Optimization of northern blotting on Immobilon-Ny+

Tech Note - TN110

Summary

For optimum performance on northern blotting, the recommendations below should be followed with Immobilon-Ny+:

RNA fixation by UV cross-linking enhances signal relative to the baking method. The optimal energy recommended at 254 nm is 20,000 µJoules/cm2 for DNA probes and 40,000 µJoules/cm2for RNA probes.

Modified Church hybridization solution works well for northern blots. Formamide may be added to the hybridization solution at concentration of 33-50% (v/v) to prevent cross-hybridization of an RNA probe to ribosomal RNA.

The stripping method using hot 0.1% (w/v) SDS is recommended for northern blots. Do not use sodium hydroxide for stripping of northern blots. Signal intensity decreases dramatically upon the first re-probing after the blot is stripped with 50 mM NaOH.

For reprobing on northern blots, hybridization with DNA probes at 68°C produces better results than RNA probes in formamide-based buffers. Also, probes should be used in an order so that low abundance targets are analyzed first and high abundance targets later.

Introduction

Many northern blotting analysis studies have been published (1), however, few examples are provided to support the various conditions specified for RNA fixation (2, 3), reprobing, and stripping (4, 5). Therefore, we systematically determined the conditions that consistently produce the best results on northern blots. In this technical note, quantitative data are presented to support the recommended conditions for UV cross-linking, probe stripping, and re-probing on northern blots using Immobilon-Ny+ positively charged nylon membrane (6,7,8,9). Experimental data were quantified by phosphor imaging analysis. A general, step-by-step protocol for northern blotting is provided at the back of technical note as an appendix.

Materials and Methods (See appendix for general protocol)

RNA Electrophoresis and Capillary Blotting (4). Mouse liver total RNA (Ambion, Inc. Austin, Texas, USA) was diluted in sample diluent (10 mM MOPS, pH 7.0, 4 mM sodium acetate, 0.5 mM EDTA, 2.2 M formaldehyde, 50% (v/v) formamide). The diluted RNA samples were denatured at 65°C for 15 minutes, chilled on ice, and then mixed with a 1/10 volume of loading buffer (50% (v/v) glycerol, 1 mM EDTA, pH 8.0, 0.25% bromophenol blue, 0.25% xylene cyanol). Aliquots of 11 µL were loaded onto 1.0% agarose gels prepared in 1X formaldehyde gel-running buffer (10 mM MOPS, pH 7.0, 8 mM sodium acetate, 1 mM EDTA) and 2.2 M formaldehyde. RNA samples were resolved at 120 Volts for 1 hr 20 min using 1X formaldehyde gel-running buffer. After electrophoresis, the gels were rinsed briefly in Milli-Q® water and incubated twice in 20 x SSC for 30 min with gentle agitation. The capillary transfer was done in 20 x SSC overnight.

UV Cross-linking of RNA. RNA was fixed to the membranes by exposure to UV light at 254 nm using a Stratalinker®2400 (Stratagene® Cloning Systems, La Jolla, California, USA) using one UV bulb at the center and one at each end. Although irradiation with fewer UV bulbs takes longer, this ensures precise energy control of UV irradiation at low energy levels. The side of the membrane with the bound RNA faced the bulbs. By using the energy mode on the Stratalinker, drift in power output of the UV bulbs was compensated for. This was considered essential for low energy exposures, as the energy output of the UV bulbs was approximately 1500 µJoules/cm2 per second.

Probe labeling. RNA probes were labeled with -32P-UTP (800 Ci/mmol) by in vitro transcription (10). The antisense probe templates (cyclophilin, -actin, GAPDH: glyceraldehyde 3-phosphate dehydrogenase and 18S-rRNA) were purchased from Ambion, Inc. DNA probe templates (Ambion, Inc.) were labeled with -32P-dCTP (3,000 Ci/mmol) by random primer labeling (11). For chemiluminescent detection, a DNA probe template of GAPDH was labeled using DIG-High Prime Labeling Kit (Boehringer Mannheim, Indianapolis, IN).

Pre-hybridization and Hybridization. Membranes were wet in Milli-Q water and placed in a single layer in glass hybridization tubes. For pre-hybridization, 10 mL of hybridization solution (0.5 M sodium phosphate, pH 7.1, 2 mM EDTA, 7% (w/v) SDS,

0.1% (w/v) sodium pyrophosphate) modified from Church and Gilbert (12, 13) was added to each tube of 30 cm length. For RNA probes, formamide was used at a final concentration of 50% (v/v). The tubes were placed in a rolling bottle hybridization oven and incubated for 1 to 2 h at 68°C. The pre-hybridization solution was poured from the tubes and replaced with 3 mL of hybridization solution containing 32P-labeled probe at a ratio of 10^5 cpm per cm2 of membrane surface area. For chemiluminescent detection, 10mL of hybridization solution containing digoxigenin-labeled DNA probe was used at a final concentration of 10ng/mL. The tubes were returned to the hybridization oven and incubated overnight at 68°C. The blots were rinsed in wash solution I (1 x SSPE, 0.5% (w/v) 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 x SSPE, 0.1% (w/v) SDS) and washed twice in fresh wash solution II for 15-20 min at 68°C. All washing steps were done in the hybridization bottles. All solutions were filtered through Express™ GP membrane filter units (0.22 µm, Millipore) to remove any particles.

Chemiluminescent Detection

Detection of digoxigenin-labeled probe hybridized on the membrane was done using a CSPD® substrate (Boehringer Mannheim, Indianapolis, IN) according to the manufacturer's protocol except for addition of a quick rinse prior to washing (9). The membrane was exposed to an X-ray film for 10 minutes.

Stripping (4,5) and Re-probing

Hot 0.1% SDS Method: Hybridized probe was stripped from the blots by two incubations in hot 0.1% (w/v) SDS for 15 minutes with gentle agitation. The SDS solution was heated to boiling, removed from the heat, and the blots were added.

Alkaline Method: Hybridized probe was stripped from the blots by incubation in 50 mM NaOH for 30 minutes at 45°C followed by neutralization in 50 mM Tris-HCl, pH 7.5, 0.1 x SSC, 0.1% (w/v) SDS for 15 min. The completeness of stripping was assessed by autoradiography for the standard exposure time.

Imaging. Radioactivity on the membranes was visualized by phosphor imaging on a Storm™ 840 Phosphor Imaging System (Molecular Dynamics, Sunnyvale, California, USA). ImageQuaNT™ analysis software was used to quantify bands on images generated with the Storm 840 System.

Results and Discussion

Effect of UV Cross-linking on Sensitivity.

Recommendation: Use UV cross-linking at 254 nm to maximize hybridization signal. UV cross-linking enhanced the hybridization signal up to 15 times as compared to no fixation.

Results: Efficacy of UV cross-linking on northern blots was compared with baking (Fig. 1, Graph 1). Although a common belief is that a positively charged nylon membrane does not require special fixation of DNA or RNA on the blot, fixation by UV cross-linking enhanced the sensitivity of RNA detection up to fifteen times above no fixation. The signal enhancement may result from covalent attachment of RNA molecules to the membrane surface (2,3). Fixation by baking at 80C for 1 hr improved the signal five to six times over no fixation. The data suggested that non-covalent interaction of RNA with the membrane imparted by baking is not strong enough to withstand repeated stripping and re-probing (see Assessment of Re-probability on Northern Blots section below).

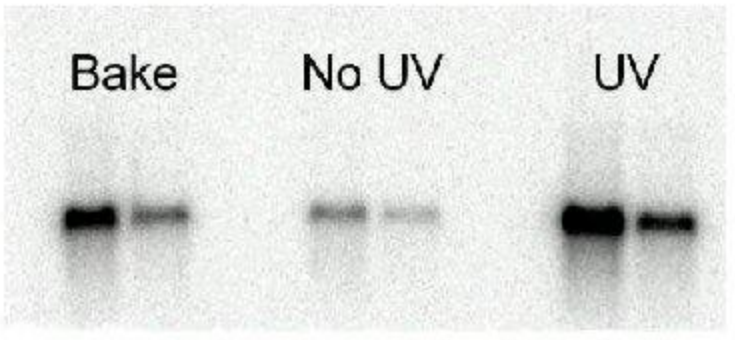
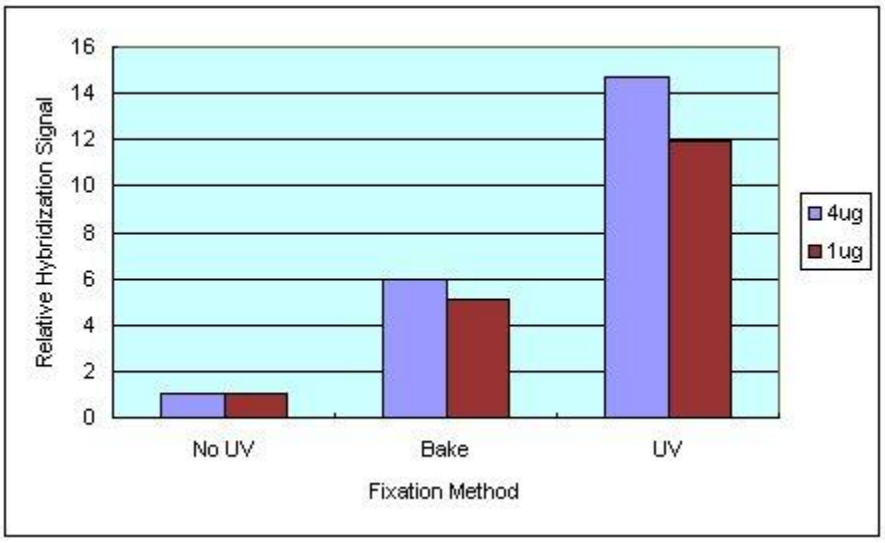


Fig. 1. Effect of Fixation Methods on Northern Hybridization using Immobilon-Ny+.
Mouse liver total RNA was applied to Immobilon-Ny+ by capillary blotting from a formaldehyde agarose gel. After drying the membrane, the RNA was fixed to the membranes by baking at 80°C under vacuum for 1 hr (Bake) or by UV cross-linking with 30,000 µJoules/cm2 at 254 nm (UV). The blots were hybridized using 32P-labeled GAPDH RNA probe. The amount of total RNA loaded was 4 (left lane of each pair) and 1 µg (right lane).

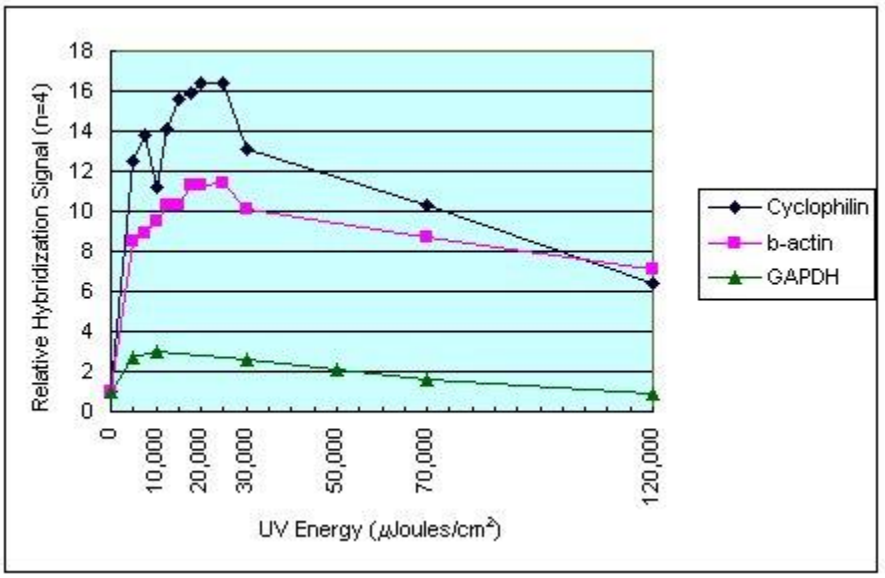


Graph 1. Quantification of Hybridization Signals after Various Fixation Protocols.
The hybridization signals were normalized to the respective signals at 0 µJoules/cm2 ("No UV"). Bake: 80°C under vacuum for 1 hr: UV: 30,000 µJoules/cm2 at 254 nm.

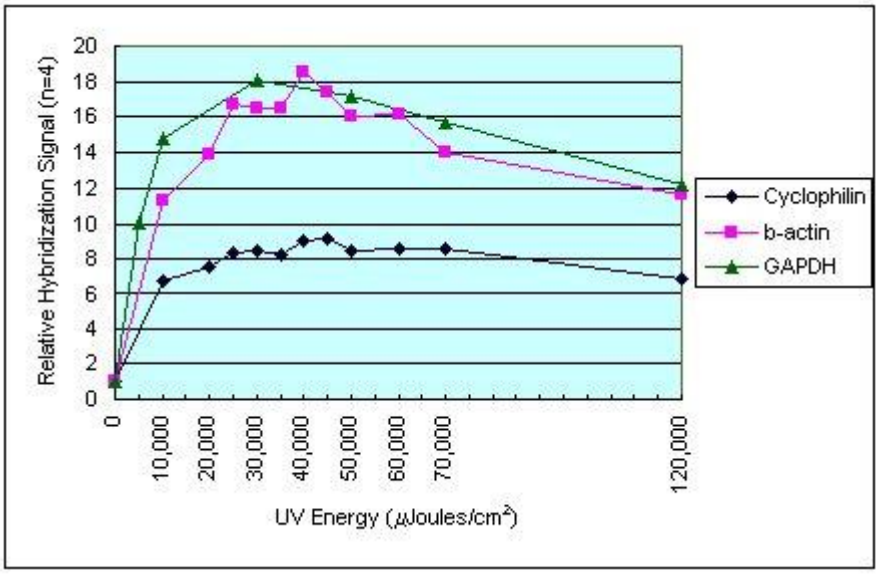
Optimization of UV Cross-linking Energy.

Recommendation: For maximum hybridization signal, cross-link either with 20,000 µJoules/cm^2 at 254 nm for DNA probes or 40,000 µJoules/cm2 at 254 nm for RNA probes.

Results: The effect of UV energy at 254 nm on hybridization of total RNA blotted to Immobilon-Ny+ was examined using three types of DNA and RNA probes. The most intense hybridization signals were observed between 10,000 and 30,000 µJoules/cm2 for the DNA probes (Graph 2) and between 30,000 and 45,000 µJoules/cm2 for the RNA probes (Graph 3). A three- to eighteen-fold signal enhancement was observed with the optimal UV cross-linking, as compared to no UV cross-linking. The intensity profiles were similar for each set of probes. Unlike the effect of UV energy of signal intensity reported previously for DNA/DNA hybridization (6), signal intensity did not decrease rapidly at higher UV energy levels.



Graph 2. Relationship of Hybridization Signal to UV Energy for DNA Probes.
Mouse liver total RNA (1 g) was resolved in a formaldehyde agarose gel and transferred to Immobilon-Ny+ by capillary blotting. The blots were dried completely, and then UV cross-linked with varying UV energies at 254 nm. The blots were hybridized using a 32P-labeled DNA probe, and the signals were quantified (n=4). The hybridization signals were normalized to the respective signals at 0 µJoules/cm2.



Graph 3. Relationship of Hybridization Signal to UV Energy for RNA Probes.
Mouse liver total RNA (1 µg) was resolved in a formaldehyde agarose gel and transferred to Immobilon-Ny+ by capillary blotting. The blots were dried completely, and then UV cross-linked with varying UV energies at 254 nm. The blots were hybridized using a 32P-labeled RNA probe, and the signals were quantified (n=4). The hybridization signals were normalized to the respective signals at 0 µJoules/cm2.

Effect of formamide in hybridization solution.

Recommendation: A concentration of 33 to 50% formamide in Church hybridization solution is recommended for hybridization, if necessary.

Results: Since impurities introduced with additives (e.g., BSA or nucleic acids) to hybridization solutions can cause unexpected problems such as background or RNase contamination, a modified Church hybridization solution (10, 11), which does not require additives, is preferable for northern blotting. Cross-hybridization of RNA probes to ribosomal RNA may occur when total RNA is probed or if the probe is “sticky”. Therefore, the effect of formamide concentration in the Church hybridization solution was evaluated for its impact on non-specific hybridization. A GAPDH RNA probe showed a high level of cross-hybridization to 18S and 28S ribosomal RNA with no formamide and slight cross-hybridization at a concentration of 25% (Fig. 3). Above a concentration of 33%, no cross-hybridization was observed and no signal loss was observed.

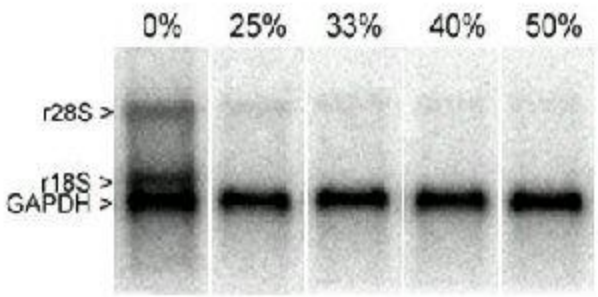


Fig. 2. Effect of Formamide Concentration in Modified Church Hybridization Solution on Hybridization.
Blots of 1 µg of mouse liver total RNA were hybridized at 68°C with a 32P-labeled GAPDH RNA probe using different formamide concentrations.

Chemiluminescent Detection.

Recommendation: Use UV cross-linking for chemiluminescent detection. UV cross-linking produces stronger signal intensity than baking.

Results: Detection sensitivity on Immobilon-Ny+ and a competitive, positively charged nylon membrane using chemiluminescent detection was evaluated. Fixation of the RNA by UV cross-linking enhanced the signal intensity when compared to baking. Immobilon-Ny+ shows better sensitivity than competitor A. Detection sensitivity was equivalent to ^{32}P-labeled DNA probe.

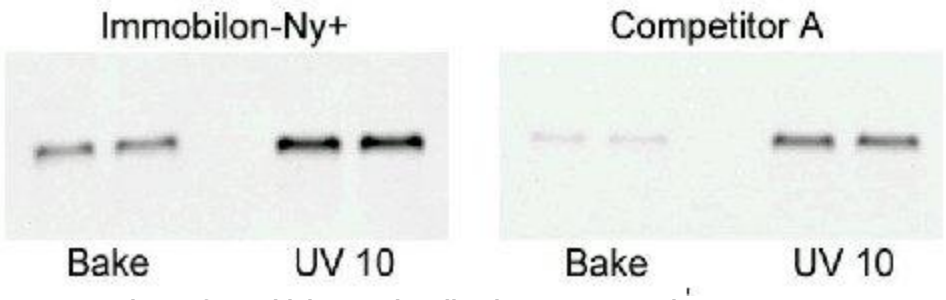


Fig. 3. Comparison of Sensitivity on Chemiluminescent Detection.
Mouse liver total RNA (1µg) was resolved on a formaldehyde agarose gel and transferred to Immobilon-Ny+ (Ny+) and a competitor's positively charged nylon membrane "A" by capillary blotting. The blots were dried completely, and then fixed by either baking or UV cross-linking with 10,000 µJoules/cm2 at 254 nm. The blots were hybridized with DIG-labeled GAPDH DNA at a concentration of 10 ng/mL and washed with same condition of 32P-labeled probe. The probe was detected according to the manufacturer's protocol then exposed to an X-ray film for 10 min. Bake: 80°C under vacuum for 1 hr: UV 10: 10,000 µJoules/cm2 at 254 nm

Comparison of Stripping Methods on Northern Blot.

Recommendation: Use hot 0.1% SDS for stripping of northern blots. To strip hybridized probe, incubate the blots in hot 0.1% SDS solution (w/v) twice for 15 min with gentle agitation.

CAUTION. Do not continue to heat the 0.1% SDS solution containing radioactive membranes while stripping. Radioactive contamination can occur by aerosolization or boiling over.

Results: Because RNA molecules can degrade in alkali, two stripping methods were compared. Stripping in hot 0.1% SDS was preferable for northern blots (Fig. 4). The second round of probing with the DNA probe gave 99% of the original signal intensity, while the RNA probe gave only 59% (Graph 5). The residual signal of 5-6% of initial intensity after stripping in hot 0.1% SDS (Graph 4) should not affect reprobing studies as long as target RNAs are probed in order of low abundance to high abundance. Although stripping in 0.4 M NaOH is suitable for Southern blots (5,8), it is not recommended for northern blots as the signal disappeared in subsequent reprobing (data not shown). High concentrations of NaOH are assumed to destroy the target RNA molecules blotted to the membrane. Even decreasing the NaOH concentration to 50 mM resulted in only 9-10% of the initial signal intensity with the second probe (Graph 5).

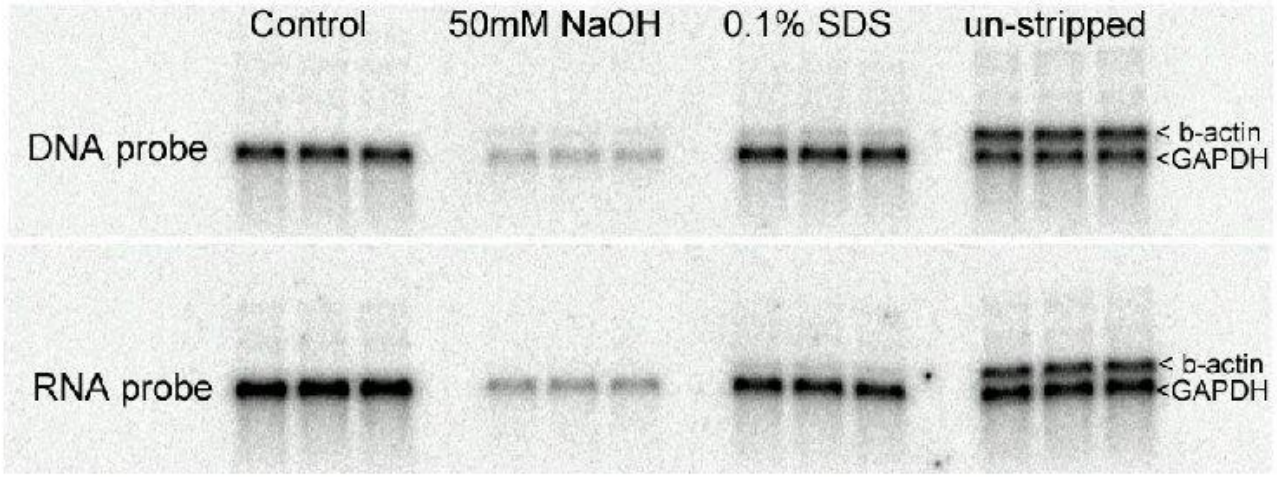
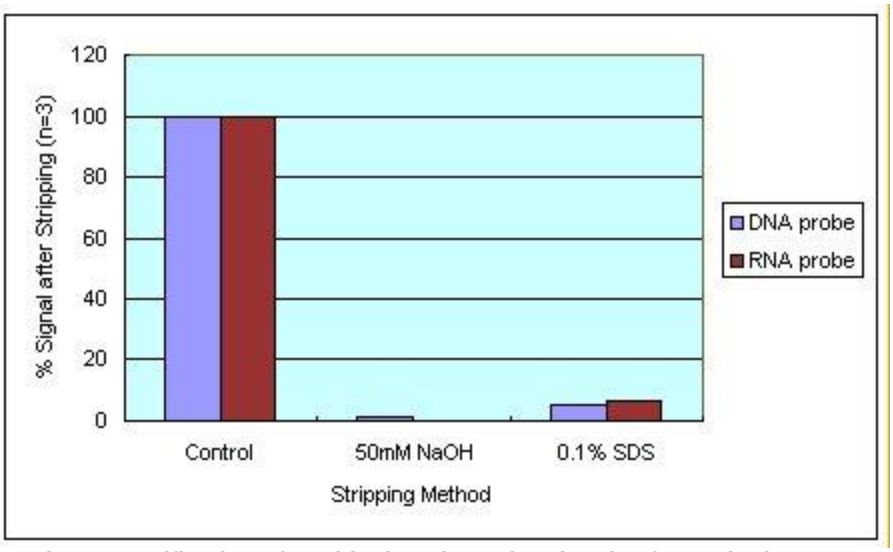
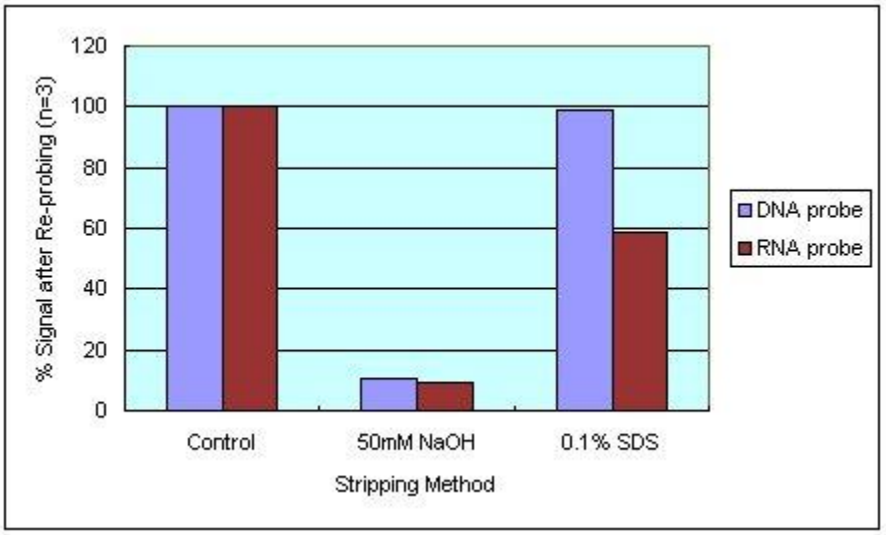


Fig. 4. Comparison of Stripping Methods.
Mouse liver total RNA (1µg) was resolved in a formaldehyde agarose gel and transferred to Immobilon-Ny+ by capillary blotting. The blots were dried completely, and then UV cross-linked with 10,000 and 30,000 µJoules/cm2 at 254 nm for DNA probes and RNA probes, respectively. The blots were hybridized using DNA or RNA -actin probes. The blots were stripped in 50mM NaOH or hot 0.1% SDS. After stripping, the blots were re-probed with DNA or RNA GAPDH probe. The "Control" blots were initially probed with GAPDH probes. The "un-stripped" blots were re-probed without stripping the -actin probe.



Graph 4. Quantification of Residual -actin Probe Signals after Stripping.
After stripping, the residual -actin probe signals were quantified and compared to un-stripped blots prior to re-probing.



Graph 5. Quantification of GAPDH Hybridization Signal after Re-probing.
This graph illustrates the loss of the hybridization signal caused by different stripping methods. The GAPDH hybridization signals shown in Fig.4 were quantified after re-probing in comparison to “Control” which was set at 100%. Each “Control” bar represents the relative signal intensity at initial probing of GAPDH using a duplicate blot transferred from the same gel.

Assessment of Re-probability on Northern Blot.

Recommendation: For reprobing northern blots, it is best to use DNA probes without formamide. Apply probes in order of anticipated low to high abundance targets.

Results: Reprobing efficiency on Immobilon-Ny+ and a competitor’s positively charged nylon was compared using DNA and RNA probes for three different mRNAs: cyclophilin (first probing), b-actin (second probing), and GAPDH (fifth probing). The third and fourth probing cycles were mock hybridizations using the same hybridization, washing, and stripping protocols, except that no probe was added. Formamide was added to the hybridization solution to a final concentration of 50% for RNA probes. In this comparison, DNA probes worked better than RNA probes (Figs. 5, 6, 7; Graphs 6, 7, 8). When RNA is used as a probe, the RNA blotted on the membrane may be less accessible. This phenomenon was also observed on three other nylon membranes tested (data not shown). Immobilon-Ny+ showed equivalent or better performance than the competitor’s nylon membranes in this analysis. A northern blot that was UV cross-linked with 60,000 µJoules/cm2 exhibited slightly better signal intensity than 40,000 µJoules/cm2 when an RNA probe was used for re-probing.

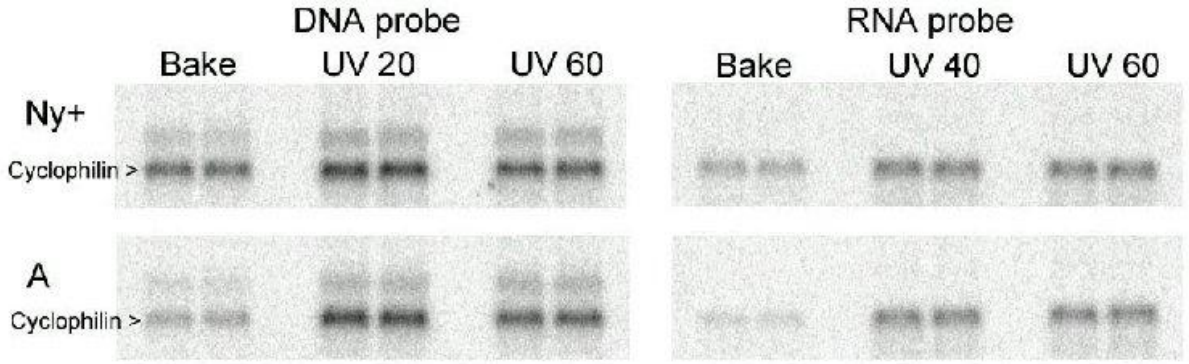
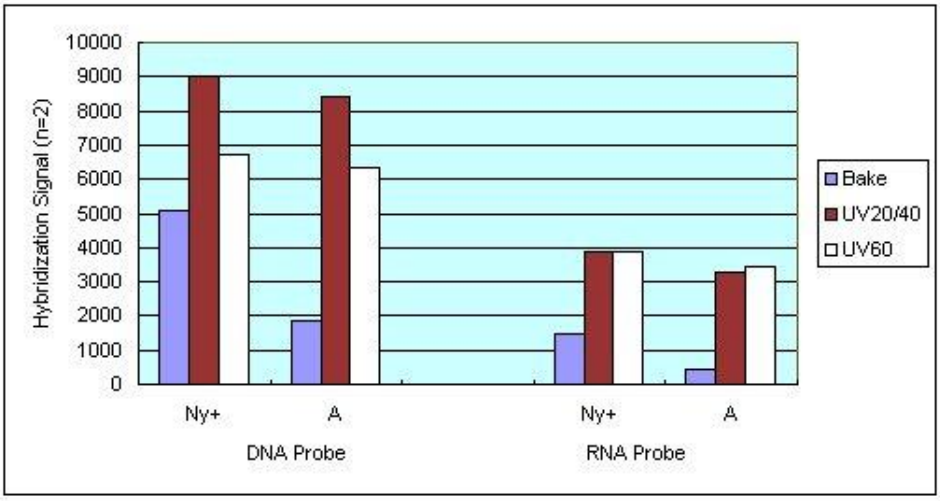


Fig. 5. First Probing with Cyclophilin Probe.
Mouse liver total RNA (1µg) was resolved in a formaldehyde agarose gel and transferred to Immobilon-Ny+ and competitor's A by capillary blotting. The blots were dried completely then fixed with either baking (Bake: 80°C for 1 hr) or UV cross-linking (UV 20, UV 40, and UV 60: 20,000, 40,000, and 60,000 µJoules/cm2 at 254 nm, respectively). The blots were hybridized using either DNA or RNA of cyclophilin probe and exposed to a phosphor screen for 6 hr. Ny+: Immobilon-Ny+. A: competitor's positively charged nylon membrane.



Graph 6. Quantification of Cyclophilin Signal after First Probing.
The bands shown in Fig.5 were quantified. The bars labeled UV20/40 in the graph refer to UV cross-linking with the optimal UV energy for either DNA (20,000 µJoules/cm2) or RNA (40,000 µJoules/cm2) probes. Ny+: Immobilon-Ny+. A: competitor's positively charged nylon.

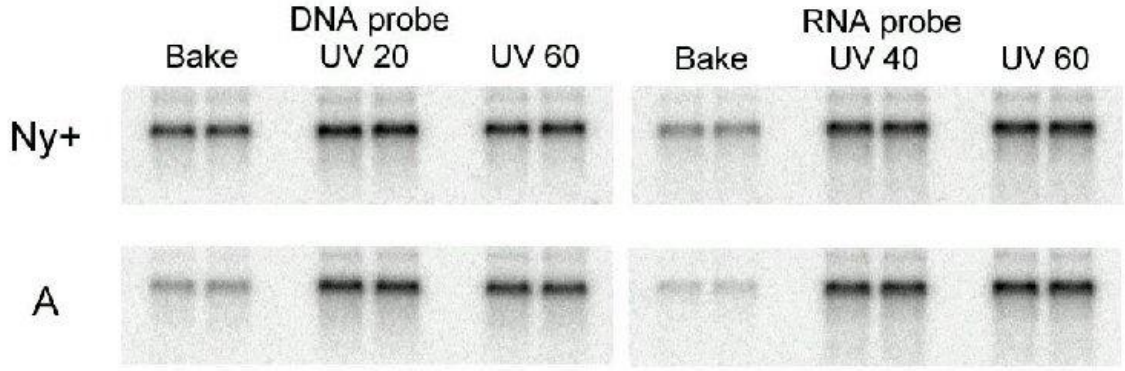
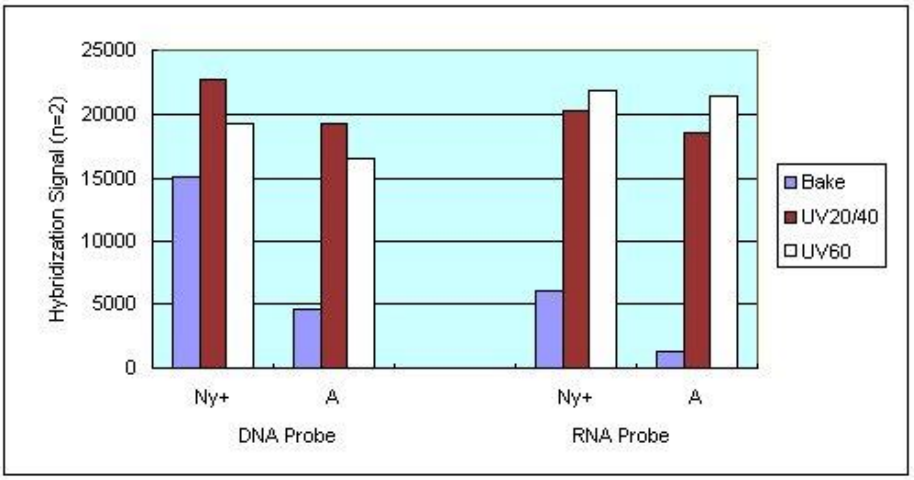


Fig. 6. Second Probing with b-Actin Probe.
After detection of cyclophilin, the blots were stripped (2 x 15 min in hot 0.1% SDS) and re-probed with DNA or RNA -actin probes. Then blots were exposed to a phosphor screen for 6 hr. Bake: 80°C for 1 hr. UV 20, UV 40, and UV 60: 20,000, 40,000, and 60,000 µJoules/cm2 at 254 nm, respectively. Ny+: Immobilon-Ny+. A: competitor's positively charged nylon membrane.



Graph 7. Quantification of b-actin Signal after Second Probing.
The bands shown in Fig.6 were quantified. The bars labeled UV20/40 in the graph refer to UV cross-linking with the optimal UV energy for either DNA (20,000 µJoules/cm2) or RNA (40,000 µJoules/cm2) probes.

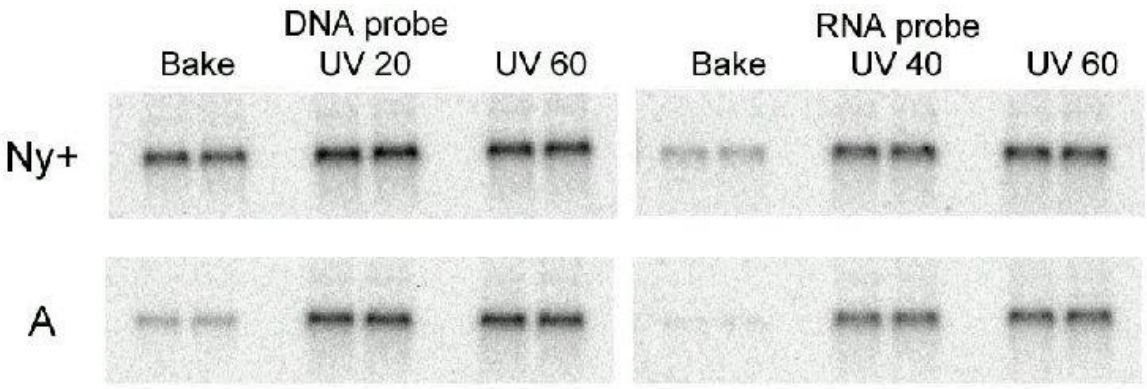
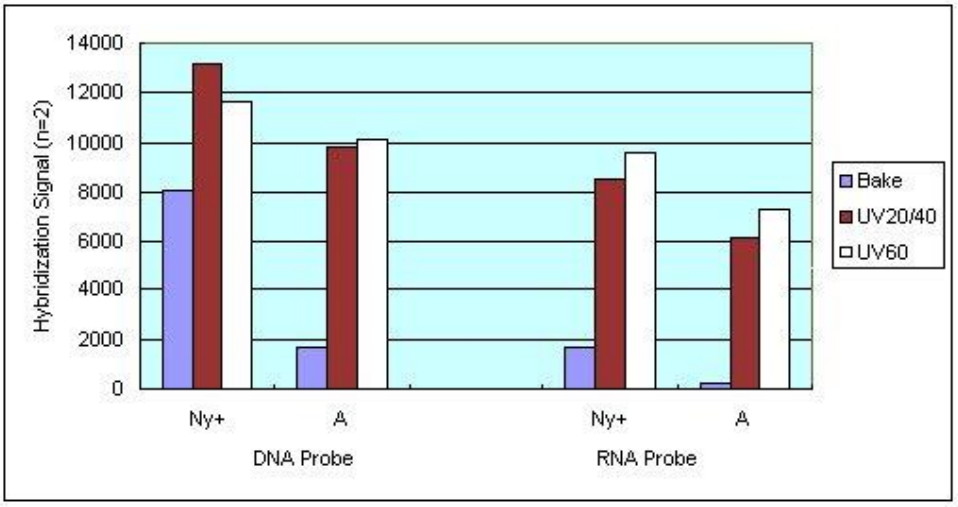


Fig. 7. Fifth Probing with GAPDH Probe.
After stripping of the -actin probe, the blots were processed through two cycles of mock hybridization and stripping. For the fifth probing cycle, the blots were re-probed with DNA and RNA GAPDH probes and exposed to a phosphor screen for 6 hr. Bake: 80°C for 1 hr. UV 20, UV 40, and UV 60: 20,000, 40,000, and 60,000 µJoules/cm2 at 254 nm, respectively. Ny+: Immobilon-Ny+. A: competitor's positively charged nylon membrane.



Graph 8. Quantification of GAPDH Signal after Fifth Probing.
The bands shown in Fig.7 were quantified. The bars labeled UV20/40 in the graph refer to UV cross-linking with the optimal UV energy for either DNA (20,000 µJoules/cm2) or RNA (40,000 µJoules/cm2) probes.

References

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Appendix

Northern Blotting Protocol

Prepare the Membrane for a 20 x SSC Transfer

1. Cut a piece of membrane to the dimensions of the agarose gel.
2. Wet the membrane by carefully laying it on top of Milli-Q water in a shallow tray.
3. Agitate the tray gently once the membrane is wet to completely immerse the membrane.
4. Transfer the membrane to a second tray containing transfer buffer (20 x SSC).
5. Equilibrate the membrane at least 5 minutes. Then continue on to the next section to process the gel.

Process the Gel and Membrane for RNA Transfer

1. Resolve the RNA on an agarose gel. Trim away areas of the gel without any RNA as long as the membrane has been cut to match the size of the gel.

2. Rinse the gel containing formaldehyde several times in Milli-Q water. Then continue on to the next section to assemble the blotting stack.

Assemble a Capillary Blotting Stack

1. Fill a tray with 500 to 1000 mL of Transfer solution (20 x SSC). Then suspend a support (for example, a glass plate) across the sides of the tray.
2. Wet two sheets of Whatman® 3MM filter paper in 20 x SSC. Then lay the sheets across the support with the ends soaking in the 20 x SSC in the tray.
3. Place the gel on top of the filter paper wicks carefully. Then place the membrane on top of the gel. Do not leave any of the gel exposed.
   NOTE: To prevent the flow of buffer around the edge of the gel, place a strip of Parafilm® along each edge of the gel. This acts as a barrier between the wicks and the absorbent material on top of the stack.
4. Wet three sheets of Whatman 3MM filter paper, cut to the size of the membrane, in 20 x SSC. Then place them on top of the membrane. Roll out any trapped bubbles between the gel, membrane, and filter paper layers with a pipette or gloved fingers.
5. Place a 10 to 20 cm high stack of absorbent material (for example, paper towels) on top of the filter paper. Place a glass plate on top of the stack. Then place a 250 to 300 g weight on top of the plate to evenly distribute the downward force.
   CAUTION: Do not use a heavy weight; it causes rapid collapse of the gel, entrapping the RNA.
6. Let the RNA transfer from the gel to the membrane for 6 to 18 hours. After this time, remove the absorbent material and filter paper.
7. Lift the blot from the gel carefully with a pair of forceps. Rinse the blot in 6 x SSC to remove any loose particles of agarose.
8. Place the blot on a sheet of filter paper to dry. Then follow the steps in the “RNA Fixation” section.

RNA Fixation

The RNA fixation process permanently binds a portion of target RNA to the membrane surface. You can fix RNA to the surface of Immobilon-Ny+ with UV cross-linking or baking. This section provides steps on both methods. The traditional method is baking under a vacuum and requires a vacuum oven. The UV cross-linking method requires a calibrated ultraviolet light source (254 nm), but it gives better sensitivity and retention in reprobing applications. Millipore recommends using the UV cross-linking after a 20 x SSC transfer for optimum sensitivity.

RNA Fixation with UV Cross-Linking

Allow the blot to dry completely.
Place the blot on a sheet of clean filter paper to prevent contamination if you plan to place the UV light source above the blotted RNA. If you plan to place the membrane on a UV transilluminator, clean the surface with Milli-Q water and a Kimwipe®.
Expose the side of the blot with the bound RNA to the UV light source (254 nm). If the light source is equipped with an internal energy detector (for example, Stratalinker®, Stratagene), the total exposure energy used should be 20,000 µJoules/cm2 for DNA probes or 40,000 µJoules/cm2 for RNA probes. Then see the “Pre-Hybridization and Hybridization” section.
NOTE: If you have a well-calibrated UV light source (254 nm), calculate an optimal exposure time using the following formula.
Exposure time (sec.) = Optimal UV energy (µJoules/cm2) Output of UV light (µwatts/cm2).
If your UV light has a power output of 1,000 µwatts/cm2 at 15cm distance, the exposure time at 15cm distance would be either 20 sec for DNA probe or 40 sec for RNA probe.
If you cannot measure the UV energy (for example, with a UV transilluminator), you need to perform a test. The blot should contain the same control sample in all lanes and then be exposed to the UV light from 5 seconds to 2 minutes using a black sheet of paper to mask the blot for the desired times. After hybridization, the exposure time giving the best signal-to-noise ratio should be used for experimental samples. Perform this test periodically since the intensity of the UV light changes with bulb age and filter polarization.
CAUTION: Exposure to UV causes a significant health hazard. Wear UV protective goggles and shield all exposed skin.

RNA Fixation with Baking

Place the blot between two pieces of clean filter paper.
Place the blot and filter papers in a vacuum oven set at 80°C for 1 to 2 hours.
Remove the blot from the oven and let cool to room temperature. Then see the “Pre-Hybridization and Hybridization” section.

Pre-Hybridization and Hybridization

After fixing the RNA with UV cross-linking or baking, follow the steps in this protocol for excellent sensitivity and minimal background. Other protocols may be equally effective, but require testing.

CAUTION: If you want to reprobe the blot, keep it wet throughout the hybridization, washing, and film exposure steps. If it dries, the probe becomes irreversibly bound to the membrane.

Prepare the buffer and wash solutions as follows:

1. Hybridization Buffer: 0.5 M Sodium Phosphate (pH 7.1), 2 mM EDTA, 7% (w/v) SDS, 0.1 % (w/v) Sodium Pyrophosphate. Formamide may be added at concentration of 33 to 50%, if necessary.
2. Wash Solution I: 1 x SSPE, 0.5% (w/v) SDS
3. Wash Solution II: 0.2 x SSPE, 0.1% (w/v) SDS

   NOTE: For the stringency washes, you can lower the temperature and increase the salt concentration to accommodate higher degrees of mismatch between the probe and target sequence.
4. Wet the blot by laying it on top of Milli-Q water in a shallow tray. Let the water move into the pores of the blot by capillary action. Once the blot is fully wet, agitate the tray gently to completely immerse it.
5. Place the blot into a hybridization bottle with the RNA oriented toward the center of the tube. (You can also use heat-sealable plastic bags.)
6. Add the amount of hybridization buffer recommended for your hybridization bottle (or bag) to pre-hybridize the blot. Incubate the blot for 0.5 to 2 hours at 68°C.
7. Pour off the Hybridization buffer and add fresh Hybridization buffer containing labeled probe. (Nylon membranes are suitable for radioactive and non-radioactive probes.) Then incubate the blots over night at 68°C.
8. Pour off the Hybridization buffer containing the probe.
9. Fill the tube halfway with Wash Solution I. Incubate for 5 minutes at room temperature with mixing. Then pour off the Wash solution. Repeat this step once. (Transfer blots hybridized in plastic bags to a glass dish for washing.)
10. Fill the tube halfway with Wash Solution II pre-heated to 68°C. Incubate for 15 minutes at 68°C. Pour off the Wash Solution. Repeat this step once.

For non-radioactive detection, proceed according to the specific instructions for your system. For radioactive detection, expose the blot to autoradiographic film. If you plan to reprobe the blot, seal it in a plastic bag for film exposure. If the blot dries, the probe becomes permanently bound to the membrane. See the following section for details on stripping. If you do not plan to reprobe the blot, let it air-dry. Then mount the blot on a piece of filter paper, wrap it in plastic wrap, and expose it to autoradiographic film.

Stripping Protocols

As described in the previous section, do not allow nylon membranes to dry during the hybridization, washing, and film exposure steps if you plan to reprobe them. Drying causes the probe to irreversibly bind to the membrane. This section describes how to perform stripping on RNA blots for subsequent reprobing.

CAUTION: Do not continue to heat the 0.1% SDS solution containing radioactive membranes while stripping. Radioactive contamination can occur by aerosolization or boiling over.

1. Prepare this stripping solution: 0.1% (w/v) SDS.
2. Heat the 0.1% SDS solution in a glass baking dish until boiling. Remove it from the heat after boiling.
3. Place the blot with forceps in the SDS solution. Incubate the blot for 15 minutes with gentle agitation. Repeat this step once.
4. Seal the blot in a plastic bag. For radioactive probes, expose the blot to film for the normal exposure time to check for the removal of the probe. For non-radioactive probes, repeat your specific detection protocol. If the probe has not been completely removed from the blot, repeat the stripping process. When all probe has been removed from the blot, you can reprobe it beginning with the pre-hybridization step.