

Immobilon™-Ny+ Nucleic Acid Blotting Membrane: An Advanced Nylon Membrane Optimized for Superior Fixation and Reprobing

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ABSTRACT

The use of charged nylon membranes in nucleic acid blotting applications has become an important factor in the success of hybridization-based assays. Retention of nucleic acids on these membranes is promoted by baking at 80°C under vacuum or by exposure to short wavelength UV light, with the latter method preferred. Immobilon[®]-Ny+ is an advanced, positively charged nylon membrane that has been optimized to show superior retention of target DNA and RNA under hybridization conditions. Higher signal levels are obtained in these assays compared to competitive membranes, even after 13 cycles of probing. This report illustrates the superior performance of Immobilon-Ny+ in ³²P and chemiluminescent hybridization assays on blotted DNA and RNA.

INTRODUCTION

Southern blotting of DNA to a microporous membrane (18) has opened up many important applications in molecular biology. Early studies were fraught with issues relating to the nitrocellulose membranes first used that were somewhat brittle and difficult to reprobe. Nylon membranes cast from nylon 66 polymer (6,8) offered improved handling, were able to withstand reprobing and retained the nucleic acid well. Several fixation approaches have been suggested, including (i) baking at 80°C under vacuum and (ii) exposure to short wavelength UV light to cross-link the DNA through the generation of thymine-thymine and thymine-cytosine dimers or covalent interaction with the primary amines on the membrane surface (16). The latter method of fixation seems effective on certain types of nylon membrane, presumably ones with available primary amines on the nylon 66 surface (7). There is considerable variability in performance of these nylon membranes

in nucleic acid blotting. Immobilon™-Ny+ has been developed to address these issues and optimized for UV-induced fixation of target nucleic acid to give a superior hybridization signal and retention of the target for reprobing.

MATERIALS AND METHODS

Membranes and Reagents

Immobilon-Ny+ was obtained from Millipore Corporation (Bedford, MA, USA). Reagents were molecular biology grade, and solutions were prepared in Milli-Q[®] water (Millipore).

Electrophoresis of DNA and RNA

Lambda *Hind*III DNA digest (New England Biolabs, Beverly, MA, USA) was diluted in sample loading buffer and resolved in 0.8% agarose gels prepared in Tris-borate-EDTA (TBE) running buffer (0.09 M Tris-borate, 2 mM EDTA) following standard methodology (17). DNA samples were separated at 120 V for 2.5 h using 1× TBE running buffer.

Mouse liver total RNA (Ambion, Austin, TX, USA) was diluted in sample buffer and resolved on 1.0 % agarose gels containing formaldehyde and following a standard methodology (17). RNA samples were separated at 120 V for 80 min using 1× formaldehyde gel-running buffer (10 mM MOPS, pH 7.0, 8 mM sodium acetate, 1 mM EDTA).

DNA Transfer to Membrane

DNA resolved on agarose gels was transferred to membrane as described by Southern (18) using Whatman[®] 3MM filter paper and 20× standard saline citrate (SSC) as the trans-

fer buffer.

For standard alkaline transfer, Immobilon-Ny⁺ and Competitor A, gels were incubated for 10–15 min in 0.25 mol/L HCl with gentle agitation. The gels were then rinsed briefly in Milli-Q water and incubated in 0.4 mol/L NaOH for 15–30 min with gentle agitation. Capillary transfer stacks (18) were prepared using 0.4 mol/L NaOH as the transfer buffer. After overnight transfer, the stacks were disassembled; the blots were rinsed in 2× SSC and dried at room temperature.

For alkaline transfer under modified conditions (Competitor B), gels were incubated for 15 min in 0.4 mol/L NaOH, 0.6 mol/L NaCl, rinsed briefly in Milli-Q water and then soaked in 0.25 mol/L HCl for 15 min. After a second brief rinse in Milli-Q water, they were incubated for 30 min in 0.4 mol/L NaOH, 0.6 mol/L NaCl. Capillary transfer stacks (18) were prepared using 0.4 mol/L NaOH, 0.6 mol/L NaCl, as the transfer buffer. After overnight transfer, the stacks were disassembled; the blots were neutralized in 0.5 mol/L Tris-HCl, pH 7.0, 1 mol/L NaCl, for 15 min before drying at room temperature.

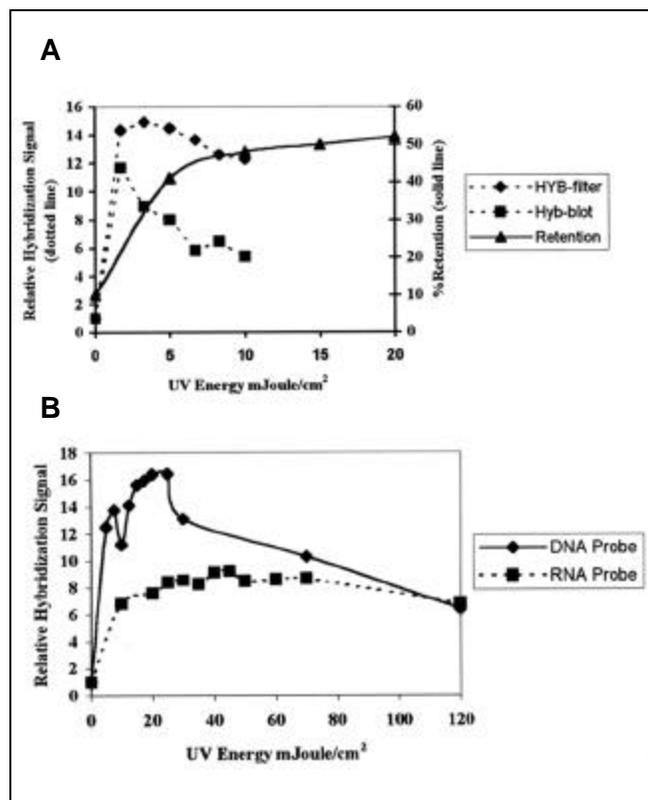


Figure 1. Influence of UV energy at 254 nm on retention and hybridization of DNA and RNA blotted to Immobilon Ny⁺. (A) Effect of UV energy at 254 nm on retention and hybridization of DNA blotted to Immobilon-Ny⁺. DNA was applied to Immobilon-Ny⁺ by blotting from an agarose gel (1 ng, Hyb-blot) or by filtration (20 pg, HYB-filter). After exposure to UV light at 254 nm, the blots were hybridized using random-primer-labeled λ *Hind*III fragments, and the signals were detected. Hybridization signals were normalized against the untreated control samples (i.e., no UV exposure). Retention results are expressed as a % of the original amount of DNA applied. (B) Effect of UV energy at 254 nm on hybridization of RNA blotted to Immobilon-Ny⁺. Mouse liver total RNA (1 μ g) was transferred to Immobilon-Ny⁺ by capillary blotting and fixed with varying UV energies at 254 nm. Data are shown for the cyclophilin mRNA using ³²P-labeled DNA (solid line) and RNA (dotted line) probes. Hybridization signals were normalized to untreated control samples (i.e., no UV exposure).

RNA Transfer to Membrane

After electrophoresis, the gels were rinsed briefly in Milli-Q water and incubated twice in 20× SSC for 30 min with gentle agitation. Capillary transfer (18) was done in 20× SSC overnight.

UV Cross-linking

DNA or RNA was fixed to dry membranes by exposure to UV light at 254 nm or 312 nm, using a Stratalinker[®] (Stratagene, La Jolla, CA, USA) by interchanging wavelength-specific bulbs. The side of the membrane with the bound DNA or RNA faced the lamps. For additional details on DNA and RNA cross-linking, see References 13 and 14, respectively.

DNA Hybridization Probe Preparation—³²P Label

*Hind*III restriction fragments of λ phage DNA (0.5–23.0 kbp) were used in these studies. For retention assays, radioactively labeled DNA was prepared by filling in the sticky ends with the Klenow fragment of DNA polymerase using α -³²P-CTP as one of the dNTPs. For hybridization assays, probe DNA was prepared by random primer labeling (17) of the λ DNA *Hind*III fragments.

DNA Hybridization Probe Preparation—Nonradioactive

Probe DNA was prepared by random primer labeling (17) of the λ *Hind*III DNA fragments using DIG-High Prime Labeling and Detection Starter Kit II from Roche Molecular Biochemicals (Indianapolis, IN, USA). The concentration of DIG-labeled probe DNA was estimated from signal intensities compared to a control-labeled DNA in a dot blot assay.

RNA Hybridization Probe Preparation

The antisense RNA probe was generated from a plasmid insert containing a 103 bp transcript from cyclophilin (Ambion) labeled with α -³²P-UTP (800 Ci/mmol) by in vitro transcription (9). DNA probe for cyclophilin was generated from a 721 bp fragment (DECA[™] templates; Ambion) labeled with α -³²P-dCTP (3000 Ci/mmol) by random primer labeling (4).

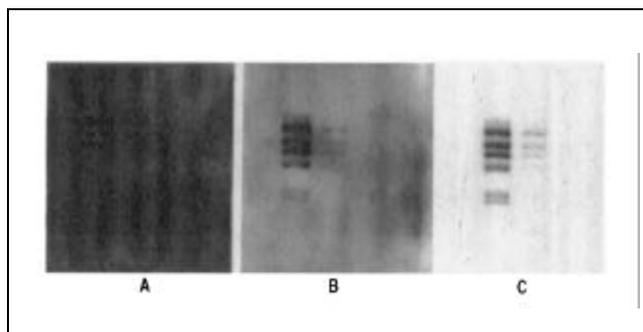


Figure 2. Effect of hybridization solution on chemiluminescent detection. Blots were exposed to film for 30 min. (A) Original hybridization solution (as supplied by vendor) unfiltered. (B) Original hybridization solution, filtered. (C) Church hybridization solution, filtered. Amounts of DNA loaded, from left to right on each blot, were 1, 0.1 and 0.01 ng.

Southern Hybridization

Prehybridization, hybridization and washes were performed in glass tubes in a rolling bottle hybridization oven. Membranes were wet in Milli-Q water and transferred to the hybridization tubes. For pre-hybridization, 10 mL of hybridization solution (5× saline-sodium phosphate-EDTA [SSPE], 5× Denhardt's solution, 0.1% SDS, 100 µg/mL sheared DNA) was added to each 30 cm tube. The tubes were incubated for 1–2 h at 68°C. The pre-hybridization solution was poured off and replaced with 3 mL of hybridization solution at 68°C containing ³²P-labeled probe at a ratio of 1.1 × 10⁵ cpm per cm² of membrane surface area. The tubes were incubated for 16–20 h at 68°C. Membranes were then washed twice for 5 min each in 2× SSC, 0.1% SDS, at room temperature and twice for 15 min each in 0.2× SSC, 0.1% SDS, at 68°C. Blots to be used for stripping and reprobing were sealed in plastic bags and never allowed to dry out. Otherwise, the blots were dried at room temperature. Radioactive DNA retained on the blots was imaged as described below.

Northern Hybridization

Northern blots were wet in Milli-Q water and placed in a single layer in glass tubes. For pre-hybridization, 10 mL of hybridization solution (0.5 M sodium phosphate, pH 7.1, 2 mM EDTA, 7% [wt/vol] SDS, 0.1% [wt/vol] sodium pyrophosphate) modified from Church and Gilbert (3) was added to each 30 cm tube. For RNA probes, formamide was

used at a final concentration of 50% (vol/vol). The tubes were processed as already described. Hybridization was carried out using ³²P-labeled probe at a concentration of 1.0 × 10⁵ cpm per cm² of membrane surface area. For chemiluminescent detection, the concentration of DIG-labeled DNA probe was 10 ng/mL. Following incubation overnight at 68°C, the blots were rinsed in wash solution I (1× SSPE, 0.5% SDS) and washed twice in fresh wash solution I for 5 min at room temperature. The blots were then rinsed briefly in pre-heated wash solution II (0.2× SSPE, 0.1% SDS) and washed twice in fresh wash solution II for 15–20 min at 68°C. All solutions were filtered through Express™ GP membrane filter units (0.22 µm; Millipore) to remove particles.

DNA Retention Assay

End-labeled DNA, diluted in 20× SSC containing unlabeled λ DNA fragments, was applied to membranes by vacuum filtration using a slot blot manifold. The DNA was then fixed to the membrane by drying at room temperature for at least 60 min, baking at 80°C under vacuum for 1 h or cross-linking with UV light. The membranes were re-wet in Milli-Q water and transferred to glass hybridization tubes with 10 mL of hybridization solution (5× SSPE, 5× Denhardt's solution, 0.1% SDS, 100 µg/mL sheared DNA) per tube. The tubes were incubated at 68°C for 16–20 h. Membranes were then washed twice for 5 min each in 2× SSC, 0.1% SDS, at room temperature and twice for 15 min each in 0.2× SSC, 0.1% SDS, at 68°C. Radioactive DNA retained on the blots was imaged as described below.

Stripping and Reprobing

Probe DNA was stripped from DNA blots by incubation in 0.4 mol/L NaOH for 30 min at 45°C with gentle agitation followed by neutralization in 0.1× SSC, 0.1% SDS, 0.2 M Tris-HCl, pH 7.5, for 15 min at room temperature. These optimal conditions were derived from data presented in the Results and Discussion section. For RNA blots, the probe was stripped by two incubations in hot 0.1% SDS for 15 min with gentle agitation. The SDS solution was brought to a boil, removed from the heat and the blots were added. The completeness of stripping for both methods was measured by phosphor imaging.

Phosphor Imaging

Radioactivity on the membranes was visualized by phosphor imaging on a Storm™ 840 Phosphor Imaging System (Molecular Dynamics, Sunnyvale, CA, USA) using Image-QuaNT™ analysis software. On gel blots the 2.0/2.2 kbp doublet was chosen for quantification because it was distinct from the other bands and its signal did not appear to overlap between adjacent lanes (Figure 3). Hybridization signals were normalized against untreated controls.

Chemiluminescent Detection and Imaging

The DIG-labeled probe was detected using a CSPD substrate (Roche Molecular Biochemicals) according to the manufacturer's protocol except for the addition of a quick rinse before washing. The membrane was exposed to XAR-X2 film for 10 min–2 h. Molecular Analyst® software (Bio-Rad Laboratories, Hercules, CA, USA) was used for quantification.

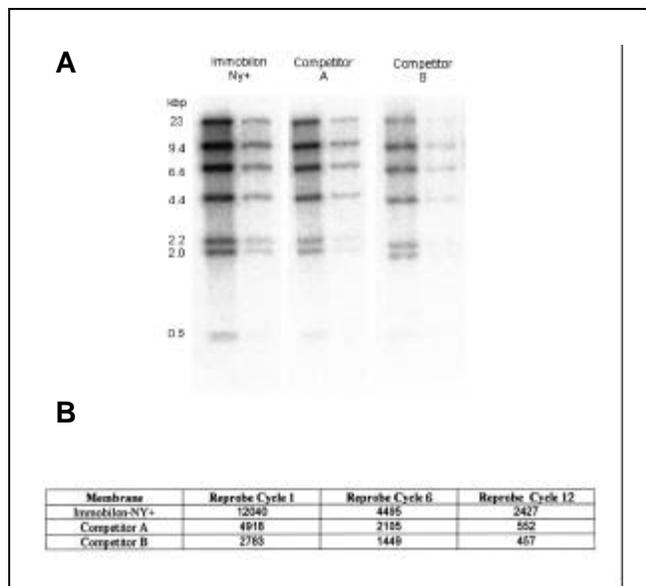


Figure 3. Signal intensity on charged nylon membranes after round 12 of reprobing (cycle 13 of hybridization). (A) Comparison of charged nylon membranes after 13 cycles of reprobing. Blots were prepared with 10 and 1 ng, respectively, of λ HindIII DNA fragments per lane on the agarose gel. After transfer in 20× SSC and UV cross-linking (254 nm, 5 mJ/cm²), the blots were hybridized with ³²P-labeled probe and then stripped in 0.4 mol/L NaOH at 45°C for 30 min. The blots were then subjected to the following cycles of reprobing: (a) 2 cycles with ³²P-labeled probes, (b) 4 mock cycles with no probe, (c) a seventh cycle with ³²P-labeled probe, (d) 5 mock cycles and (e) a final thirteenth cycle with ³²P-labeled probe. Between each cycle the blots were stripped as outlined above. After the final round of ³²P probing, signals were detected by phosphor imaging for 2 h. (B) Calculated band volumes for the 2.0/2.2 kb doublet. Gel blots were quantified as described in Materials and Methods for the 10 ng loading of the λ HindIII DNA fragments.

RESULTS AND DISCUSSION

In hybridization assays on microporous membranes, transfer and retention of the target nucleic acid influences detection sensitivity. The target is usually fixed onto the membrane by baking at 80°C under vacuum or exposure to short wavelength UV light, the latter being most commonly used with charged nylon membranes. Immobilon-Ny⁺ was developed to be optimal for UV cross-linking after transfer of nucleic acid from agarose gels or filtration (i.e., slot blotting). Optimal performance in hybridization is achieved by balancing two parameters: (i) the physical retention of the target on the membrane surface and (ii) the accessibility of the probe to the target during hybridization. Figure 1, panel A shows an example of a study investigating the relationship between these parameters and signal intensity. DNA was well retained by the Immobilon-Ny⁺ surface, approaching 50% of total at UV exposures of 20 mJ/cm². Maximum retention of >60% was achieved at UV exposures >60 mJ/cm². However, hybridization signals decline rapidly at exposures >5 mJ/cm² for slot-blotted DNA and >1.7 mJ/cm² for gel-resolved DNA. Above these energy levels, presumably significant structural cross-linking of the target DNA sterically hinders hybridization. DNA transferred in a slot blot format is presumably located deeper in the porous structure of the membrane in contrast to a surface location in passive transfer. These observations suggest that Immobilon-Ny⁺ exhibits optimal performance at lower UV irradiation lev-

els than reported in the literature (1,7). UV cross-linking at 312 nm was not as effective in promoting retention of target DNA and did not lead to a higher hybridization signal (13); it is not recommended for Immobilon-Ny⁺.

In contrast, RNA fixation required a higher UV energy to achieve optimal hybridization efficiency: 20 mJ/cm² for DNA probes and 40 mJ/cm² for RNA probes (Figure 1, panel B). Presumably, this is related to the presence of uracil instead of thymidine in the RNA structure and the consequential reduction of covalent bonds.

It has been reported that UV cross-linking should be done on wet blots (3,7,17). Moist blots, having water only in the pores, gave the lowest hybridization signal (data not shown; see Reference 13), while wet blots, having a thin layer of water across the surface, and dry blots produced 1.6-fold and 3.1-fold higher signals, respectively. Immobilon-Ny⁺ should be dry for optimal UV fixation.

Transfer under alkaline conditions has been reported (17) to fix DNA to the nylon surface without the need for UV exposure. Under alkaline conditions, DNA transferred to Immobilon-Ny⁺ showed a lower hybridization signal (data not shown, see Reference 13) compared to the 20× SSC control with UV fixation. UV treatment after alkaline transfer gave only a marginal improvement of the signal. In contrast, UV treatment after 20× SSC transfer gave a signal 4.6-fold higher than that seen with alkaline transfer (no UV exposure). Alkaline transfer to Immobilon-Ny⁺ is not recommended for maximal hybridization signal.

Alkaline transfer on nylon membranes showed (data not shown; see Reference 12) that Immobilon-Ny⁺ performed about two-fold better in both standard (Competitor A) and modified protocols (Competitor B) compared to the competitor membrane. As an example of the variability seen with the range of nylon membranes on the market, Competitor B failed to show any bands on initial transfer using the standard protocol and required the vendor's optimized protocol as outlined in Materials and Methods.

Nonradioactive detection methodologies are becoming widely accepted as an alternative to radioisotopes but are prone to low detection sensitivity and background issues, especially on charged nylon membranes. Using DIG-labeled probes for chemiluminescent detection on Immobilon-Ny⁺, it was possible to visualize a 4.4 kbp band of a λ HindIII digest that corresponded to 0.89 pg of DNA, with a 2 h film exposure. This was slightly less sensitive than detection with a ³²P-labeled probe (15). The original hybridization solution recommended by the kit manufacturer and one based on SDS (Church buffer; Reference 3) were compared in this application (Figure 2). The recommended hybridization solution caused high background (Figure 2, panel A), which was reduced by filtration through a Millex[®] GP filter unit (0.22 μ m; Millipore) (Figure 2, panel B) to remove particulate matter that presumably interfered with efficient washing of the blot or caused nonspecific adsorption of the probe. SDS-based hybridization buffer without blocking agents gave the highest signal-to-noise ratio (Figure 2, panel C). SDS presumably interacts with the positively charged nylon surface and minimizes hydrophobic interactions between the probe and the membrane. Optimization of UV fixation gave the maximum hybridization signal between 3.5 and 10 mJ/cm² and a decline in signal at exposures >15 mJ/cm² (data not shown; see Reference 15). After transfer, baking the membrane at 80°C under vacuum produced 75% of the signal obtained with optimal UV fixation.

Immobilon-Ny⁺ was compared to two competitive membranes following transfer from an agarose gel in 20 \times SSC and optimal UV fixation for each membrane (as recommended by the vendor). Immobilon-Ny⁺ exhibited two-fold higher signal than competitors A and B when detected with the ³²P-labeled probe (12). After reprobing, Immobilon-Ny⁺ maintained a higher level of signal compared with the competitor membranes through 12 cycles of reprobing (Figure 3). In reprobing analysis, there was a marked loss of hybridization signal after the first round of stripping, most likely reflecting the loss of target nucleic acid from the blot because of inadequate UV-induced cross-linking. This is to be expected as the optimal UV exposure for maximum hybridization signal is less than optimal for DNA retention (Figure 1, panel A). Relative performance differences between the membranes remained constant through all cycles of reprobing (Figure 3, panel B).

In reprobing analysis, a background signal can be generated from the nonspecific binding of a probe or residual signal left from an earlier round of hybridization. Immobilon-Ny⁺, when used with the recommended SDS-based hybridization buffer, showed a consistently low background signal in ³²P and chemiluminescent detection systems (10). Four stripping protocols were evaluated on Immobilon-Ny⁺ and Competitor A membranes (data not shown; see Reference 11). All protocols left <3% of the initial ³²P probe on the Immobilon-Ny⁺ blots when used at a concentration of 10 ng probe per mL during hybridization. Immobilon-Ny⁺ had less residual probe signal than Competitor A.

In summary, data have been presented to illustrate the optimal performance of Immobilon-Ny⁺ in nucleic acid blotting and hybridization. This advanced charged nylon membrane retains target nucleic acid after UV fixation and produces a superior hybridization signal with both radioactive and chemiluminescent detection systems. After multiple cycles of reuse, it maintains a high signal level through 12 cycles of reprobing. This next generation of optimized nylon blotting membrane will provide consistent and superior performance in this hybridization assay for nucleic acids.

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