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Robust and Sensitive Nylon Hybridization Membrane Suitable for High-Throughput Robotic Arraying Applications

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ABSTRACT

An important aspect of automated macroarraying is the suitability of the nylon membrane selected on which samples are to be arrayed. PerForma is a positively charged nylon membrane that has been developed specifically for automated macroarraying. Tests using fluorescent hybridization detection methods have shown that immobilized DNA amounts as low as 0.25 pg can be detected and that positive signals are obtainable after 21 stripping cycles. This report describes the improved colony growth, improved handling characteristics, increased hybridization detection sensitivity, and increased stripping and reprobing capability obtained using PerForma.

INTRODUCTION

Analysis of gene libraries through hybridization can yield various types of valuable information—from searching for particular DNA sequences within a library to the characterization of gene expression profiles (5,7). The last decade has seen rapid advances in methods for constructing and analyzing gene libraries (2). A major influence has been the automation of labor-intensive steps (3). The advantages of automated macroarraying offers over older colony lift techniques for gene library analysis are the high-density array of bacterial colonies produced and the creation of an addressable microplate array of the library before arraying on nylon. The libraries are picked by robot and transferred into 96- or 384-well microplates. The resulting cultures are arrayed robotically on nylon membranes in a high-density format. This allows an ordered and high-throughput approach to li-

brary screening. DNA samples (usually PCR products) are also robotically arrayed on nylon membranes to form macroarrays. Although the arraying is fully automated, several manual manipulations still remain. These include aligning the nylon membrane correctly on the robot bed, before arraying, and processing the membrane to remove bacterial debris and to immobilize the DNA on the membrane.

At present, no commercially available membrane has been optimized specifically for automated macroarraying. A nylon membrane ideal for this application must possess favorable handling characteristics, promote well-defined colony growth, exhibit a high hybridization signal-to-noise ratio, and be durable enough to enable numerous stripping and reprobing cycles. Currently available membranes exhibit a number of drawbacks. Some do not support optimal colony growth, thus reducing the amount of DNA immobilized on the membrane, leading to poor hybridization sensitivity. Others possess poor handling characteristics, making it both more difficult and time consuming to manipulate the membrane during the arraying and subsequent hybridization procedures. PerForma has been optimized for automated macroarraying, through addressing these various disadvantages. During the development process, several different prototypes were evaluated. These differed in the type of nylon, the surface chemistry, and the supporting substrate. PerForma is a proprietary nylon membrane designed to optimize wet handling characteristics and with a surface chemistry optimized for chemifluorescent detection. This report describes the results that were obtained using the PerForma membrane, showing that PerForma is more sensitive for nonradioactive (chemifluorescent) detection than existing membranes.

MATERIALS AND METHODS

Molecular Biology

Standard methods were followed, as described in Sambrook et al. (6).

Membranes

PerForma was compared with competitor membranes, which are commercially available positively charged nylon membranes available in approximately 22 × 22 cm sizes.

Preparation of Dot Blots

An approximately 500-bp region of a pSport plasmid (Invitrogen, Paisley, UK) was amplified using PCR, and the concentration determined by gel electrophoresis against DNA quantification standards (Invitrogen). Serial dilutions of the PCR product were prepared and spotted in triplicate onto membrane samples, allowed to air-dry, and then U/V cross-linked (120 mJ/cm²; CL-1000 Ultraviolet Cross Linker; UVP, San Gabriel, CA, USA). The dot blots were stored in sealed hybridization bags (Genetix Ltd., Hampshire, UK) at room temperature before hybridization.

Robotic Arraying of PCR Products

Serial dilutions of PCR product (500 bp) were transferred to 384-well microplates (Genetix Ltd.). Sterile blotting paper (1.5 mm, Genetix Ltd.) was soaked in denaturing solution (1.5 M NaCl, 0.5 M NaOH), placed on sterilized filter blocks (Genetix Ltd.), and rolled with a sterile disposable 10-mL pipet to remove excess liquid. The membrane samples were soaked in denaturing solution, laid upon the blotting paper, and rolled, as above, to remove excess liquid and air bubbles. The PCR products were arrayed in a 5 × 5 pattern (i.e., each pin makes a 5 × 5 array of spots producing a total of 57 600 spots on a 22.2 × 22.2 cm nylon membrane) with a QBot (Genetix Ltd.) equipped with a 384 × 250 μm pin gridding tool. After arraying, the membranes were placed on blotting paper soaked in denaturing solution, for 10 minutes. The membranes were then transferred on to blotting paper, soaked in neutralizing solution (1.5 M NaCl, 1 M Tris), for 5 min. The membranes were allowed to air-dry and then UV cross-linked (120 mJ/cm²). Arrayed membranes were stored in sealed hybridization bags at room temperature before hybridization.

Arraying of Transformed Bacterial Colonies

Colonies of *E. coli* (JM109:pUC18) were picked into LBA broth [LB (Merck, Northampton, UK) + 50 μg/mL ampicillin (Sigma, Poole, UK)] in 384-well microplates and grown overnight at 37°C. Certain wells were replaced with *E. coli* carrying I.M.A.G.E clone 50117 (HGMP, Hinxton, UK). Blotting paper (1.5 mm) was soaked in LBA broth and placed on filter blocks, as described for the PCR product arraying. Membranes were soaked in LBA and placed on the blotting paper, as previously described. Bacteria were arrayed in a 5 ×

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5 format using a QBot, fitted with a $384 \times 250 \mu\text{m}$ pin tool (Genetix Ltd.). The membranes were transferred to QTrays (Genetix Ltd.) containing LBA + 3% agar, ensuring that no air bubbles formed, and incubated, inverted, at 37°C overnight. Following colony growth, the colonies were assessed for growth of even-sized, round colonies, with no convergence of the colonies. The membranes were placed on blotting paper soaked in denaturing solution, for 4 min at room temperature. The membranes were then transferred to another sheet of blotting paper soaked in denaturing solution and placed for 4 min on a glass plate supported above a boiling water bath. Membranes were transferred to blotting paper soaked in neutralizing solution and incubated for 4 min at room temperature. The membranes were allowed to air-dry for 10 min and were transferred, colony side down, to freshly prepared Proteinase K solution (0.05 M Tris-HCl, pH 8.0, 0.1 M NaCl, 0.05 M EDTA, 1% Sarkosyl, 0.1 mg/mL Proteinase K) and incubated at 37°C for 50 min (4). Following this, the membranes were placed between two sheets of dry blotting paper and compressed by rolling with a disposable 10-mL pipet over the sandwiched membranes. The membranes were allowed to air-dry overnight, UV cross-linked ($120 \text{ mJ}/\text{cm}^2$), and then sealed in hybridization bags before use in hybridization.

Nonradioactive Hybridization

Digoxigenin (DIG)-labeled probes were prepared using PCR, quantified as detailed in the manufacturer's instructions (Roche Applied Science, Lewes, UK), and then stored at -20°C until required. All incubation steps were carried out in hybridization boxes (Genetix Ltd.), under gentle agitation, in 200-mL volumes. Membranes were prehybridized in preheated, modified Church Buffer (0.25 M Na_2HPO_4 , 5% SDS, 2 mM EDTA, pH 8.0) (1), filtered using a $0.4\text{-}\mu\text{m}$ syringe filter (Nalge Nunc International, Roskilde, Denmark), and incubated at 65°C for approximately 120 min. The membranes were transferred to fresh, preheated Church Buffer containing 10 ng/mL DIG-labeled probe and incubated for 16–20 h at 65°C . The membranes were washed in 0.02 M Na_2HPO_4 , 0.1% SDS for 20 min at room temperature and then for 20 min at 65°C . The membranes were subsequently blocked in milk solution (5% skimmed milk powder in $1\times$ PBS, pH 7.4; Sigma) for 45 min at room temperature. The membranes were then transferred to centrifuged milk solution ($1250\times g$ for 15 min) containing a 1:20 000 dilution of anti-DIG antibody (Roche Applied Science) and incubated at 37°C for 60 min. Membranes were then washed twice in $1\times$ PBS for 30 min at room temperature and twice in 0.1 M Tris-HCl, pH 9.5, 1 mM MgCl_2 for 10 min at room temperature. AttoPhos[®] (1 mM; JBL Scientific) in 0.48 M DEA, 0.08 M Tris-HCl, pH 9.5, 0.8 mM MgCl_2 was sprayed evenly ($5 \mu\text{L}/\text{cm}^2$ membrane) onto each membrane using a motorized spray gun (0.2 mm nozzle diameter; model 1101, Kager, Mannheim, Germany). The membranes were sealed in hybridization bags, wrapped in aluminum foil, and incubated for approximately 16 h at room temperature. The membranes were imaged using a Fuji Las-1000 dark box (Fujifilm, Tokyo, Japan), and the images were analyzed using ArrayVision[™] (Imaging Research, Ontario, Canada). Following imaging, the membranes were stripped in 1% SDS, 0.4 M

NaOH for 30 min at 65°C in 0.1 M Tris-HCl, pH 7.4, $2\times$ SSC, 0.1% SDS for 45 min at room temperature and overnight in Church Buffer at 65°C . Successful stripping was determined by imaging as described above. The membranes were stored in sealed hybridization bags in $2\times$ SSC at 4°C .

Radioactive Hybridization

Probe was prepared and quantified using the Rediprime II[™] ^{32}P labeling kit as detailed by the manufacturer's proto-

Figure 1. Comparison of hybridization detection sensitivity using both (A) chemifluorescence and (B) radioactive detection methods. Serial dilutions of the pSport PCR product were spotted manually, in triplicate, on different membranes and hybridized using both fluorescent and radioactive detection, as described in the Materials and Methods section. The amount of DNA in each spot is indicated.

Figure 2. Detection of robotically arrayed PCR product using both (A and B) chemifluorescent and (C and D) radioactive detection methods. Serial dilutions of the pSport PCR product (concentrations spotted are indicated) were robotically arrayed onto both PerForma and Membrane A, as described in the Materials and Methods section.

Figure 3. Comparison of signal intensity exhibited by a bacterial PerForma array after 21 stripping cycles. Bacterial colonies transformed with pUC18 or I.M.A.G.E 50117 were arrayed onto membranes, as described in the Materials and Methods section, and hybridized with a probe against the I.M.A.G.E. 50117 insert.

col (Amersham Biosciences, Little Chalfont, UK). Unless otherwise stated, all steps were carried out, under agitation, in approximately 30 mL solution in hybridization bottles. The membranes were prehybridized in preheated hybridization solution (2.5× Denhardt's solution, 6× SSC, 0.1% SDS, 0.1 mg/mL Herring Sperm DNA) at 65°C for 60 min. Sufficient radiolabeled probe was added to a volume of hybridization buffer to give 70 $\mu\text{L}/\text{cm}^2$ of membrane, to give 1.5×10^6 cpm/mL, and incubated at 65°C overnight. The membranes were washed three times in preheated 2× SSC, 0.1% SDS for 20 min at 65°C and once in 0.2× SSC, 0.1% SDS for 20 min at room temperature. The membranes were imaged using a TyphoonTM 8600 (Amersham Biosciences). The membranes were stripped by being placed in 0.1% boiling SDS for approximately 30 s, removed, placed in fresh boiling 0.1% SDS, and allowed to cool to room temperature under gentle agitation. The stripped membranes were stored in sealed bags at 4°C or frozen at -20°C for long-term storage.

RESULTS AND DISCUSSION

Comparison of Nonradioactive and Radioactive Hybridization

The degree to which a nylon membrane binds and retains arrayed DNA, after subsequent stripping cycles, affects the utility of the membrane. Figure 1 illustrates the detection sensitivity of PerForma using both radioactive and fluorescent detection. When used with the DIG/AttoPhos fluorescent detection system (Figure 1A), comparison of the detection sensitivity between PerForma and other commercially available membranes demonstrates that immobilized DNA amounts as low as 0.25 pg can be consistently detected. This is due to two factors. First, the intrinsic fluorescence of the nylon 6,6 incorporated into PerForma has been greatly reduced to obtain a greater signal-to-noise ratio when probed and to produce a much lower, more uniform background. Second, PerForma

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has been developed to enhance the binding of DNA through the treatment of the membrane with a positively charged surface chemistry.

When used with the RediPrime II radioactive detection system (Figure 1B), results show that PerForma has at least equivalent performance to other commercially available membranes in terms of detection sensitivity, while exhibiting a more uniform background. Figure 1b shows that amounts as low as 0.25 pg can be detected using PerForma with radioactive detection. The results show that PerForma is more sensitive when used with chemifluorescent, as opposed to radioactive, detection. We have speculated that this could be due to the novel surface chemistry of PerForma undergoing a free radical change caused by components present in the solutions used in the fluorescent detection steps, but not present in the radioactive detection solutions.

DNA Arraying

It is becoming increasingly common for researchers to bypass the labor-intensive and sometimes inconsistent approach of arraying bacteria and instead to array PCR products directly onto nylon membranes. Therefore, it was crucial to be able to array PCR products onto PerForma successfully. In-house optimization experiments (data not shown) have demonstrated that the phenomenon of "comet tailing" is virtually eliminated through arraying onto membranes moistened with a denaturing solution rather than onto dry membranes. This also eliminates the need to add denaturant to microplates containing PCR products, thus increasing the storage shelf life of potentially valuable PCR products.

Figure 2, A and B, compares the detection sensitivity of Competitor A versus PerForma using arrayed PCR products and chemifluorescent detection. Using PerForma, 10 pg/ μ L can be consistently detected, compared to 100 pg/ μ L using Competitor A. Figure 2, C and D, compares the detection sensitivity of Competitor A versus PerForma using the RediPrime II radioactive detection system: 10 pg/ μ L can be detected using PerForma.

Colony Arraying

The automated arraying of bacterial colonies remains the most commonly employed approach to analyze gene libraries. During the development process, great emphasis was placed on selecting a suitable substrate on which the nylon 6,6 is cast. The eventual choice possesses a low intrinsic fluorescence, supports good colony growth, is easy to handle, and is more robust than existing membranes. Assessment of colony growth after arraying showed that PerForma produced well-defined colony growth and that the colonies also grew faster than on other membranes, thus reducing incubation time by 4–5 h. The improvement is observed particularly with bacterial strains, such as the *E. coli* strain SOLR, which we have observed to give slower growth when arrayed onto nylon membranes. Good colony growth is presumed to improve the amount of DNA present for immobilization on the membrane following cell lysis.

A commonly experienced problem associated with nylon membranes is the susceptibility to folding and creasing dur-

ing the handling and processing steps. The novel substrate used in PerForma eliminates these problems by making the membrane stiffer and more robust so that it is resistant to folding and tearing. These features increase the ease with which the membrane can be used and saves time, especially for high-throughput applications.

Multiple Hybridizations

The number of cycles of stripping and reprobing that a membrane will withstand is an important economic consideration when selecting an appropriate membrane. As Figure 3 illustrates, PerForma can be successfully stripped and reprobed at least 21 times without losing significant signal intensity, although this will vary depending on the nature of the probe being used. The analysis software ArrayVision was used to compare signal intensities between the first and subsequent twenty-first hybridization cycles. Figures 3, A–D, shows hybridization to a colony array with positive target colonies spotted in duplicate. Parts A and C from Figure 3 are from the first cycle of hybridization, and parts B and D from Figure 3 are the same membrane after 21 stripping cycles rehybridized with the same probe. The percentage ratio of signal intensity of positives does not decrease by less than 75% of the original intensity. The stripping and reprobing robustness exhibited by PerForma can be attributed to a combination of the novel components comprising the membrane and the surface chemistry with which it is treated.

REFERENCES

1. Church, G.M. and W. Gilbert. 1984. Genomic sequencing. *Proc. Natl. Acad. Sci. USA* 81:1991-1995.
2. Clark, M.D., G.D. Panopoulou, D.J. Cahill, K. Bussow, and H. Lehrach. 1999. Construction and analysis of arrayed cDNA libraries. p. 205-233. *In* S.M. Weissmann (Ed.), *Methods in Enzymology*. Academic Press, San Diego.
3. Jaklevic, J.M., H.R. Garner, and G.A. Miller. 1999. Instrumentation for the genome project. *Ann. Rev. Biomedical Eng.* 1:649-678.
4. Nizetic, D., R. Drmanac, and H. Lehrach. 1990. An improved bacterial colony lysis procedure enables direct DNA hybridisation using short (10, 11 bases) oligonucleotides to cosmids. *Nucleic Acids Res.* 19:182.
5. Rast, J.P., G. Amore, C. Calestani, C.B. Livi, A. Ransick, and E.H. Davidson. 2000. Recovery of developmentally defined gene sets from high-density cDNA macroarrays. *Dev. Biol.* 228:270-286.
6. Sambrook, J., E.F. Fritsch, and T. Maniatis. 1989. *Molecular Cloning: A Laboratory Manual*, 2nd ed. CSH Laboratory Press, Cold Spring Harbor, NY.
7. Schummer, M., N.V. Wailap, R.E. Bumgarner, P.S. Nelson, B. Schummer, D.W. Bednarski, L. Hassel, R.L. Baldwin, et al. 1999. Comparative hybridisation of an array of 21 500 ovarian cDNAs for the discovery of genes overexpressed in ovarian carcinomas. *Gene* 238:375-385.

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