots/anchors in the four corners of 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.

Primary grid:



Secondary Grid:

1		**1**	
	2		**2**
3		**3**	
	4		**4**

3. Look up the spot coordinates in the Excel Worksheet ***(ARRAY ORDER).*** 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 ORDER)*** you can see that this corresponds to spot number **1060**.

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. 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.
2. Type in the spot number in the space that says *"Enter spot number here"* and hit the return key. For example, type **1060.**
3. All the information concerning the gene should now be displayed. Example, *spsE.*

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 (F). Note that alternate gene names may be found under the **Gene Description** column (column G) or under the **New Name** column (column H).
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 *dnaK*.
5. The row with the gene name will now be deselected.
6. Note down the spot number corresponding to the gene name. For example, **2010** for *dnaK.* 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 press return. For example, enter **2010**.
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 **2010** would be Field **2**, Row **C**, Column **24**, Spot Position **1**.
6. IMPORTANT: Use the ***Zoom*** function to zoom in or out on the array layout as desired.

Appendix E - Stripping the arrays

Stripping Solution:

	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 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.

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