Schleicher & Schuell
APPLICATIONS NOTES
FOR BIOMOLECULAR SCREENING

Methods for Preparing PCR Based Arrays on Nytran® N and SuPerCharge Nylon Membranes.

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Introduction to Array Technology

The use of Northern blots for performing gene expression analysis has been a powerful tool in research laboratories since its inception in 1977 (1). It has allowed researchers to answer questions about the expression of a specific RNA species; such as in which cell or tissue is the gene expressed, how much transcript is present, and what conditions or events influence its expression. Information gained from such studies has helped elucidate the mechanisms of several disease processes and has served as a predictor of disease progression. However, as powerful a technique as it is, the labor-intensive nature and time-demands of the traditional Northern blot limit what the researcher can learn about the response and interaction of multiple genes in an experimental sample.

Presently, science is in the midst of a "genomic revolution" that is rapidly changing the way scientists think about and study genes (2, 3, 4). Large-scale sequencing and the application of expressed sequence tag (EST) technology has led to the identification of hundreds of thousands of genomic sequences from various microbes to multicellular eukaryotes and plants (5). Expression analysis of each known gene or EST using traditional Northern analysis would be an enormous challenge. Clearly, a technique that allowed for the simultaneous analysis of thousands of genes would tremendously expedite this process. The solution has been a mixture of old and new technology; applying traditional molecular techniques to cutting-edge array technology.

DNA array technology enables the simultaneous expression analysis of hundreds to thousands of genes (6, 7, 8). Genes of interest are typically isolated as purified cDNA clones or as PCR amplicons and placed into 96 or 384 well microtitre plates (source plate). Arrays can then be constructed with the aid of either a hand-held or robotically controlled arraying pin tool. The pin tool transfers nanoliter volumes of purified cDNA or PCR products from the source plate to a solid support surface. Each spot in the array has a specific address and represents a distinct gene or EST. Surfaces such as nitrocellulose or nylon are ideal for arrays since nucleic acid can be easily immobilized, hybridized, and detected using standard molecular techniques. The arrays, when complete, can be used immediately as a tool to analyze the expression patterns of a defined set of genes in a cell or tissue type (7). These arrays can also be used for detecting single-nucleotide polymorphisms (SNP) as well as sequence polymorphisms resulting from insertions/deletions of two or more bases (9, 10).

To perform a differential gene expression application, total cell RNA is isolated from both control (normal) and experimental (biologically stressed, toxic or drug compound treated, etc.) tissues. Reverse transcriptase is used to make cDNA copies of the isolated RNA to produce a cDNA probe that is more stable without altering the relative concentrations of RNA. In addition, radioactive nucleotides, chemiluminescent, or fluorescent reporter molecules can be inserted into the cDNA during synthesis to provide probes for array hybridization. The cDNA probes from the two tissues are hybridized to an arrayed membrane and subsequently detected using the appropriate detection systems. When individual genes display differences in reporter intensity between the control and experimental tissues, it reflects differential expression of that gene; greater intensity in experimental tissue indicates that the gene is up-regulated, lower intensity indicates down regulation. While array technology provides valuable yes/no information about gene expression, quantitative information extracted from the arrays should be viewed with caution and requires careful confirmation via Northern analysis and/or qRT-PCR techniques such as TaqMan® (Applied BioSystems-PE).

The popularity and sheer power of array technology is rapidly making it a necessary laboratory tool for all those involved in any type of gene discovery

and analysis. As the pool of scientists utilizing this technology grows, the need is rapidly burgeoning for a set of guidelines to steer the researcher though the basics of array construction. There are several types of robotic systems available for spotting molecules onto membrane surfaces. How these robots perform depend on many factors. The volume of liquid transferred from the source plate to the surface depends in large part on the type of pin tool being used by the robot, the chemistry of the solution, and the condition of the membrane surface. How large or small can a PCR fragment or oligonucleotide be for efficient spotting and immobilization? Should the nucleic acid be denatured before or after spotting and what are the recommended methods? This technical applications note was designed to answer many of the questions posed above and to provide some basic guidelines for arraying onto Schleicher & Schuell Nytran nylon membranes using a BioRobotics (Cambridge, U.K.) Total Array System (TAS™).

Robotic Arrayer and Materials

All of the information presented in this note was generated with the aid of a BioRobotics BioGrid 600 arraying robot. The gridding tool contains 384, free-floating, independent 0.7mm solid pins. The nylon membranes used for the arrays were Schleicher & Schuell 0.45µm Nytran SuPerCharge (SPC) and 0.45µm & 0.2µm Nytran N. The ß-globin, vimentin, and α-tubulin PCR products were amplified from human K562 high molecular weight DNA (GIBCO BRL), and the pUC amplicon was from pUC19. The size of each amplicon is detailed in Table 1.

Table 1. Target DNA/PCR amplicons

Template	Amplicon Designation	Primer Ln; source	Amplicon size
pUC 19	pUC	20 mers; Genosys	116 bp
K562	ß-globin	20 mers; Perkin Elmer	268 bp
K562	α-tubulin	26 mers; CLONTECH	527 bp
K562	Vimentin	20 mers; Genosys	1.3 kbp

K562: high molecular weight DNA from K562 cell line. GIBCO BRL

Table 2.

Dispensed Solution	Calculated Volume Dispensed; nl/0.7mm pin	Amt of DNA spotted if source plate [DNA] = 100 ng/µl; ng
Water	62 ± 2 (n=4*)	6.2
6X SSC (0.9M NaCl/90 mM Na Citrate)	50 ± 0 (n=2)	5
150 mN NaOH/5X SSC	53 ± 7 (n=2)	5.3
150 mN NaOH/5X SSC/10 % glycerol	51 ± 0 (n=2)	5.1
0.4N NaOH	45 ± 3 (n=2)	4.5
0.4 N NaOH/0.5 M NaCl	40 ± 2 (n=2)	4
0.4 N NaOH/1.5 M NaCl	41 ± 3 (n= 4)	4.1
0.4 N NaOH/1.5 M NaCl/10 % glycerol	39 ± 0 (n=2)	3.9

* = number of experiments; each experiment had 6-8 wells/solution.

Methods and Results

Pin Volume Determination

When preparing an array for expression analysis, it is essential that the PCR product is on the membrane in excess. For PCR amplicons between 350 - 700 base pairs spotted with a 0.7mm solid pin, this translates into spots approximately 700 - 750µm in diameter containing 1 - 5 ng of DNA. Since the concentration of the PCR amplicon in the source plate is easily controlled, it is essential to know how much fluid is actually being transferred to the membrane. The solid pins used in this study rely on passive transfer to move PCR products from the source plate to the membrane. The amount of fluid transferred can be influenced by several conditions including humidity, chemistry of the solution, and surface energies of the membrane. Table 2 shows the results of several experiments designed to determine the per-spot-volume of fluid transferred by a 0.7mm solid pin and the influence of solution chemistry.

To perform this experiment, a known volume of each solution (from table 2) was dispensed into 8 different wells of a 384 well Nunc source plate. The solutions were gridded robotically onto 0.45µm Nytran SPC membrane using a 0.7mm pin tool from BioRobotics. Specifically, the solutions were arrayed in a 4x4 double offset configuration onto four, 22cm x 22cm membranes. The pins were dipped into the solution and transferred to the membrane as 1 pin strike per application per spot. After 384 individual applications, the volume remaining in the well was measured. The calculated pin volume was determined as: (starting volume - remaining volume) ÷ 384 = volume

delivered per pin per transfer. The data in the far right hand column of Table 2 gives a close approximation of the concentration of DNA transferred to the membrane surface as a function of source plate chemistry. In general, increasing the ionic strength and pH of the solution results in less volume transferred to the membrane surface.

Pre-grid and Post-grid Denaturing Methods

There are many factors that need to be considered when making decisions about array preparation and processing. Detailed below are two protocols that have worked extremely well with Nytran nylon membranes. The first protocol calls for denaturing the DNA prior to spotting onto the membrane, while the second spots duplex DNA onto the membrane followed by a denaturation step.

A. Pre-Grid Protocol: The procedure that we recommend for pre-grid denaturation was adapted from Jennifer Ng at the Thirteenth International Histocompatibility Workshop (11). In this procedure, 100µg/ml of each PCR amplicon is dispensed into the appropriate wells of a microtitre plate (source plate). Freshly prepared 5X denaturation solution (2N NaOH/50 mM EDTA) is placed in a separate container. Both the source plate and denaturation solution are placed into a 37°C incubator for 30 minutes. Upon completion of the incubation period, denaturation solution is added to the wells of the microtitre source plate so that the final concentration of denaturation solution is 1X (0.4 N NaOH/10 mM EDTA). The plate is incubated for an additional 30 minutes at room temperature to complete the denaturation process. The data displayed in this application note was generated using the above procedure; however, we have been able to generate comparable arrays by omitting the pre-incubation step. We recommend that each procedure be evaluated under arraying conditions to determine which variation, pre-incubation or not, provides the optimal results.

The denatured amplicon is spotted onto Nytran nylon membranes and immobilized by UV crosslinking at 120 mjoules/cm^2 using a Stratalinker 1800 (Stratagene). This method spots approximately 4.5 nanograms of PCR amplicon per pin strike when using a 0.7mm diameter solid pin. It's important to note that 2N NaOH should be freshly prepared from NaOH pellets. The percentage of DNA that is denatured decreases when "old" (greater than 2 weeks) NaOH is used to prepare the denaturation solution.

Figure 1.

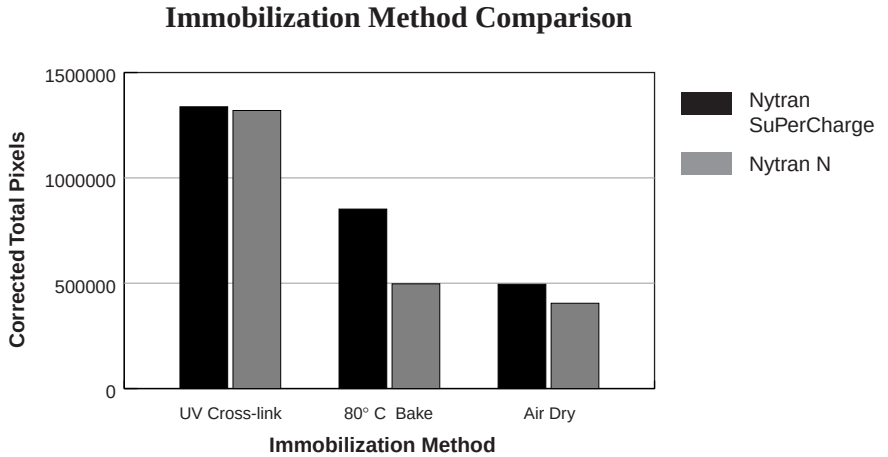

Figure 1: *A comparison of methods for immobilizing DNA in arrays onto Nytran nylon membranes using UV cross-linking, baking at 80° C, and air-drying.*

Figure 2.

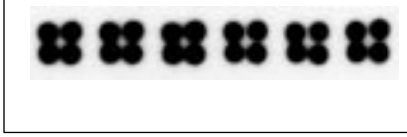

Figure 2: *An array spotted as a series of 2x2 grids. This set of arrays represents ß-globin amplicons spotted with 0.7 mm solid pin from BioRobotics and hybridized to a complementary probe labeled with psoralen-biotin.*

B. Post-Grid Protocol: The post-grid denaturation protocol calls for placing 100µg/ml of PCR amplicon, diluted in TE, into the appropriate wells of a microtitre plate (source plate). The PCR amplicons are spotted (arrayed) onto Nytran nylon membrane, after which they are denatured by incubating the membrane on S&S GB004 paper soaked with 0.4N NaOH/3M NaCl for 5 minutes, followed by neutralization on paper soaked with 6X SSC for 5 minutes. As an alternative to the paper method, the process can also be carried out by dipping the arrayed membranes directly into containers with denaturant and neutralization solutions. The arrayed DNA is immobilized via UV crosslinking at 120 mjoules/cm^2 using a Stratalinker 1800 (Stratagene). With this method, a single pin strike with a 0.7mm diameter solid pin yields PCR amplicon spots of approximately 6.0 nanograms when the DNA is in a solution of TE.

Immobilization Method Comparison

UV crosslinking, baking at 80°C, and air-drying are all methods for attaching DNA to nylon membranes. When comparing these methods under the same arraying and detection conditions, it was determined that the optimal method for immobilizing DNA to Nytran nylon membranes is through UV crosslinking (Figure 1). The data in Figure 1 was generated as follows:

1. PCR amplicons were spotted immediately after heat denaturing using a 0.7mm pin tool. They were spotted onto 0.2µm Nytran N and 0.45µm Nytran SuPerCharge nylon membranes in a series of 2 x 2 double offset arrays (Figure 2).

2. The membranes were cut into three sections in order to compare three immobilization methods. One section was air dried for 3 hours, another section was baked for 30 minutes at 80°C, and the third section was UV cross-linked, 120 mjoules/cm^2, using a Stratalinker 1800 (Stratagene).

3. The membranes were prehybridized for one hour at 42°C (50% formamide, 5X SSC, 5% casein, 1% SDS) followed by hybridization overnight at 42°C with 15 ng/ml of probe. The probe represented a single, psoralen-biotin labeled amplicon (ß-globin), or a mixture of all four, psoralen-biotin labeled amplicons (pUC, ß-globin, α-tubulin, and vimentin; each at 15 ng/ml). The psoralen-biotin labeled amplicons were purified by QIAquick™ 96 well PCR purification-plate (Qiagen), and then labeled using S&S's psoralen biotin procedure (12).

4. Membranes were washed 2 times in 2X SSC/0.1 % SDS, for 5 minutes each, at room temperature, followed by 2 times in 0.1X SSC/0.1 % SDS, for 5 minutes each, at 68°C.

5. Membranes were blocked for 1 hour in 1X TBS/3 % casein/ 0.1 % SDS at room temperature.

6. Incubated for 1 hour at room temperature in blocking solution containing S&S's streptavidin-alkaline phosphatase conjugate at the suggested 1:1650 dilution.

7. Free conjugate was removed by washing 3 times in 1X TBS/0.1 % SDS, for 10 minutes each, at room temperature and then 1 time in 1X TBS for 5 minutes at room temperature. Excess buffer was removed from the membranes, and then CDP Star® (Tropix) substrate was added for 5 minutes. Excess substrate was removed, and the membranes were sealed in a reaction folder. The reaction folder was placed in a cassette, and exposures were taken at 37°C and room temperature using Kodak BioMax film. Different exposures were collected to insure that all data collected and digitized was within the linear range of the film.

8. The film was scanned with a Mustek 600 III EP Plus flat bed scanner. Images were acquired with PhotoMagic (Micrografx Inc.), digitized by Un-Scan-It (Silk Scientific Corp.) and analyzed using Prism (GraphPad Software).

Figure 3.

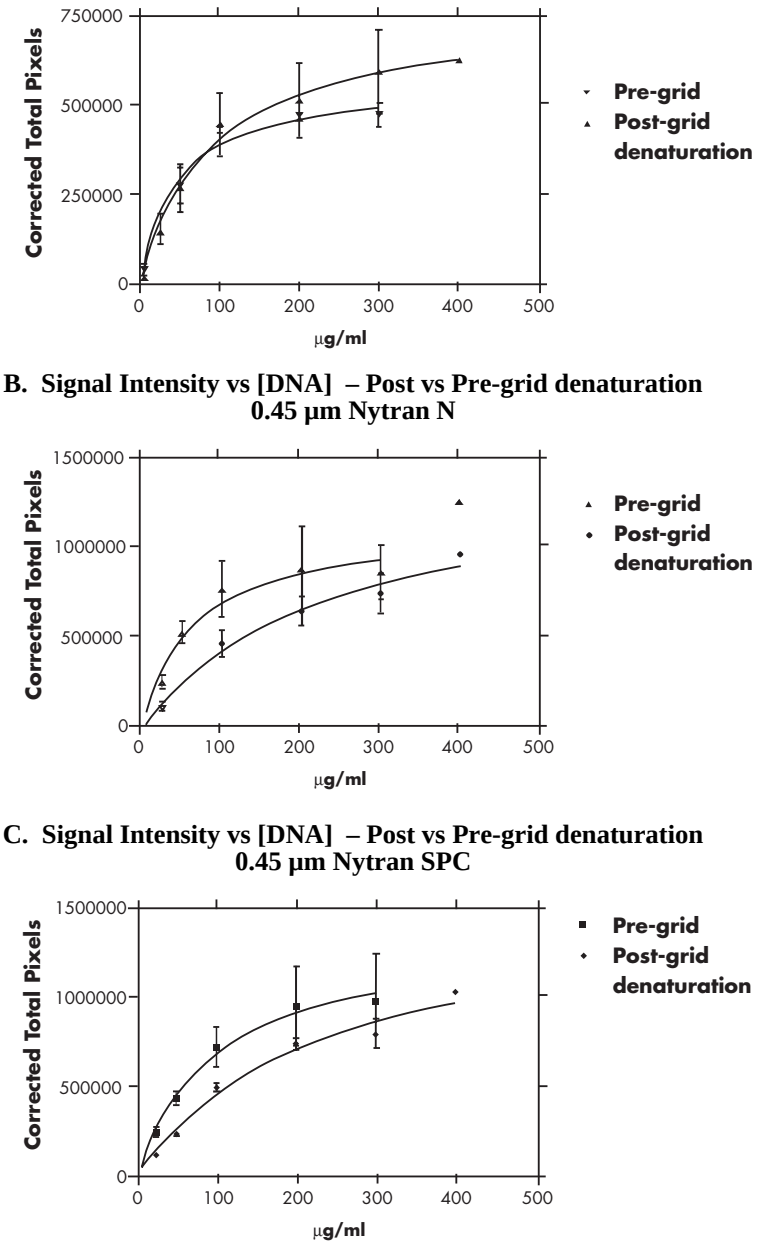

Figure 3: *Compares source plate DNA concentration to hybridization signal intensity for both Post and Pre-grid denaturation procedures.*

The graph in figure 1 was generated using the pUC amplicon data and is representative of data collected for the other 3 amplicons. The results show that the optimal immobilization method for both Nytran SuPerCharge and N membranes is UV crosslinking (Figure 1.)

Effect of DNA Concentration on Hybridization Efficiency

Optimal detection characteristics can be influenced, in part, by the concentration of DNA on the membrane. Either low or high concentrations of DNA can give rise to poor detection signal. When too much DNA is gridded onto the membrane, hybridization efficiency is reduced due to steric interference of "DNA on top of DNA". Using both pre-grid and post grid denaturation procedures, we determined the optimal concentration of DNA that should be present in the source plate. The experiments were performed exactly as described above, except that the source plate contained a titration series of PCR amplicons ranging from 5 to 400µg/ml (Figure 3a-c). The data shows that the optimal concentration for source plate PCR amplicons is 100 µg/ml. This concentration provides the best balance between signal intensity and amplicon utilization.

An additional experiment was performed to evaluate the affect of multiple pin strikes on signal intensity. Using a titration series ranging from 0.5 to 500µg/ml, PCR amplicon (pUC amplicon) was spotted onto each of three membranes with 1, 2, 5, or 10 strikes. A strike is defined as the number of times that the pin tool dips into the source plate and transfers fluid to a single spot on the membrane. Figure 4 shows the effect of multiple pin strikes at different DNA concentrations in the source plate. The data suggests that multiple pin strikes do not lead to an additive increase in signal intensity, but that optimizing the source plate DNA concentrations together with a single pin strike provide the most efficient method of spotting arrays.

Figure 4.

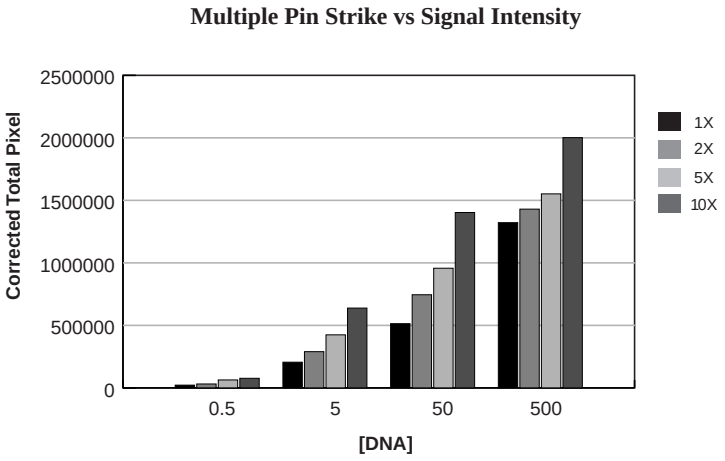

Figure 4: *The effect of increasing pin strikes vs. source plate DNA concentration. A pin strike is defined as a single transfer of DNA from the source plate to a spot on the membrane surface. Multiple pin strikes involve multiple transfers from the same well in the source plate to the same spot on the membranes. Pin strikes of 1, 2, 5, and 10 were evaluated for DNA concentrations of 0.5, 5, 50, and 500 µg/ml.*

Discussion

Schleicher & Schuell recommends two protocols for building arrays on Nytran nylon membranes. Pre-grid denaturation allows for denatured DNA to be placed directly onto the membrane obviating the need for any post-grid processing. Post-grid denaturation requires that DNA be spotted onto the membrane followed by denaturation. There are several factors that must be considered when deciding which method to use. Pre-grid denaturation requires the disposal of valuable source plate PCR amplicons after the array (s) has been completed. Denatured DNA remaining in the source plate cannot be re-used and should be discarded since DNA stored under basic, denaturing conditions will deteriorate over time. Post-grid denaturation does not waste valuable PCR amplicons, but it does require extra time and effort to denature and process the filter. Both methods are especially robust and prepare arrays that are reproducible with similar hybridization and signal intensities regardless of amplicon size, i.e., amplicons between 116 and 1,300 base pairs behaved similarly.

We have also evaluated other array methods in addition to our recommended procedures. Heat denaturing the amplicon prior to arraying works quite well if the array can be spotted immediately. Otherwise, as a function of time, "snapback" or re-annealing of the DNA prior to spotting can occur. This phenomenon reduces the amount of single-stranded DNA available for hybridization. With this procedure, there is no PCR amplicon waste nor is there any post denaturation process, but spotting must be completed within 30 minutes of denaturation.

Other denaturation methods include a variety of chemical denaturation procedures. In general, those that use NaOH to denature DNA have produced decent arrays as long as the NaOH used is freshly prepared. Our recommended pre-grid chemical denaturation protocol also requires the use of NaOH. Although we recommend the 30 minute 37°C pre-incubation step, we have recently shown that this protocol can be successfully executed without the pre-incubation step with PCR amplicons between 113 and 1,300 base pairs in length. In our hands, the pre-incubation step has been a highly reproducible procedure providing excellent denaturation of source plate DNA, since heat in combination with base produces a more uniform and complete denaturation than either heat or base by itself.

DNA concentration in the source plate is another important consideration when planning an array. Clearly, DNA concentrations below 100µg/ml in the source plate will lead to weaker signal intensities. Those greater than 100µg/ml don't produce enough of an increase in signal intensity to justify the extra effort and cost required for concentrating the DNA. In fact, very high DNA concentrations of 400µg/ml and above tend to produce decreases in signal intensity. As DNA concentrations increase without an increase in spot size, the DNA begins to layer on top of itself. This decreases the percentage of DNA accessible to hybridization. The data in Figure 4 agrees with this explanation since multiple pin strikes on the same spot placing DNA on top of DNA fail to produce the expected additive signal intensity increases. It's also possible that multiple pin-strikes at the same spot on the membrane may produce enough damage to decrease the hybridization efficiency, thus lowering signal intensity.

In conclusion, PCR amplicons and/or cDNAs can be spotted in arrays on Nytran N or SuPerCharge nylon membranes and detected using chemiluminescent or radioactively labeled probes. Both the pre-grid or post-grid denaturation procedures provide robust methods for array preparation. The source plate DNA concentration and solution chemistry will determine the amount of DNA in each array spot. The optimal DNA source plate concentration should be determined for each application and pin type. When using BioRobotics 0.7mm (or 0.4mm) solid pin tools, we recommend a source plate DNA concentration of 100µg/ml and a single pin strike. UV light provides optimal DNA immobilization to the membrane surface.

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