Critical Evaluation of Membrane Supports for Use in Quantitative Hybridizations

LUTGARDE RASKIN,^{1*} WILLIAM C. CAPMAN,^{2†} MATTHEW D. KANE,^{2‡}
BRUCE E. RITTMANN,³ AND DAVID A. STAHL^{1,2,4§}

*Environmental Engineering and Science, Department of Civil Engineering, Newmark Civil Engineering Laboratory,¹
Department of Veterinary Pathobiology,² and Department of Microbiology,⁴ University of Illinois at
Urbana-Champaign, Urbana, Illinois 61801, and Department of Civil Engineering,
Technological Institute, Northwestern University, Evanston, Illinois 60208³*

Received 10 July 1995/Accepted 26 October 1995

The quantification of 16S rRNA by oligonucleotide probe hybridization was investigated with MagnaGraph (Micron Separation, Inc. [MSI]), Magna Charge (MSI), Magna (MSI), Immobilon-N (Millipore Corporation), and Nytran (Schleicher & Schuell, Inc.) membranes as supports for nucleic acid immobilization. The levels of detectability provided by the Magna Charge and Immobilon-N membranes were 20 to 50 times better than those obtained with the MagnaGraph, Magna, and Nytran membranes. The variability of the signal response for individual membranes ranged from 10 to 50%, with the Magna Charge and Immobilon-N membranes demonstrating the lowest variability.

Detection of RNA (rRNA and mRNA) by hybridization has become a routine technique in molecular biology (7). Most hybrid detection techniques involve immobilization of nucleic acid on nylon or nitrocellulose membranes prior to hybridization with a probe (7, 8, 10). In most cases, hybridization conditions are optimized only as much as is necessary for hybrid detection. However, when hybridization is used for quantification of low-abundance species present in natural environments, experimental conditions must be optimized simultaneously for detectability and precision. Quantitative hybridization techniques employed in our laboratory are generally based on hybridization of ³²P-labeled probes to rRNA (most commonly the 16S rRNA) recovered from environmental samples (5, 9).

Over a period of approximately 5 years, we have experienced significant variations in hybridization detectability and precision within and among batches of nylon membranes. This has often compromised quantification and has served as a reliable source of frustration. Therefore, in this study, we discuss some of our observations with respect to membrane variability and present the evaluation of signal intensity (to study detectability) and variability of the signal response (to study precision) with a variety of membranes: Magna, a 0.45- μ m-pore-size amphoteric ("uncharged") nylon membrane from Micron Separation, Inc. (MSI) (Westboro, Mass.); MagnaGraph, a 0.45- μ m-pore-size "chemically optimized" (slightly positively charged) nylon membrane from Micron Separation, Inc.; Magna Charge, a 0.45- μ m-pore-size charge-modified nylon membrane from Micron Separation, Inc.; Immobilon-N, a

0.45- μ m-pore-size charge-modified polyvinylidene difluoride membrane from Millipore Corporation (Bedford, Mass.); and Nytran, a 0.45- μ m-pore-size charged nylon membrane from Schleicher & Schuell, Inc. (Keene, N.H.).

The RNA used in the hybridizations was extracted from a variety of gram-negative bacteria by a hot phenol extraction procedure (9), and the recovered RNA was precipitated by addition of 2 volumes of ethanol (0.3 M sodium acetate, final aqueous concentration) or 0.6 volumes of isopropanol (2.5 M ammonium acetate, final aqueous concentration). RNA was denatured by addition of 3 volumes of 2% glutaraldehyde in 50 mM sodium phosphate (pH 7.0) to 1 volume of nucleic acid. After incubation (10 min) at room temperature, the nucleic acid was diluted [with double-distilled water (ddH₂O) containing 1 μ g of poly(A) per ml and 0.02 μ l of 2% bromophenol blue per ml] and immediately applied to the membranes. To evaluate the effect of the presence of salt on hybridization performance, the addition of SSC to a final concentration of 1.5 M NaCl and 0.15 M sodium citrate (10 $\times$ SSC) after the glutaraldehyde denaturation step was investigated.

RNA samples were applied in a total volume of 50 or 100 μ l to the membranes (21.8 by 6.5 cm) with a slot blot device (Minifold II; Schleicher & Schuell, Inc.) under a slight vacuum adjusted to pull the entire sample volume through the membrane in 1 to 2 min. The membranes (except Immobilon-N) were pretreated in ddH₂O before the nucleic acid was applied. Because Immobilon-N membranes are hydrophobic, they must be pretreated in 95% ethanol (for several seconds) prior to a rinse in ddH₂O (3 min). Membranes were air dried and baked for 2 h at 80°C before hybridization.

Baked membranes were pretreated in hybridization buffer [0.9 M NaCl, 50 mM sodium phosphate (pH 7.0), 5 mM EDTA, 10 $\times$ Denhardt solution (7), 0.5% sodium dodecyl sulfate (SDS), 0.5 mg of poly(A) per ml] (the hydrophobic Immobilon-N membranes were first pretreated in 95% ethanol and rinsed in ddH₂O) and placed in screw-cap hybridization tubes (Robbins Scientific, Sunnyvale, Calif.), and 10 ml of hybridization buffer was added to each tube. Membranes were incubated (prehybridized) for 2 h at 40°C in a rotating incubator (Robbins Scientific) before addition of 5'-end ³²P-labeled probe (8) in a volume of less than 1 ml. Incubation was

* Corresponding author. Mailing address: Department of Civil Engineering, University of Illinois at Urbana-Champaign, 3221 Newmark Civil Engineering Laboratory, 205 North Mathews Ave., Urbana, IL 61801. Phone: (217) 333-6964. Fax: (217) 333-9464. Electronic mail address: lraskin@cern.ce.uiuc.edu.

† Present address: Augsburg College, Minneapolis MN 55454.

‡ Present address: Laboratory of Molecular Systematics, National Museum of Natural History, Smithsonian Institution, Washington, D.C. 20560.

§ Present address: Department of Civil Engineering, Northwestern University, Evanston, IL 60208-3109.

FIG. 1. Coefficients of variation [$CV = 100(\text{standard deviation}/\text{mean})$] for complete membranes and average CVs for three neighboring slots of different lots (MO9801, MO5201, MO5231, and MO3131) of MagnaGraph. *C. crescentus* RNA (precipitated with 2 volumes of ethanol and sodium acetate) was denatured (1.5% glutaraldehyde, 10-min incubation time at 24°C), and 50 μl of a 1-ng/ μl solution was applied to the membranes (30 replicate slot blots). Probe S-Univ-1390-a-A-18 (11) was used in the hybridization, and the final wash temperature was 46°C. Bound probe was quantified by laser densitometry.

continued for 14 to 18 h at 40°C, and the membranes were subsequently washed for 30 min in 1% SDS-1× SSC at the previously determined temperature of dissociation (6, 8).

The membranes were dried and exposed to preflashed film (Kodak XRP; Eastman Kodak Co., Rochester, N.Y.) with intensifying screens (Cronex Lightning-Plus; E. I. du Pont de Nemours & Co., Inc., Wilmington, Del.) at -80°C. Bound probe was quantified by scanning the developed film with an Ultrascan laser densitometer and Gelscan XL software package (Pharmacia LKB Biotechnology, Uppsala, Sweden). The film response was linear for peak heights between approximately 0.1 and 1.4 absorbance units (AU) (total film absorbance minus background film absorbance). If the AU were not within this range, new exposures were made. Alternatively, bound probe was quantified directly by scintillation counting of the hybridized slot blots.

To study the magnitude of signal response variability associated with individual membranes, we applied 30 replicate slot blots of a *Caulobacter crescentus* RNA sample to membranes from four different lots of MagnaGraph and hybridized them as described above. The coefficients of variation for each membrane (30 replicates) ranged from 20.7 to 52.9% (Fig. 1). Since we routinely apply three replicate RNA samples when performing quantitative hybridizations, we also calculated the average coefficient of variation of three neighboring slots for each membrane. These values ranged from 11.8 to 46.3% (Fig. 1). The application of three replicate nucleic acid samples provides the minimum level of replication necessary to perform statistical analysis of the hybridization results, while still allowing for a manageable experiment with state-of-the-art hybridization and quantification procedures. In this context, it should be noted that we have experienced an increase in precision and detectability when quantifying bound probe with storage phosphorimaging (model 400 series PhosphorImager; Molecular Dynamics, Sunnyvale, Calif.) compared with quantification with X-ray film autoradiography combined with laser densitometry or scintillation counting, which were the methods employed for this study. A discussion of the improved quantification with storage phosphorimaging does not fall within the scope of this study (for more information, see reference 4).

The high coefficients of variation observed with MagnaGraph membranes, combined with significant variations in lot-

to-lot performance, both with respect to detectability and precision, prompted our evaluation of a variety of alternative membrane supports. We tested the influence of application and denaturation conditions on detectability and precision for the following membrane samples: Magna (samples 1 and 2), Magna Charge (samples 5 and 6 and lot S66543), Immobilon-N (lots AOSM8625 and AOSM8644), Nytran (lots 69/8 and 7/1), and MagnaGraph (lot 63242). The sample numbers refer to manufacturer's lot numbers for membrane samples obtained from the manufacturer free of charge, while the lot numbers refer to the manufacturer's lot number provided with purchased membranes.

It is generally accepted that preparation of nucleic acid in high-ionic-strength buffers increases hybridization signals with uncharged membranes and that high salt concentrations inhibit nucleic acid binding to charged membranes (2). However, as our results obtained with MagnaGraph membranes suggest (Fig. 1), the amount of charge applied to commercial membranes appears to vary among lots of the same membrane type. In addition, only a qualitative indication of the amount of charge applied to the various membranes is provided by the manufacturers (described above). Consequently, it is difficult to determine optimal salt concentrations for nucleic acid application a priori. Therefore, we evaluated the effect on hybridization performance of the addition of salt after the glutaraldehyde denaturation step and prior to blotting. After denaturation, half of a *Caulobacter* sp. strain FWC2 RNA sample was diluted with dilution water (low-salt condition) and the other half was adjusted to a final concentration of 10× SSC (high-salt condition). The average hybridization responses from 42 replicate slot blots for the low-salt and high-salt samples are shown in Fig. 2. For low-salt conditions, the charge-modified Magna Charge (samples 5 and 6) and Immobilon-N (lot AOSM8625) membranes gave a signal 300 to 500 times greater than those of the other membranes (MagnaGraph lot 63242, Magna samples 1 and 2, Nytran lot 69/8). The responses for the Nytran membranes for low-salt conditions are not shown in Fig. 2. Nytran lot 69/8 demonstrated a response

FIG. 2. Average hybridization response (for 42 replicate slot blots) for RNA applied under low-salt and high-salt conditions. AU for different exposure times on different days were standardized for exposure during 24 h on the same day and normalized by the low-salt Immobilon-N response. *Caulobacter* sp. strain FWC2 RNA (precipitated with 2 volumes of ethanol and sodium acetate) was denatured (1.5% glutaraldehyde, 10-min incubation time at 24°C), and 50 μl of a 0.5-ng/ μl solution was applied to two Magna membranes (samples 1 and 2 [bars 1 and 2, respectively]), two Magna Charge membranes (samples 5 and 6 [bars 3 and 4, respectively]), one Immobilon-N membrane (lot AOSM8625 [bar 5]), two Nytran membranes (lots 69/8 and 7/1 [bars 6 and 7, respectively]), and one MagnaGraph membrane (lot 63242 [bar 8]). Probe S-Univ-1390-a-A-18 (11) was used in the hybridizations, and the final wash temperature was 46°C. Bound probe was quantified by laser densitometry.

FIG. 3. Coefficients of variation (CV) for complete membrane (42 replicate slot blots) for RNA applied under low-salt and high-salt conditions. Membranes and experimental conditions are as specified in the legend to Fig. 2.

similar to that of the low-signal membranes; Nytran lot 7/1 was highly variable, with the response of one portion of the membrane comparable to those of the other low-signal membranes, whereas the other portion gave a much greater response. The results obtained with the Nytran membranes suggested a substantial variation in the amount of charge associated with the membrane within a lot, as well as between lots of membranes. When SSC was added, the responses of the low-signal membranes improved (were 10 to 15 times greater), while the responses for Magna Charge and Immobilon-N membranes decreased considerably (20- and 4-fold, respectively).

In addition to comparing the hybridization responses of Magna Charge, Magna, MagnaGraph, Immobilon-N, and Nytran membranes under different conditions, we examined the variability between replicate slots on each of these membranes with and without addition of SSC. Forty-two replicate slot blots of a nucleic acid sample with and without SSC were applied to the different membranes. After hybridization, the coefficients of variation for each membrane were determined. The results of this study (Fig. 3) revealed that membranes that demonstrate a high-level hybridization response do not necessarily demonstrate less variability. For the low-salt condition, the coefficients of variation were approximately 10% for Immobilon-N and Magna Charge. (Data for Nytran membranes are not reported because of the high level of variation in response between two portions of the same membrane [described above].) The addition of SSC increased the variability in all cases (except for Nytran lot 7/1, which demonstrated a very high level of variability without addition of SSC).

The experiments described above confirmed that the signal response for uncharged or slightly charged membranes can be improved by increasing the ionic strength of nucleic acid samples. However, a consequence of improved detectability is that the variability of the signal response increases as well. If detectability is not a concern (i.e., if the different 16S rRNAs are present in concentrations well above the detection limit of the method), it may be desirable not to increase the ionic strength of the nucleic acid solution in order to keep the variability as low as possible.

RNA denaturation (1.5% glutaraldehyde, 10-min incubation at room temperature) and precipitation conditions were compared for the charge-modified Magna Charge (lot S66543) and Immobilon-N (lot AOSM8644) membranes with *Fibrobacter succinogenes* RNA and oligonucleotide probe S-Univ-1392-a-A-15 (9). This oligonucleotide is complementary to virtually all known 16S-like rRNAs. For both membranes, the detectability

determined by averaging the responses of 30 replicate slot blots with nondenatured nucleic acid was slightly better (15 to 20%) than the detectability with denatured nucleic acid. For probe S-Univ-1392-a-A-15, the reduction in signal associated with denaturation is likely a reflection of the character of the target site within the 16S rRNA molecule. The target site for probe S-Univ-1392-a-A-15 apparently does not participate significantly in intramolecular structure (1). Thus, target accessibility is not improved by glutaraldehyde denaturation, and denaturation is actually associated with signal loss. This is in contrast to hybridization results obtained with *F. succinogenes* RNA and oligonucleotide probe S-Ss-F.suc-0628-a-A-22 (3), for which the target site is located in a more structured region of the molecule (1). Figure 4 shows the hybridization response versus the glutaraldehyde concentration used for RNA denaturation at room temperature (Fig. 4a) and at 50°C (Fig. 4b). Generally, these results indicate that the signal intensity, and hence the detectability, can be improved considerably with increased denaturation (increased glutaraldehyde concentration, incubation temperature, and incubation time) up to a maximum point beyond which further denaturation has no effect or even decreases the signal (reflecting either reduced retention by the membrane or destruction of the target 16S rRNA). Since intermediate denaturing conditions yielded maximum response, the increase in response with increased dena-

FIG. 4. Influence of RNA denaturation conditions on hybridization response (AU). *F. succinogenes* RNA (precipitated with 2 volumes of ethanol and sodium acetate) was denatured with different concentrations of glutaraldehyde and different incubation times. After dilution, 100 μ l of a 0.2-ng/ μ l solution was applied to MagnaGraph membranes. Probe S-Ss-F.suc-0628-a-A-22 (3) was used in the hybridization, and the final wash temperature was 58°C. Bound probe was quantified by laser densitometry. (a) Denaturation at room temperature (24°C). (b) Denaturation at 50°C.

FIG. 5. Hybridization response for the experiment examining RNA precipitation with Magna Charge (lot S66543) and MagnaGraph (lot 63242) membranes with oligonucleotide probe S-Univ-1392-a-A-15 (9). Fifty microliters of different concentrations of denatured (1.5% glutaraldehyde, 10-min incubation time at 24°C) *Fibrobacter intestinalis* RNA was applied to the membranes (approximate amounts of 16S rRNA are given in nanograms). (a) Magna Charge membrane. Nucleic acid was precipitated with 2 volumes of ethanol and sodium acetate. (b) Magna Charge membrane. Nucleic acid was precipitated with 0.6 volumes of isopropanol and ammonium acetate. (c) MagnaGraph membrane. Nucleic acid was precipitated with 2 volumes of ethanol and sodium acetate. (d) MagnaGraph membrane. Nucleic acid was precipitated with 0.6 volumes of isopropanol and ammonium acetate.

turation is most consistent with an increase in the amount of target able to participate in hybridization. The decreased response under stronger denaturation conditions is consistent with target destruction as observed for probe S-Univ-1392-a-A-15.

The retention of isopropanol (amended with 2.5 M ammonium acetate)-precipitated samples was compared with the retention of ethanol (amended with 0.3 M sodium acetate)-precipitated samples with charge-modified Magna Charge membranes (lot S66543). The responses were comparable for both precipitation conditions, and nucleic acid was detectable at amounts as low as 0.1 ng of 16S rRNA (Fig. 5a and b). A similar experiment, in which the slightly charged MagnaGraph membranes (lot 63242) were used, demonstrated detection limits for ethanol (amended with 0.3 M sodium acetate)- and isopropanol (amended with 2.5 M ammonium acetate)-precipitated samples of approximately 10 and 0.1 ng of 16S rRNA, respectively (Fig. 5c and d). It is likely that residual salt carried over from the isopropanol precipitation step, which employed a high concentration of ammonium acetate, improved RNA retention on the slightly charged MagnaGraph membranes (the level of coprecipitation of salts is higher with isopropanol than with ethanol [7, 10]). This is consistent with our earlier observations presented in Fig. 2: a 10- to 15-fold signal increase was observed for the MagnaGraph membrane when the ionic strength of the RNA sample was increased. Thus, RNA precipitation conditions, which apparently affect the final salt concentration in the RNA solution, do not appreciably influence hybridization detectability for charge-modified membranes but do have a significant effect on the RNA detection limit for slightly charged (and presumably uncharged) membranes.

In conclusion, Magna Charge and Immobilon-N membranes gave a much higher (300 to 500 times) level of hybridization response than the other membranes tested (Magna, MagnaGraph, and Nytran). Both Magna Charge and Immobilon-N membranes apparently have a very high nucleic acid binding capacity, which decreases considerably when high-ionic-strength nucleic acid solutions are used but which does not vary much under different denaturation conditions or with different nucleic acid precipitation methods (nucleic acid precipitation

was only evaluated for Magna Charge membranes). Although the hybridization detectability with uncharged and slightly charged membranes was improved (10 to 15 times) by increasing the ionic strength of the nucleic acid samples, the hybridization response was still much lower than that for Immobilon-N and Magna Charge membranes. However, since many hybridization techniques are only used for hybrid detection, nylon membranes produced by standard manufacturing processes (such as Magna, MagnaGraph, and Nytran) should serve well, providing that quantification of low-abundance species is not a concern and that nucleic acid preparation and application protocols are optimized for each membrane type. (Sometimes, optimization may even be necessary for each membrane lot.)

In addition to the evaluation of detectability, the assessment of precision is equally important as part of a quantitative hybridization assay. In this study, coefficients of variation for hybridization responses for individual membranes ranged from approximately 10% (Immobilon-N and Magna Charge membranes) to 50% (some MagnaGraph membranes). This relatively large variability in local membrane properties appears to be the greatest source of experimental error in quantitative hybridization experiments. Therefore, in order to estimate variability in each experiment, it is critical to apply multiple blots of the same sample. (We have found that the application of three blots is often the upper practical limit in state-of-the-art hybridization and quantification procedures.) In addition, because of the relatively large amount of variability in local membrane properties, it is important to estimate the concentration of target RNA in environmental samples by applying RNA samples and a reference series of known amounts of target RNA to the same membrane.

This research was supported by grant N00014-88-K-0093 from the Office of Naval Research and cooperative research agreement CR812496-01-3 from the U.S. Environmental Protection Agency. M.D.K. was the recipient of a Postdoctoral Fellowship in Environmental Biology (BSR-9001832) from the National Science Foundation.

REFERENCES

- Gutell, R. R., N. Larsen, and C. R. Woese. 1994. Lessons from an evolving rRNA: 16S and 23S rRNA structures from a comparative perspective. *Microbiol. Rev.* 58:10-26.
- Khandjian, E. W. 1987. Optimized hybridization of DNA blotted and fixed to nitrocellulose and nylon membranes. *Bio/Technology* 5:165-170.
- Lin, C., B. Flesher, W. C. Capman, R. I. Amann, and D. A. Stahl. 1994. Taxon specific hybridization probes for fiber-digesting bacteria suggest novel gut-associated *Fibrobacter*. *Syst. Appl. Microbiol.* 17:418-424.
- Raskin, L., W. C. Capman, R. Sharp, L. K. Poulsen, and D. A. Stahl. Molecular ecology of gastrointestinal ecosystems. In R. I. Mackie, B. A. White, and R. E. Isaacson (ed.), *Ecology and physiology of gastrointestinal microbes*, vol. 2. Gastrointestinal microbiology and host interactions, in press. Chapman & Hall, Ltd., London.
- Raskin, L., L. K. Poulsen, D. R. Noguera, B. E. Rittmann, and D. A. Stahl. 1994. Quantification of methanogenic groups in anaerobic biological reactors by oligonucleotide probe hybridization. *Appl. Environ. Microbiol.* 60:1241-1248.
- Raskin, L., J. M. Stromley, B. E. Rittmann, and D. A. Stahl. 1994. Group-specific 16S rRNA hybridization probes to describe natural communities of methanogens. *Appl. Environ. Microbiol.* 60:1232-1240.
- Sambrook, J., E. F. Fritsch, and T. Maniatis. 1989. *Molecular cloning: a laboratory manual*, 2nd ed. Cold Spring Harbor Laboratory Press, Cold Spring Harbor, N.Y.
- Stahl, D. A., and R. Amann. 1991. Development and application of nucleic acid probes, p. 205-248. In E. Stackebrandt and M. Goodfellow (ed.), *Nucleic acid techniques in bacterial systematics*. John Wiley & Sons, Ltd.
- Stahl, D. A., B. Flesher, H. R. Mansfield, and L. Montgomery. 1988. Use of phylogenetically based hybridization probes for studies of ruminal microbial ecology. *Appl. Environ. Microbiol.* 54:1079-1084.
- Tijssen, P. 1993. Hybridization with nucleic acid probes, part I: theory and nucleic acid precipitation. Elsevier Science Publishers B.V., Amsterdam.
- Zheng, D., L. Raskin, E. W. Alm, and D. A. Stahl. Unpublished data.