

GENETIC ENGINEERING GEN NEWS

Northern Hybridization Analysis

Tutorial: Comparing Different Membranes and Fixation Methods

Rich Sobel, Ph.D.,
Andrew Dubitsky, Ph.D., and
Yardenah Brickman, Ph.D.

The cellular response to experimental or environmental stimulus can be more readily understood when subpopulations of RNA are harvested and characterized. This gives the researcher an insight into the differential expression of genes and possibly the cellular level at which these genes are modulated. There are numerous methods of analyzing RNA once it is isolated, including Northern blot analysis, nuclease-protection assays, and nuclear runoff assays.

The successful production of Northern blots depends on minimal RNA degradation, efficient transfer out of the gel and immobilization on the membrane, and the ability to reliably detect the sequence of interest. This tutorial compares the effectiveness of different

membranes, in terms of transfer efficiency, sensitivity, and reproducibility of results.

Nylon Membranes

The methods used in this report can serve as an example of how Northern blotting with nylon membranes has been optimized for measuring gene expression in a prostate cancer cell line. The basic protocol for nylon membranes requires that the RNA be denatured in the presence of formaldehyde (most often in the gel) and does not require any additional treatment for efficient transfer.

Immobilized RNA should be cross-linked to the membrane with UV light for highest possible sensitivity. It is then available for detection using a variety of labeled probes, including radiolabeled synthetic oligonucleotides, DNA labeled by nick translation, and random primed DNA prepared in the presence of labeled nucleotides.

The most common method of detec-

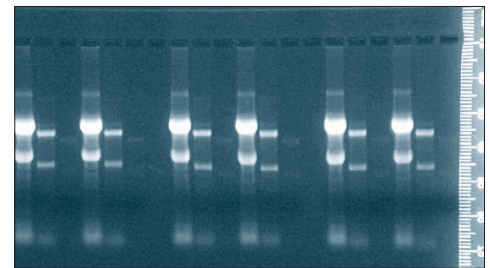


Figure 1. Ethidium-bromide-stained RNA after electrophoresis in a formaldehyde-agarose gel. Six series of lanes were loaded with 10 g, 1 g, and 0.1 g of total RNA. Band intensity confirmed equivalent loading to each series on the gel.

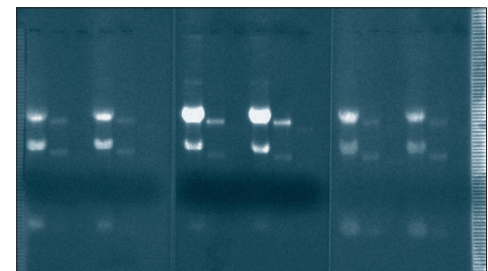


Figure 2. Ethidium-bromide-stained RNA after alkaline transfer to nylon membranes, visualized with a UV trans-illuminator. Biodyne B membrane shows higher levels of RNA than do competitor membranes under these transfer conditions.

tion is radioactivity, although in recent years, chemiluminescent detection has been gaining wider acceptance, given the decreased hazards associated with non-radioactive detection techniques.

A variety of buffers for transfers have

been described in the literature.¹ It is widely accepted that charged nylon membranes retain nucleic acids in alkaline solutions², which was thought to eliminate the need of post-transfer fixation (via baking or exposure to ultraviolet irradiation).

While transfer in a weak alkali solution showed that RNA is captured and retained by the membranes, there is compelling evidence that with all membranes tested, UV cross-linking enhances the ability to detect low-level transcripts.

Results presented here indicate that the Biodyne® B membrane (Pall Corp.; East Hills, NY) has a better signal-to-noise ratio than do other positively charged nylon membranes.

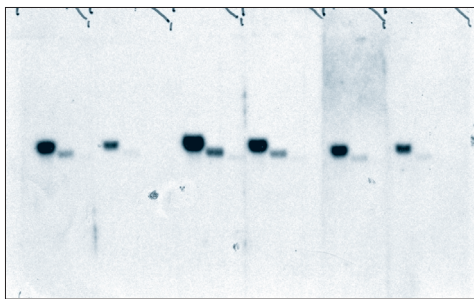


Figure 4. Phosphorimager scan (3-hour screen exposure) after hybridization with ³²P-labeled GAPDH probe; high-stringency wash (62°C). Signal is significantly higher on the Biodyne B membrane than on the competitor nylon membranes. 0.1 g RNA produced faint signal only on the Biodyne B membrane following UV fixation.

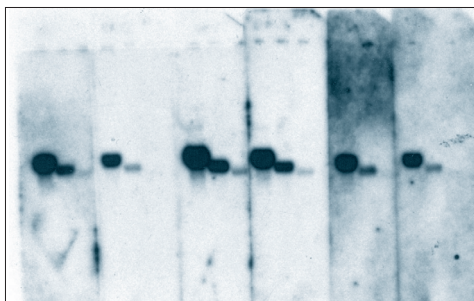


Figure 5. Overnight autorad film exposure, membranes as shown in Figure 4 (hybridized with ³²P-labeled GAPDH probe followed by high-stringency washes); 0.1 g RNA produces clear signal only on the Biodyne B membrane; signal is stronger when UV fixation is used. High background levels on Nylon membrane 2 interfere with the ability to analyze the blots and achieve results suitable for publication.

Results also show that higher signal-to-noise affords better sensitivity, since low-level signals can be seen more clearly.

Biodyne B membrane is a hydrophilic nylon microporous membrane with quaternary ammonium surface chemistry. The membrane is strongly cationic. This positive charge is maintained over a broad pH range and promotes strong ionic binding of nucleic acids, making it ideal for rapid transfer techniques. In addition, this strong binding makes it suitable for prolonged transfer procedures, without the risk of nucleic acid diffusion from the membrane.

Nylon 1 (competitor membrane) is a neutral nylon membrane, with typical binding capacities for nucleic acids of up to 600 µg/cm². Similar to Biodyne B membrane, it is hydrophilic and therefore requires no prewetting. Nylon Membrane 2, from a second manufacturer, is a positively charged nylon membrane that was designed to give optimum signal-noise ratios when used with radioactively labeled probes.

Methods

Total RNA from LNCaP (a prostate cancer cell line) was pre-

pared from log-phase tissue culture cells. The culture media (RPMI with 5% FBS/pen-strep) was removed by aspiration, and cells were removed directly into TRIzol reagent (Life Technologies, a division of Invitrogen Corp.; Carlsbad, CA) for isolating RNA. RNA was then quantified by spectrophotometry.

Lanes of 10, 1.0, and 0.1 µg of total RNA were loaded in six replicates on one 1.2% agarose/ MOPS/ formaldehyde gel and electrophoresed at 6 V/cm in 1X MOPS/ formaldehyde buffer. Loading buffer contained ethidium bromide to visualize samples. Gel was transferred directly after electrophoresis with no prior treatment, except for a brief 2- to 3-second exposure to UV for acquiring a photograph at the conclusion of electrophoresis, to confirm equivalent loads in respective lanes.

Overnight capillary transfer in 10 mM NaOH was performed for ~18 hours onto three separate membranes, Pall Biodyne B membrane, noncharged Nylon 1, and positively charged Nylon 2. Each membrane covered two replicates (six lanes) of the three different amounts of RNA loaded.

Following transfer, membranes were

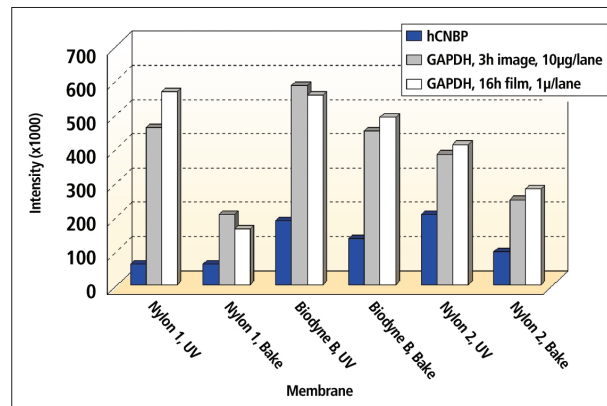


Figure 6. Relative signal intensity on images. This plot shows highest signal strength on Biodyne B membrane. Values for Nylon membrane 2 are artificially high, as background signal is included in the band analysis.

briefly exposed to a UV trans-illuminator to facilitate accurate cutting in half (three lanes per half), and either cross-linked in a Stratalinker® (Stratagene, Inc.; La Jolla, CA) at the manufacturer's recommended setting, or baked for two hours at 80°C. Membranes were photographed on the transilluminator to confirm transfer, as was the gel to confirm completeness of transfer.

Membranes were prehybridized at 42°C in ULTRAhybe™ (Ambion, Inc.; Austin, TX) for several hours. Membranes were then hybridized overnight with random prime radiolabeled probe in a hybridization oven with rotating bottles (Thermo Hybaid; Franklin, MA). 1.5×10^6 cpm/mL of random primed labeled probe was used for both hCNBP and hGAPDH probes.

After the initial probing and visualization with hCNBP, membranes were stripped in vigorously boiling 0.5% SDS for 10 minutes and then shaken in the same solution until it reached room temperature (several hours). Successful stripping of the membranes was confirmed by loss of signal as detectable by a Geiger counter. Membranes were then reprobed with hGAPDH, a high-copy message, for additional normalization of hCNBP signals.

Membranes were washed with three series of buffers with increasing stringency. Low-stringency washes (2X SSC, 0.1%SDS) consisted of two washes for 10 minutes each, followed by optional two medium-stringency washes (1X SSC, 0.1%SDS) for 15 minutes each.

Finally, two final high-stringency washes (0.1X SSC, 0.1%SDS) were performed for 20–30 minutes each at the hybridization temperature or higher (as indicated in the figure legends). All washes were performed in hybridization

bottles rotating at high speed. Membranes were then enclosed in plastic and exposed to a phosphorimager screen and then to Kodak BMS film (Kodak; Rochester, NY). Band intensities on electronic images were quantified with ImageQuant software (Molecular Dynamics; Sunnyvale, CA).

Results

A UV photograph of the ethidium bromide stained gel is shown in Figure 1. There are similar intensities for the 28S and 16S rRNA bands in all series, indicating that equal amounts of RNA were available for transfer to the different membranes.

After transfer, a photograph of the membrane was taken with UV illumination (Figure 2). Signal strength is now clearly higher on the Biodyne B membrane (see table). Hybridization with the hCNBP probe shows clear bands only on the Biodyne B membrane, most clearly following UV fixation (Figure 3). Background signal on Nylon Membrane 2 interferes with quantification, producing an artificially high reading.

Stripping and reprobing with GAPDH probe indicates that target RNA remains on the membrane after high-stringency washing and stripping. Signal is clearly highest on Biodyne B

membrane in the 3-hour phosphorimage (Figure 4). A faint band can be seen in the 0.1- μ g lane on the UV-fixed Biodyne B membrane.

An extended exposure of these membranes with Kodak BMS film again shows background intruding onto the signal for Nylon Membrane 2. Biodyne B membrane provides a strong clear signal, even at 0.1 μ g per lane (Figure 5).

A comparison chart of signal strength for major bands is shown in Figure 6. Biodyne B membrane has the highest overall signal in each instance, except for hybridization with hCNBP probe. Here, Nylon Membrane 2 produced a higher signal, due to background artifacts.

Discussion and Conclusions

The traditional approach in analyzing gene expression—isolating full-length, nondegraded RNA—is only measurable once the RNA has been transferred to a solid support and detected. Nylon membranes are suited for these applications because of irreversible binding of nucleic acids, accessibility to labeled probes, and the ability to withstand multiple processing cycles.

The researcher can assess the integrity of the sample by probing for a control transcript and the transcript from a gene of interest. Once on the membrane, it is advantageous to be able to reprobe the

Band Intensity (ImageQuant Volume / 1,000)

| Membrane/ Fixation: | All bands in lane | | | Major Band | | | |
|------------------------|--------------------|---------------------|---------------------|---------------------|---------------------|---------------------|--------------------|
| | Fig 1 1 μ g | Fig 1 10 μ g | Fig 1 10 μ g | Fig 2 10 μ g | Fig 3 10 μ g | Fig 4 10 μ g | Fig 5 1 μ g |
| Nylon 1, UV | 160 | 388 | 121 | 100 | 60 | 458 | 563 |
| Nylon 1, Bake | 164 | 386 | 116 | 104 | 60 | 204 | 162 |
| Biodyne B, UV | 173 | 393 | 117 | 142 | 186 | 582 | 554 |
| Biodyne B, Bake | 173 | 391 | 115 | 137 | 134 | 449 | 488 |
| Nylon 2, UV | 179 | 416 | 122 | 109* | 205* | 381* | 409* |
| Nylon 2, Bake | 182 | 394 | 115 | 96 | 96 | 248 | 279 |
| NOTES | lane on gel | lane on gel | on gel | post- transfer | hCNBP | GAPDH | GAPDH |

* Values artificially inflated due to presence of high background signal in the area of the band.

RNA sample for various target RNA species without loss of target, so that the task of isolating new RNA is not repeated unnecessarily.

Since the isolation process is the most time-consuming and labor-intensive part of the procedure and there is extensive handling of the RNA (providing opportunity for RNase degradation), the number of times RNA is isolated from a given sample must be minimized. This is greatly aided by a membrane that allows repeat screenings from a single RNA-isolation process.

In these experiments, three different amounts of total RNA were run on an agarose gel and used as targets for a control transcript, GAPDH, and for the transcript of interest, hCNBR. The original gel shows that approximately equal amounts of RNA were added to replicate wells. Exposure of the gel to UV light after the transfer step indicated that all detectable levels of RNA had left the gel.

Most protocols for RNA transfer specify the use of 10X or 20X SSC transfer buffer; however, this study indicates that effective transfer and immobilization will also take place if 10-mM NaOH is used. Immediately after the transfer step, trans-illumination showed that the Biotodyne B membrane bound higher levels of RNA than did either the competitor neutral or positively charged membranes. Visual detection of RNA in this manner is not sensitive, so the experiment continued with radiolabelled probes to both the control RNA transcript and the hCNBP.

Two methods of fixation were tested in this study. UV cross-linking was more effective in binding the RNA to the membrane than oven baking at 80°C. This is evident in the case of all three membranes, as the ability to detect the lower levels of RNA is better on all of the membranes that had been cross-linked. These results indicate that cross-linking the nucleic acids to the membrane before hybridization can enhance sensitivity.

Signal-to-noise ratio plays an important role in the endpoint sensitivity of a given membrane. The neutral membrane (Nylon Membrane 1) produced a similar noise level to that with the Pall Biotodyne B membrane in both of the hybridizations, but signal is significantly stronger on the Biotodyne B membrane. This allows for the clear visualization of the band at 0.1 µg RNA on both the cross-linked and baked Pall membranes.

The signal from this band could be clearly seen on either of the competitor membranes. All of these results indicate that Biotodyne B membrane binds more completely, providing greater sensitivity, and therefore is suited for detection of low-level transcripts.

Nylon Membrane 2, the other positively-charged membrane, also shows good sensitivity as faint signal is visible in the lanes with lower amounts of RNA. It has, however, a much higher noise component than does the Biotodyne B membrane. Background signal in the area of the band of interest artificially inflated the intensity value for that band. Further, high background levels

make it more difficult to achieve results suitable for publication. This is obvious with both the control (GAPDH) and the test transcript (hCNBP).

Finally, there does not appear to be any problem with stripping the first probe from the membrane (GAPDH) and reprobing it for the gene of interest (hCNBP), with respect either to sensitivity or increased background. Stripping and reprobing minimizes the number of times RNA isolation must be performed. Similar data has been confirmed for Biotodyne B membrane and its use in Southern blotting, where the membrane can be reprobed multiple times (six probes are used for standard RFLP analysis) without loss of target.

Biotodyne B membrane thus shows high sensitivity for both DNA and RNA sequences immobilized on the membrane. When appropriate blocking agents are used, background levels can be reduced to insignificant levels. Because of these properties, Biotodyne B membrane is suited for samples with limited biomolecular content. **GEN**

Rich Sobel, Ph.D., is a senior scientist at the Vancouver General Hospital Prostate Centre. Andrew Dubitsky, Ph.D., is a technical director at Pall Corp. (East Hills, NY), and Yardenah Brickman, Ph.D., is the market manager at Pall Corp., based in Mississauga, Canada. Please address correspondence to Yardenah Brickman, Pall Corp., 7205 Mill Creek Drive, Mississauga, Ontario, Canada L5N 3R3. E-mail: Yardenah_Brickman@pall.com.

References

1. Sambrook, J., Fritsch, E.F., & Maniatis, T. *Molecular Cloning: A Laboratory Manual*. (Cold Spring Harbor Laboratory Press, NY, 1989).
2. Reed, K.C. and Mann, D.A. Rapid transfer of DNA from agarose gels to nylon membranes. *Nucleic Acids Res.* **13**, 7207 (1985).