COMMUNICATION

EVALUATION OF THE PERFORMANCE OF DIFFERENT PLASTICS USED TO SEAL NYLON cDNA ARRAYS

Avaliação da performance de diferentes plásticos usados para selar arranjos de cDNA em náilon

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ABSTRACT

cDNA arrays are a powerful tool for discovering gene expression patterns. Nylon arrays have the advantage that they can be re-used several times. A key issue in high throughput gene expression analysis is sensitivity. In the case of nylon arrays, signal detection can be affected by the plastic bags used to keep membranes humid. In this study, we evaluated the effect of five types of plastics on the radioactive transmittance, number of genes with a signal above the background, and data variability. A polyethylene plastic bag 69 μm thick had a strong shielding effect that blocked 68.7% of the radioactive signal. The shielding effect on transmittance decreased the number of detected genes and increased the data variability. Other plastics which were thinner gave better results. Although plastics made from polyvinylidene chloride, polyvinyl chloride (both 13 μm thick) and polyethylene (29 and 7 μm thick) showed different levels of transmittance, they all gave similarly good performances. Polyvinylidene chloride and polyethylene 29 mm thick were the plastics of choice because of their easy handling. For other types of plastics, it is advisable to run a simple check on their performance in order to obtain the maximum information from nylon cDNA arrays.

Index terms: Signal detection, nylon arrays, sensitivity.

RESUMO

Os arranjos de cDNA são uma poderosa ferramenta para o estudo de padrões de expressão gênica. Os arranjos em membranas de náilon apresentam ainda a vantagem de poderem ser reutilizados diversas vezes. Porém, um ponto bastante delicado em estudos de expressão gênica em larga escala é a sensibilidade. No caso de arranjos em membranas de náilon, a detecção dos sinais pode ser afetada pelo envoltório plástico utilizado para manter as membranas úmidas. Nesse estudo, nós avaliamos os efeitos de cinco tipos de plásticos na transmissão radioativa detectada, no número de genes com sinal acima da emissão de fundo e na variabilidade dos dados. O plástico produzido com polietileno com 69 μm de espessura apresentou uma forte interferência na emissão radioativa, bloqueando 68.7% do sinal detectado. Este bloqueio na transmitância diminuiu o numero de genes detectados e aumentou a variabilidade dos dados. Outros plásticos mais finos tiveram resultados melhores. Apesar de plásticos feitos de cloreto de polivinilideno e cloreto de polivinila (ambos com 13 μm de espessura) e polietileno (29 e 7 μm de espessura) terem diferentes níveis de transmitância, todos apresentaram performances semelhantes nos testes realizados. Cloreto de polivinilideno e polietileno com 29 μm de espessura foram os plásticos escolhidos devido à facilidade de manuseio. Para outros tipos de plásticos, é recomendável realizar um teste de suas performances antes de utilizá-los para envolver membranas de náilon, de forma a obter o máximo de informação dos experimentos com arranjos de cDNA.

Termos para indexação: Detecção de sinal, arranjos de náilon, sensibilidade.

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Around one-third of the genes found in every genome project has an unknown function, and studies aimed to determine the physiological roles of gene products are one of the main tasks in genomics. cDNA array technology is the main strategy for assessing gene expression profiles on a large scale. cDNA clones corresponding to the genes under study are arrayed on a solid support, such as glass or nylon. The arrays are then hybridized with labeled probes

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obtained from the samples under study. The amount of labeled probe bound at the cDNAs on the arrays is proportional to the number of mRNA molecules present in the initial population of cells. Because each array contains thousands of genes, the expression profile of a relevant part of the cell transcriptome can be easily assessed.

cDNA arrays based on glass slides (microarrays) are by far the most common way to study gene expression on a large scale, whereas cDNA arrays on nylon filters (macroarrays) are an alternative to glass slides. Although the latter accomodate a lower number of genes, they are cheaper and more robust since they are based on the hybridization of radiolabeled probes to filters, a routine procedure in most molecular biology laboratories. Using macroarrays, we have successfully identified several genes that are induced by cold stress in sugarcane (Nogueira et al., 2003), and several recent studies on gene expression profiling have also used macroarrays, including the identification of angiogenesis factors in lung cancer tissues (Ohira et al., 2002), the response to sulfur starvation in *Arabidopsis thaliana* (L.) Heynh. plants (Hirai et al., 2003), and the identification of genes involved in ceramide-dependent neuronal apoptosis (Decraene et al., 2002).

One of the key issues in cDNA array analysis is the detection of genes that are expressed at low levels, particularly because these correspond to the largest part of the transcriptome in most cells (Bishop et al., 1974; Jongeneel et al., 2003). Thus, finding ways to increase the signal detection is of great importance, since the higher the signal the greater the reliability of the measurements.

During our studies using macroarrays we noted that little attention has been given to the plastic bags used to seal the nylon filters (the latter can be re-used after stripping, but keeping the membranes wet is essential for subsequent rounds of hybridization). We found that a significant fraction of the radioactivity is blocked by the most commonly used plastic material. We have therefore compared several plastics to determine which one provides the best transmittance and evaluated their effect on the detection of gene expression and other aspects of cDNA array analysis.

The maize inbred line Cat 100-6 (Al-tolerant) was obtained from the Germplasm Bank of the Universidade Estadual de Campinas, Campinas, Brazil. The plants were grown in the field and self-pollinated. Seeds were surface-sterilized with 70% ethanol for 1 min and with 0.5% (v/v) sodium hypochloride for 20 min rinsed four times with sterile water and germinated between layers of moist filter paper at 28 °C for 60 h. After 3 days, the seedlings were transferred to polystyrene holders that were floated on an aerated nutrient solution (Moon et al., 1997).

Nylon array hybridization was done using plasmids containing sugarcane ESTs that were obtained from the SUCEST RT1 library made from sugarcane root RNA (Vettore et al., 2001). Plasmidial DNA obtained by alkaline lysis was denatured with 0.2 M NaOH. DNA was spotted on nylon filters (Hybond-N, AP-Biotech, USA) with a hand-held 96-pin printhead tool which typically deposited 0.1µl (around 10 ng) of DNA solution (V&P-Scientific, USA), as described by Schummer et al. (1999). The DNA was fixed to the filters by baking at 80°C for 2 h. The filters contained 768 ESTs in duplicate, to give 1536 spots per filter. ^{33}P-Labelled cDNA was produced from 30 µg of total RNA from maize roots of seedlings exposed to several Al activities (0, 5, 15, 50 and 85 x 10^{-6}) and hybridized to nylon arrays essentially as described by Nogueira et al. (2003). Membranes were washed with 1x SSC and 0.1% SDS for 15 min at 65 °C, and twice with 0.1x SSC and 0.1% SDS at 65 °C (15 min each).

For detection of gene expression we compared five different plastics sold in supermarkets: polyvinyl chloride (PVC, 13 µm thick, *MajiPack*™, Inproco, Brazil), polyvinylidene chloride (PVDC, 13 µm thick, *Saran Wrap*™, Johnson & Son, USA) and three polyethylene plastics 69, 29 and 7 µm thick (PE-69, PE-29 and PE-7, Garoa, Brazil). Twenty replica nylon filters were used and four filter were sealed with each plastic. Four additional filters without sealing were used as controls. After sealing, the filters were exposed for 96 h to imaging plates in an FLA3000-G screen system (Fuji Photo Film, Japan). The intensity of each spot on the digitalized images was quantified using *Arrayvision*™ software (Imaging Research, Canada). The local background was subtracted from the intensity of each spot and the data were rearranged into MS Excel (Microsoft, USA) files for further analysis. We used four replica nylon filter for each plastic to correct and minimize the variability of the DNA amount fixed on the membrane.

Three parameters were used to evaluate the interference of each type of plastic on the gene expression signal detected: the total radiation on each membrane (i.e., the sum of the intensities of all spots), the number of spots that showed a signal at least 10% greater then the local background emission (around the spot) and the number of genes for which the corresponding pair of replicate spots showed signals that varied in intensity by less than two fold. For each parameter, the data from each type of plastic and from naked nylon arrays were compared in pairs. To avoid assumptions regarding the distribution of the data, statistical comparisons were done using the Wilcoxon-Mann-Whitney rank sum test (Woolson, 1987), a nonparametric test for two independent samples.

During a study of gene expression in maize plants exposed to aluminum stress, we decided to check the effect of commonly used plastic sealing bags on signal detection. Nylon cDNA arrays have the advantage they can be reused several times, in the same way as Southern and Northern blots. We have reused nylon arrays up to seven times without noticeable loss of signal (unpublished results). However, the membranes need to be sealed to avoid drying, and this can lead to covalent linking between the immobilized probes on the array and the radioactive target probes. The inconvenience of sealing with plastics is their blocking effect on the radioactive signal.

Initially, the effects of three different plastics (PE-69, PVC and PVDC) were compared to naked membranes (NM group). Nylon membranes that had previously been exposed without plastic were wrapped in the three types of plastic and the signals were then measured again. The set of naked membranes was exposed twice without wrapping in plastic in order to control for the loss of signal resulting from radioisotope decay. The PE-69 plastic, routinely used by our group, had a strong blocking effect, with only 31.3% of the original emission from naked membranes being detected (Figure 1 and Figure 2). PVC and PVDC showed less blockage, with 70.9% and 75.5% of the emission being detected. The latter values were significantly higher ($p<0.05$) than those observed with PE-69, but were not significantly different from naked membranes.

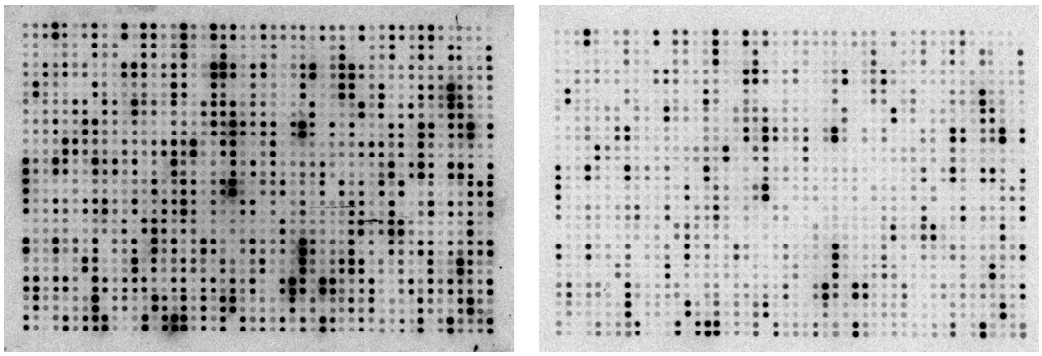

Figure 1 – Example of the effect of plastics on signal detection. Filters containing 768 ESTs fixed in duplicate, giving a total of 1536 spots per filter, were exposed with different sealing plastics. Left: image of radioactive emission of a naked membrane. Right: image of radioactive emission of a replicate membrane sealed with PE-69.

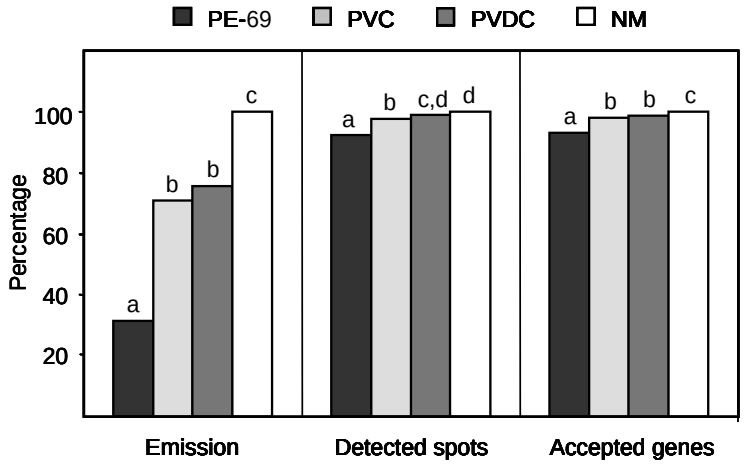

Figure 2 – Effect of sealing plastics on the geometric means of the ratios of total emission, detected spots and accepted genes. The values obtained for each sealed filter were divided by those previously detected for the same filter without sealing. In the NM group, the values were determined twice without sealing in order to control for radioisotope decay. The geometric mean of four ratios is shown for each group. Letters at the top of each column indicate significantly different groups ($p<0.05$).

One of the main problems with the decreased signal caused by the interference of plastics is that genes with low levels of expression are not detected. Since several regulatory genes are expressed at low levels, this loss must be minimized. The number of detected spots with a signal at least 10% higher than the local background was used as a parameter to assess this effect. This percentage is usually used as a cut-off value in cDNA array experiments, and only signals above this level are considered in gene expression analysis. Compared to the naked membranes, a very similar number of spots was detected when using PVC and PVDC (97.5% and 98.9%, respectively; Figure 2). The lowest detection was observed with PE-69 (92.3%). In a typical assay with 3000 genes, the difference between PE-69 and PVDC would represent a loss of 198 genes, which is high. The largest difference in the number of detected spots (between PE-69 and NM) was 7.7%, much lower than the difference between the total emission (68.7%; Figure 2).

In cDNA array experiments, each gene is usually spotted twice in the same array. Random fluctuations during spotting can affect the amount of DNA in each spot. Although in glass arrays these fluctuations are not a problem (a control target is usually also hybridized with the test target), in nylon arrays, which are hybridized with only one target at a time, these fluctuations may result in false positives, i.e. genes that are not up-regulated, but give higher signals in one array because of different amounts of DNA (the same is true for false negatives). To avoid this type of fluctuation in nylon array experiments, it is usual to eliminate from subsequent analysis all genes whose signals from different spots on the same membrane vary more than two-fold (Larkin et al., 2002). Since the shielding effect of sealing plastics decreases the signal intensities, it could also increase data variability, causing more spots to be discarded by this criterion. To evaluate this effect, we analyzed the differences in the number of genes discarded by this criterion in nylon filters wrapped in different sealing plastics. The percentage of accepted genes in nylon arrays sealed with PVC and PVDC were only 2% lower than the percentage accepted in naked membranes (Figure 2). In contrast, on arrays sealed with PE-69, 7% of the spots were rejected.

These results showed that PVC and PVDC were the best sealing plastics for arrays compared to PE-69. However, working with 13 μm thick PVC can be troublesome because the plastic easily wrinkles and the hybridization solution sometimes leaks from the terminally sealed edges. Since PVDC is also not easily available in countries other than the USA, we tested additional polyethylene-based plastics which are used to produce plastic bags worldwide. These plastics were thinner than PE-69 (7 μm and 29 μm) and were compared with PVC (Figure 3). The radioactive emission was the only parameter that showed significant variation among the three types of plastics. Whereas PE-7 had a similar transmittance than PVC, PE-29 clearly had a higher shielding effect, transmitting only 82% of the emission detected with PVC. The differences in the number of detected spots and accepted genes among the three plastics were very small and not significant. Based on these results, and because PE-29 is easier to handle than PE-7 and PVC, we concluded that sealing arrays with PE-

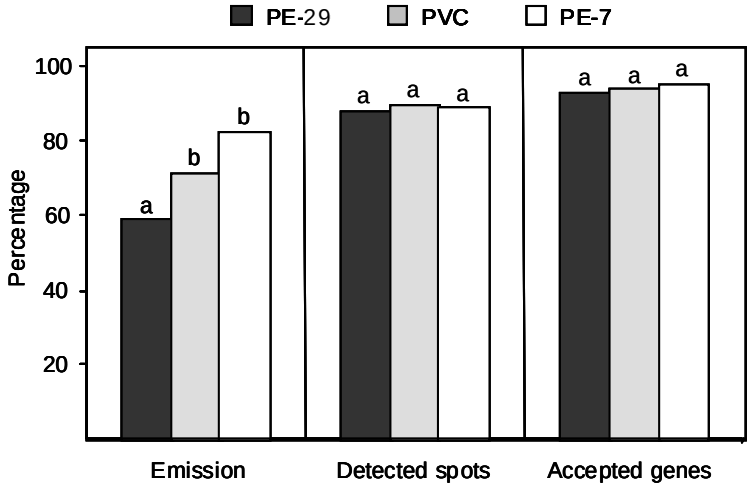

Figure 3 – Performance of polyethylene plastics compared to PVC. The ratios were determined as in figure 2. Letters at the top of each column indicate significantly different groups ($p<0.05$).

29 plastic was a good alternative for increasing the signal detection in nylon arrays.

The differences we found can be explained by the two factors that most influence the transmittance of any material, namely, chemical composition and thickness. Thus, PVDC and PVC had the same thickness and very similar performances. Among the polyethylene plastics, the lower performance of PE-69 probably reflected its greater thickness. However, although the difference in thickness between PE-29 and PE-7 affected the transmittance, no significant differences were found in the number of spots detected or accepted. This may indicate that transmittance becomes a problem only below a certain threshold level.

Our findings indicate that the choice of the sealing plastic is very important for obtaining the maximum information from nylon arrays. Among the plastics tested, PVDC showed the best performance, although in countries were this is not available, PE-29 proves a good alternative. Based on these findings, we have used PE-29 in various experiments, including the search for cold-induced genes from sugarcane (Nogueira et al., 2003), and the results from the hybridizations used in this work will be published elsewhere.

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