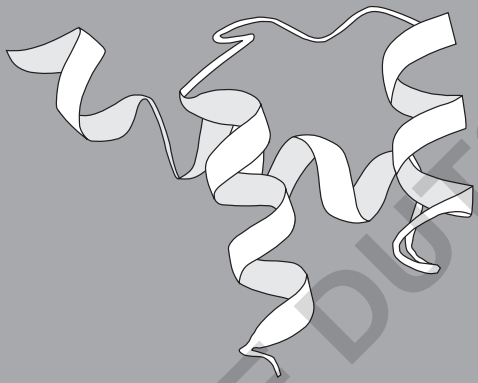
MILLIPORE



Immobilon™-Ny+

Transfer Membrane
User Guide

DOMINIQUE DUTSCHER SA

Notice

The information in this document is subject to change without notice and should not be construed as a commitment by Millipore Corporation. Millipore Corporation assumes no responsibility for any errors that may appear in this document. This manual is believed to be complete and accurate at the time of publication. In no event shall Millipore Corporation be liable for incidental or consequential damages in connection with or arising from the use of this manual.

© 2005 Millipore Corporation. All rights reserved.

Millipore is a registered trademark of Millipore Corporation.
Millex, Millipore Express, Milli-Q and Stericup are registered trademarks of Millipore Corporation.
Immobilon is a trademark of Millipore Corporation.
DIG is a trademark of Boerhringer Manheim Gmbh.
Kimwipe is a registered trademark of Kimerly-Clark Corporation.
Parafilm is a registered trademark of American National Can.
Stratalinker and Stratagene are registered trademarks of Stratagene.
Whatman is a registered trademark of Whatman Paper Ltd.
Tween is a registered trademark of ICI Americas Inc.

P36007 Rev. D, 03/05

COPYRIGHTED BY DUTSCHER SAS

Contents

Introduction 1

Southern Blotting Protocol 2
- Prepare the Membrane (Southern) 2
- Process the Gel and Membrane (Southern) 3
- Assemble a Capillary Blot Stack (Southern) 3

DNA Fixation 5
- DNA Fixation with UV Cross-linking 5
- DNA Fixation with Baking 6
- Determine the Labeled Probe Concentration (Southern) 7
- Pre-Hybridization and Hybridization (Southern) 8

Northern Blotting Protocol 10
- Prepare the Membrane (northern) 10
- Process the Gel and Membrane (northern) 11
- Assemble a Capillary Blot Stack (northern) 11

RNA Fixation 13
- RNA Fixation with UV Cross-linking 13
- RNA Fixation with Baking 15
- Determine the Labeled Probe Concentration (northern) 15
- Pre-Hybridization and Hybridization (northern) 16

COMMERCIALE DUTSCHER SAS

Detection of DIG Labeled Probe 18
Stripping Protocols 19
DNA Blot Stripping 20
DNA or RNA Blot Stripping 20
Colony Lifts 21
Grow Bacterial Colonies 21
Prepare the Membrane 21
Replicate Colonies onto Immobilon-Ny+ Membrane 22
Colony Lysis Protocol 23
DNA Fixation 24
DNA Fixation with UV Crosslinking 25
DNA Fixation with Baking 25
Hybridization to Colony Lifts on Immobilon-Ny+ Membrane 26
Product Ordering Information 28
Technical Assistance 29
Standard Warranty 29

DOMINIQUE DUTSCHER SA

Immobilon-Ny+ Transfer Membrane

Introduction

The Millipore Immobilon-Ny+ Transfer Membrane is a positively charged nylon membrane developed specifically for nucleic acid blotting applications. It is validated for the detection of subpicogram quantities of DNA and for the ability to be stripped and reprobed multiple times. The charge density and consistency of Immobilon-Ny+ membrane are optimized for sensitivity and reproducibility.

This instruction manual describes how to perform a number of different protocols. For example, it includes steps for northern and Southern blotting, DNA and RNA fixation, DIG chemiluminescent detection and hybridization procedures. It also contains steps on stripping and colony lifts. Refer to the sections you need.

NOTE: For information on plaque lift techniques, see *Molecular Cloning, A Laboratory Manual*, 2nd Edition by J. Sambrook, E.F. Fritsch, and T. Maniatis (Cold Spring Harbor Laboratory Press, Cold Spring Harbor, New York, 1989).

DOMINIQUE DUTSCHER SA

Southern Blotting Protocol

This section contains steps to prepare the membrane, process the gel and membrane for a DNA transfer, assemble a capillary transfer stack, perform DNA fixation with UV cross-linking or baking, and perform pre-hybridization and hybridization. For optimum sensitivity using Immobilon-Ny+ membrane, Millipore recommends a capillary transfer in 20 x SSC followed by DNA fixation with UV cross-linking.

CAUTION: Millipore does not recommend using an alkaline transfer method with Immobilon-Ny+ membrane. Even though this method reduces the time required to process the gel before blotting and fixes the DNA to the membrane, you may not get optimum transfer results.

Prepare the Membrane (Southern)

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.

   CAUTION: Do not immerse the Immobilon-Ny+ membrane in liquid on the first liquid exposure. You can only expose one side to liquid on the first exposure. If you wet both sides, air can become trapped in the pores and form bubbles.

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.

DOMINIQUE DUTSCHER SA

Process the Gel and Membrane (Southern)

1. Prepare the solutions as follows:
   - Depurination: 0.25 N HCl
   - Denaturing: 0.5 N NaOH, 1.5 M NaCl
   - Neutralization: 1 M Tris·Cl, pH 8, 1.5 M NaCl
   - Transfer: 20 x SSC
2. Resolve the DNA fragments on an agarose gel. Trim away areas of the gel without any DNA as long as the membrane has been cut to match the size of the gel.

   NOTE: To improve the transfer efficiency of large DNA fragments, nick the DNA by partial depurination. To do this, incubate the gel in Depurination solution for 15 minutes. Then pour off the solution and rinse the gel briefly in Milli-Q water.
3. Denature the DNA by incubating the gel in Denaturing solution for 30 minutes. Then pour off the solution and briefly rinse the gel in Milli-Q water.
4. Incubate the gel in Neutralization solution for 30 minutes. Then continue on to the next section to assemble the blotting stack.

Assemble a Capillary Blotting Stack (Southern)

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.

OMINIQUE DUTSCHER SA

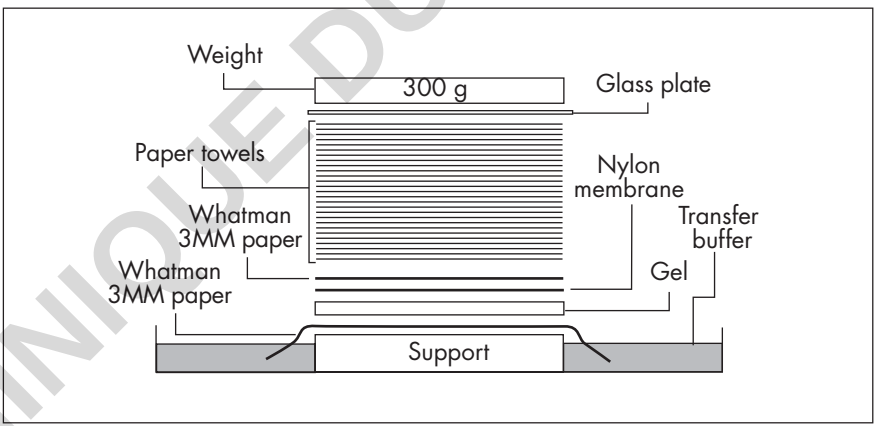
Assemble a Capillary Blotting Stack (Southern), continued

NOTE: To prevent the flow of buffer around the edge of the gel, place a strip of Parafilm® plastic 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.

   The stack should look like this:



CAUTION: Do not use a heavy weight; it causes rapid collapse of the gel, entrapping the DNA.

DOMINIQUE DUTSCHER SAS

Assemble a Capillary Blotting Stack (Southern), continued

6. Let the DNA 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 "DNA Fixation" section.

DNA Fixation

The DNA fixation process permanently binds DNA to the membrane surface. You can fix DNA to the surface of Immobilon-Ny+ membrane 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.

DNA Fixation with UV Cross-Linking

1. Allow the blot to air-dry completely or dry the blot for 10 to 20 minutes at 80 °C.

   NOTE: The blot must be completely dry before UV cross-linking. Residual water in the pores of the membrane causes less efficient cross-linking.

2. 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 DNA. If you plan to place the membrane on a UV transilluminator, clean the surface with Milli-Q water and a Kimwipe® tissue.

DOMINIQUE DUTSCHER SAS

DNA Fixation with UV Cross-Linking, continued

CAUTION: Exposure to UV causes a significant health hazard. Wear UV protective goggles and shield all exposed skin.

3. Expose the side of the blot with the bound DNA to a UV light source (254 nm). If the light source is equipped with an internal energy detector (for example, Stratalinker®, Stratagene® devices), the total exposure energy used should be **5,000 microJoules/cm²**. If you have a well-calibrated UV light source (254 nm), calculate the optimal exposure time using the following formula:

   Exposure time (sec.) = Optimal UV energy (microJoules/cm²) ÷ Output of UV light (microwatts/cm²).

   For example, if your UV light has a power output of 1,000 µwatts/cm² at 15 cm distance the exposure time at 15 cm distance would be 5.0 seconds. Then see the "Pre-Hybridization and Hybridization (Southern)" section. If you are performing DIG chemiluminescent detection, see the "Determine the Labeled Probe Concentration (Southern)" section, before the "Pre-Hybridization and Hybridization (Southern)" section.

   NOTE: 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.

DOMINIQUE DUTSCHER SAS

DNA Fixation with Baking

1. Allow the blot to air-dry completely. Then place it between two pieces of clean filter paper.
2. Place the blot and filter papers in a vacuum oven set at 80 °C. Close the oven, turn on the vacuum, and let the blot bake for 1 to 2 hours.
3. Remove the blot from the oven and let cool to room temperature. Then see the "Pre-Hybridization and Hybridization (Southern)" section.

   NOTE: If you are performing DIG chemiluminescent detection, see the next section ["Determine the Labeled Probe Concentration (Southern)"], before the "Pre-Hybridization and Hybridization (Southern)" section.

Determine the Labeled Probe Concentration (Southern)

This procedure is for users performing DIG chemiluminescent detection only. After the labeling reaction, the probe labeling efficiency must be determined for maximum results. For precise determination of DIG labeled probe concentration, Millipore recommends comparison by dot blotting with DIG labeled control probe onto Immobilon-Ny+ membrane instead of using the test strip included in the kit. Make a serial dilution series of each probe in dilution buffer, then follow these steps:

CAUTION: The probe concentration must be determined exactly. Using an improper probe concentration for hybridization causes high background or low sensitivity.

1. Spot 1 µL of the dilution series and control labeled probe onto a small piece of dry Immobilon-Ny+ membrane.
2. Dry the blots for 10 minutes at 80 °C.
3. Perform UV cross-linking with 60,000 µJoules/cm^2 at 254 nm.
4. Follow DIG probe detection procedure using BCIP/NBT.
5. Estimate the probe concentration by comparison to control probe.

DOMINIQUE DUTSCHER SAS

Pre-Hybridization and Hybridization (Southern)

After fixing the DNA 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.

1. Prepare the buffer and wash solutions as follows:

Hybridization Buffer	*Wash Solution I*	*Wash Solution II*
5 x SSPE 5 x Denhardt's 100 µg/mL sheared DNA 0.5% (w/v) SDS	2 x SSC 0.1% (w/v) SDS	0.2 x SSC 0.1% (w/v) SDS

If you are performing DIG chemilminescent detection prepare these solutions:

Hybridization Buffer	*Wash Solution I*	*Wash Solution II*
0.5 M Sodium Phosphate (pH 7.1) 2 mM EDTA 7% (w/v) SDS 0.1% (w/v) Sodium Pyrophosphate	2 x SSC 0.1% (w/v) SDS	0.2 x SSC 0.1% (w/v) SDS

NOTE: For the stringency washes, you can lower the temperature and increase the SSC concentration to accommodate higher degrees of mismatch between the probe and target sequence. Filtration of the solution is recommended to remove any particles using an appropriate high flow rate membrane, for example Millipore Stericup®-GP (0.22 µm) filter unit.

DOMINIQUE DUTSCHER SA

Pre-Hybridization and Hybridization (Southern), continued

2. 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.
3. Place the blot into a hybridization bottle with the DNA oriented toward the center of the tube. (You can also use heat-sealable plastic bags.)
4. Add the amount of Hybridization buffer recommended for your hybridization bottle (or bag) to pre-hybridize the blot. Then incubate the blot for 1 to 2 hours at 68 °C .
5. Pour off the Hybridization buffer and add fresh Hybridization buffer containing labeled probe. For DIG chemiluminescent detection, use a probe of 10 to 15 ng/mL at a final concentration. At concentrations of > 20 ng/mL the background increases rapidly. Then incubate the blots for 12 to 18 hours at 68 °C.

   CAUTION: **For users performing DIG chemiluminescent detection only:** An excessive probe concentration dramatically increases high background. A probe concentration that is too low causes a signal decrease. After the labeling reaction, the probe labeling efficiency must be determined according to the "Determine the Labeled Probe Concentration (South ern)" section.
6. Pour off the Hybridization buffer containing the probe.
7. 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.)

OMINIQUE DUTSCHER SA

Pre-Hybridization and Hybridization (Southern), continued

8. 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.
9. For radioactive detection, expose the blot to autoradiographic film. If you are performing DIG chemiluminescent detection see the "Detection of DIG Labeled Probe" section. 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.) Then see the "Stripping Protocol" 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.

Northern Blotting Protocol

This section contains steps to prepare the membrane, process the gel and membrane for a 20 x SSC transfer, assemble a capillary transfer stack, perform RNA fixation with UV cross-linking or baking, and perform pre-hybridization and hybridization. For optimum sensitivity using Immobilon-Ny+ membrane, Millipore recommends a capillary transfer in 20 x SSC followed by RNA fixation with UV cross-linking.

CAUTION: Millipore does not recommend using an alkaline transfer method with Immobilon-Ny+ membrane. Even though this method reduces the time required to process the gel before blotting and fixes the RNA to the membrane, you may not get optimum transfer results.

Prepare the Membrane (northern)

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.

COMMERCIALE DUTSCHER SAS

Prepare the Membrane (northern), continued

CAUTION: Do not immerse the Immobilon-Ny+ membrane in liquid on the first liquid exposure. You can only expose one side to liquid on the first exposure. If you wet both sides, air can become trapped in the pores and form bubbles.

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 (northern)

1. Resolve RNA fragments 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 (northern)

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 plastic along each edge of the gel. This acts as a barrier between the wicks and the absorbent material on top of the stack.

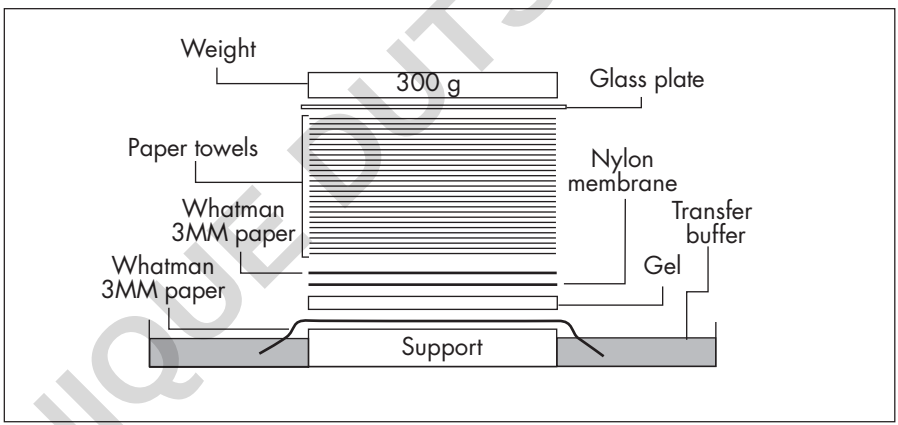
DOMINIQUE DUTSCHER SAS

Assemble a Capillary Blotting Stack (northern), continued

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.

   The stack should look like this:



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.

DOMINIQUE DUTSCHER SA

Assemble a Capillary Blotting Stack (northern), continued

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 RNA to the membrane surface. You can fix RNA to the surface of Immobilon-Ny+ membrane 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

1. Allow the blot to air-dry completely or dry the blot for 10 to 20 minutes at 80 °C.

   NOTE: The blot must be completely dry before UV cross-linking. Residual water in the pores of the membrane causes less efficiant cross-linking.

2. 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 tissue.

   CAUTION: Exposure to UV causes a significant health hazard. Wear UV protective goggles and shield all exposed skin.

DOMINIQUE DUTSCHER SAS

RNA Fixation with UV Cross-Linking, continued

3. Expose the side of the blot with the bound RNA to a UV light source (254 nm). If the light source is equipped with an internal energy detector (for example, Stratalinker, Stratagene devices), the total exposure energy used should be **20,000 microJoules/cm² for DNA probes or 40,000 microJoules/cm² for RNA probes.** Then see the "Pre-Hybridization and Hybridization (northern)" section. If you are performing DIG chemiluminescent detection, see the "Determine the Labeled Probe Concentration (northern)" section, before the "Pre-Hybridization and Hybridization (northern)" section.

   NOTE: If you have a well-calibrated UV light source (254 nm), calculate the optimal eposure time using the following formula:

   Exposure time (sec.) = Optimal UV energy (microJoules/cm²) ÷ Output of UV light (microwatts/cm²).

   For example, if your UV light has a power output of 1,000 microwatts/cm² at 15 cm distance, the exposure time at 15 cm distance would be either 20 seconds for DNA probe or 40 seconds 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.

DOMINIQUE DUTSCHER SA

RNA Fixation with Baking

1. Allow the blot to air-dry completely. Then place it between two pieces of clean filter paper.
2. Place the blot and filter papers in a vacuum oven set at 80 °C. Close the oven, turn on the vacuum, and let the blot bake for 1 to 2 hours.
3. Remove the blot from the oven and let cool to room temperature. Then see the "Pre-Hybridization and Hybridization (northern)" section.

   NOTE: If you are performing DIG chemiluminescent detection, see the next section ["Determine the Labeled Probe Concentration (northern)"] before the "Pre-Hybridiztion and Hybridization (northern)" section.

Determine the Labeled Probe Concentration (northern)

This procedure is for users performing DIG chemiluminescent detection only. After the labeling reaction, the probe labeling efficiency must be determined for maximum results. For precise determination of DIG labeled probe concentration, Millipore recommends comparison by dot blotting with DIG labeled control probe onto Immobilon-Ny+ membrane instead of using the test strip included in the kit. Make a serial dilution series of each probe in dilution buffer, then follow these steps:

CAUTION: The probe concentration must be determined exactly. Using an improper probe concentration for hybridization causes high background or low sensitivity.

1. Spot 1 µL of the dilution series and control labeled probe onto a small piece of dry Immobilon-Ny+ membrane.
2. Dry the blots for 10 minutes at 80 °C.
3. Perform UV cross-linking with 60,000 µJoules/cm^2 at 254 nm.
4. Follow DIG probe detection procedure using BCIP/NBT.
5. Estimate the probe concentration by comparison to control probe.

DOMINIQUE DUTSCHER SAS

Pre-Hybridization and Hybridization (northern)

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.

1. Prepare the buffer and wash solutions:

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

NOTE: For the stringency washes, you can lower the temperature and increase the SSPE concentration to accommodate higher degrees of mismatch between the probe and target sequence. Filtration of the solution is recommended to remove any particles using an appropriate high flow rate membrane, for example Millipore Stericup-GP (0.22 µm) filter unit.

2. 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.

3. 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.)

DOMINIQUE DUTSCHER SAS

Pre-Hybridization and Hybridization (northern), continued

4. Add the amount of Hybridization buffer recommended for your hybridization bottle (or bag) to pre-hybridize the blot. Then incubate the blot at 68 °C for 1 to 2 hours.

5. Pour off the Hybridization buffer and add fresh Hybridization buffer containing labeled probe. For DIG chemiluminescent detection, use a probe of 10 to 15 ng/mL at a final concentration. At concentrations of > 20 ng/mL the background increases rapidly. Then incubate the blots for 12 to 18 hours at 68 °C.

   CAUTION: **For users performing DIG chemiluminescent detection only:** An excessive probe concentration dramatically increases high background. A probe concentration that is too low causes a signal decrease. After the labeling reaction, the probe labeling efficiency must be determined according to the "Determine the Labeled Probe Concentration (north ern)" section.

6. Pour off the Hybridization buffer containing the probe.

7. 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.)

8. 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.

9. For radioactive detection, expose the blot to autoradiographic film. If you are performing DIG chemiluminescent detection see the "Detection of DIG Labeled Probe" section. 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.) Then see the "Stripping Protocol" 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.

COMMON DUPONT SAS

Detection of DIG Labeled Probe

1. Prepare the following buffer and wash solutions:

Blocking Solution	*DIG Washing Buffer*	*Detection Buffer*
1% Blocking reagent 0.1 M Maleic acid, pH 7.5 0.15 M NaCl	0.1 M Maleic acid, pH 7.5 0.15 M NaCl 0.3% (v/v) Tween® 20 surfactant	0.1 M Tris-HCl, pH 9.5 0.1 M NaCl

NOTE: Filtration of the solutions is recommended to remove any particles using an appropriate high flow rate membrane, for example Millipore Stericup-GP (0.22 µm) filter unit. Use a pressure driven filter unit such as Millex®-GP device for the solution containing Blocking reagent.

2. Equilibriate the blot in DIG washing buffer (1 mL/cm^2) for 5 minutes at room temperature. Then pour off the washing buffer.
3. Place the blots in a plastic bag and add blocking buffer (0.1 mL/cm^2) to the blot.
4. Agitate the blots at room temperature for 1 hour.
5. Near the end of the blocking, dilute anti-digoxigenin-alkaline phosphate conjugate (after centrifuge at 12,000 × g for 10 minutes) in fresh blocking buffer at 1:10,000.
6. Pour off the blocking buffer and add freshly diluted anti-digoxigenin-alkaline phosphate conjugate (0.1 mL/cm^2). Then incubate the blots at room temperature for 1 hour.
7. Pour off the solution and transfer the blots to a suitable plastic container.
8. Add DIG washing buffer (1 mL/cm^2) and rinse the blots for 1 minute. Then wash the blots at room temperature for 15 minutes. Pour off the washing buffer and wash again.
9. Pour off the washing buffer. Add the detection buffer (0.05 mL/cm^2). Then incubate the blots at room temperature for 5 minutes.

DOMINIQUE DUTSCHER SAS

Detection of DIG Labeled Probe, continued

10. Pour off the detection buffer. Place the blots on a sheet of transparency film. Add ready-to-use CSPD (0.01 mL/cm^2) on the surface of the blot and place a second sheet of transparency film over it.
11. Remove any air bubbles that form on the blots. Incubate at room temperature for 5 minutes.
12. Remove the excess amount of the CSPD from the blots by lifting the sandwich and allowing the excess substrate to drip from one corner. Clean the blot side of the transparent film and make sure no air bubbles are trapped between the membrane and the transparency film.
13. Incubate the blot at 37 °C for 15 minutes. Then expose the blot to an X-ray film at room temperature for an adequate time for detection (15 minutes to 3 hours). Develop the film.

Stripping Protocols

As described in the "Pre-Hybridization and Hybridization" 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 DNA blots and RNA blots for subsequent reprobing.

CAUTION: Do not use sodium hydroxide for stripping of RNA blots. Signal intensity decreases dramatically upon the first re-probing after the blot is stripped with 50 mM NaOH.

DOMINIQUE DUTSCHER SAS

DNA Blot Stripping

1. Prepare the solutions as follows:
   - Stripping: 0.4 M NaOH
   - Neutralization: 0.1 x SSC, 0.1% (w/v) SDS, 0.2 M Tris·Cl, pH 7.5
2. Heat the Stripping solution in a glass beaker on a hot plate to 45 °C. Heat enough solution to fully cover the blot.
3. Remove the blot from its plastic bag with forceps and gently place it in the heated Stripping solution. Let the blot float into the solution. Avoid pushing it into the solution with forceps to prevent damaging the membrane.
4. Incubate the blot in the Stripping solution at 45 °C for 30 minutes. Then transfer the blot to the Neutralization solution and incubate for 15 minutes.
5. 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.

DNA or RNA Blot Stripping

1. Prepare this stripping solution: 0.1% (w/v) SDS.
2. Heat the 0.1% (w/v) SDS in a glass baking dish until boiling. Remove it from the heat after boiling.

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.

OMNIQUE DUTSCHER SA

DNA or RNA Blot Stripping, continued

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 re-probe it beginning with the pre-hybridization step.

Colony Lifts

This section describes how to grow bacterial colonies, prepare the membrane and replicate colonies onto Immobilon-Ny+ membrane. It also contains a colony lysis protocol.

Grow Bacterial Colonies

1. Grow the bacteria in nutrient medium containing the appropriate antibiotic.
2. Plate the bacteria on agar plates to obtain the desired number of colonies for colony lifts.

Prepare the Membrane

1. Cut pieces of Immobilon-Ny+ membrane to fit into your agar plates (15 cm common plate size) or use pre-cut disks.
2. Label the membranes with a soft-lead pencil.
3. Wet the membranes with Milli-Q water and sandwich them between dry sheets of Whatman 3MM filter paper.

OMINIQUE DUTSCHER SA

Prepare the Membrane, continued

CAUTION: Do not immerse the Immobilon-Ny+ membrane in liquid on the first liquid exposure. You can only expose one side to liquid on the first exposure. If you wet both sides, air can become trapped in the pores and form bubbles.

4. Wrap the pile of Immobilon-Ny+ nylon membranes in aluminum foil. Then autoclave them at 15 lb./in.^{2} (1.05 kg/cm^2) at 121 °C on a liquid cycle for sterilization.
5. See the next section to replicate colonies onto the membranes.

Replicate Colonies onto Immobilon-Ny+ Membrane

1. Prepare LB agar plates that contain the appropriate antibiotic.
2. Place the bacterial suspension on the surface of the agar plates.
3. Place the plates, with their lids ajar, in a laminar flow hood to allow the surface of the agar to dry.
4. Close the lids, invert the plates, and incubate them for 12 to 14 hours at 37 °C.
5. Place the plates at 4 °C for 30 to 60 minutes to chill.
6. Use sterile, blunt-ended forceps to remove a sterile filter from the sterilized pack (prepared in the previous section, "Prepare the Membrane").
7. Place the membrane, labeled side down, on the surface of the agar and in contact with the bacterial colonies. Leave the membrane on the agar surface until it wets out completely.
8. Use an 18-gauge needle to mark several locations on the membrane by stabbing through it and the agar underneath. You will use these holes to align the membrane with the marks in the agar as described in the "Hybridization to Colony Lifts on Immobilon-Ny+ Membrane" further in these instructions.

OMINIQUE DUTSCHER SA

Replicate Colonies onto Immobilon Ny+ Membrane, continued

9. Remove the membrane from the agar plate gently with forceps. You can now do one of the following with the colony lift:
   - Immediately follow the steps in the next section, "Colony Lysis Protocol and DNA Fixation."
   - Place the colony lift, colony side up, on a fresh LB agar plate containing the appropriate antibiotic. Incubate the plate for several hours to allow the colonies to grow before lysing them as described in the following section.
10. Regenerate the bacterial colonies on the master plate by incubating it for 5 to 7 hours at 37 °C. Then seal the plate with Parafilm and store it in an inverted position at 4 °C.

Colony Lysis Protocol

1. Prepare the solutions as follows:
   - Denaturing: 0.5 N NaOH, 1.5 M NaCl
   - Neutralization: 1.5 M NaCl, 0.5 M Tris·Cl (pH 7.4)
   - 2 x SSC
2. Cut three pieces of Whatman 3MM filter paper to the appropriate size and fit them into the bottoms of three plastic or glass trays. You can use standard petri dishes for small batches of membranes.
3. Saturate a piece of 3MM filter paper in each of the solutions prepared in step 1 (for a total of three separate pieces).
4. Peel the nylon membranes from their agar plates with forceps gently. Then gently place them, colony side up, on the 3MM filter paper saturated with Denaturing solution for 5 minutes.

COPYRIGHT DOMINIQUE DUTSCHER SAS

Colony Lysis Protocol, continued

5. Transfer the first membrane from the Denaturing solution to the 3MM filter paper piece saturated with Neutralization solution. Then transfer the rest of the membranes in the order they were removed from their agar plates. Leave the membranes on the 3MM filter paper saturated with Neutralization solution for 5 minutes.
6. Transfer the membranes to the 3MM filter paper saturated with 2 x SSC. Expose each filter to the 2 x SSC for 5 minutes.
7. Place the membranes, colony side up, on a dry sheet of 3MM filter paper. Allow the filters to dry for a minimum of 30 minutes at room temperature.

DNA Fixation

The DNA fixation process permanently binds a portion of the target DNA to the membrane surface. You can fix DNA to the surface of Immobilon Ny+ membrane 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 gives better sensitivity. The optimal UV energy varies depending on colony size. Therefore, optimization of UV cross linking may be required in advance for maximum sensitivity.

DOMINIQUE DUTSCHER SA

DNA Fixation with UV Cross-linking

1. Allow the blot to dry completely at 80 °C for 10 to 20 minutes.
2. 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 DNA. If you plan to place the membrane on a UV transilluminator, clean the surface with Milli-Q water and a Kimwipe tissue.
3. Expose the side of the blot with the bound DNA to a UV light source (254 nm). Determine the optimal UV energy for your experiment. Then see the "Hybridization to Colony Lifts on Immobilon-Ny+ Membrane" section

CAUTION: **Exposure to UV causes a significant health hazard. Wear UV protective goggles and shield all exposed skin.**

DNA Fixation with Baking

1. Place the blot between two pieces of filter paper.
2. Place the blot and filter papers in an oven set at 80 °C for 1 to 2 hours.
3. Remove the blot from the oven and let cool to room temperature. Then see the "Hybridization to Colony Lifts on Immobilon-Ny+ Membrane" section.

OMINIQUE DUTSCHER SA

Hybridization to Colony Lifts on Immobilon-Ny+ Membrane

1. Prepare the buffer and solutions as follows:

Buffer	*Prewash*
2 x SSC	5 x SSC 0.5% (w/v) SDS 1 mM EDTA (pH 8.0)

Hybridization Buffer	*Wash Solution I*	*Wash Solution II*
5 x SSPE 5 x Denhardt's 100 µg/mL sheared DNA 0.5% (w/v) SDS	2 x SSC 0.1% (w/v) SDS	0.2 x SSC 0.1% (w/v) SDS

2. Wet the membranes by laying them on top of 2 x SSC in a shallow tray (after DNA fixation). Once the membranes are fully wet, agitate the tray gently to completely immerse the filters.

   CAUTION: Do not immerse the Immobilon-Ny+ membrane in liquid on the first liquid exposure. You can only expose one side to liquid on the first exposure. If you wet both sides, air can become trapped in the pores and form bubbles.

3. Transfer the membranes to a glass dish containing Prewash solution. Cover the dish with plastic wrap and incubate the filters at 50 °C for 30 minutes with gentle agitation.

4. Soak Kimwipe tissues in Prewash solution and use them to gently remove the bacterial debris from the surfaces of the membranes.

5. Carefully place the membranes into hybridization bottles with the colonies oriented toward the center of the tube. (You can also use heat-sealable plastic bags.)

OMINIQUE DUTSCHER SA

Hybridization to Colony Lifts on Immobilon-Ny+ Membrane, continued

6. Add the recommended amount of Hybridization buffer to pre-hybridize the Immobilon-Ny+ membranes. Then incubate them at 68 °C for 2 hours.
7. Pour off the Hybridization Buffer and add fresh Hybridization buffer containing labeled probe. Incubate the membranes for 12–18 hours at 68 °C. Then pour off the Hybridization buffer containing the probe. (Transfer blots hybridized in plastic bags to a glass dish for washing.)
8. Fill the tubes halfway with Wash solution I. Incubate for 5 minutes at room temperature with mixing. Then pour off the Wash solution. Repeat this step once.
9. 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.
10. Dry the membranes at room temperature on 3MM filter paper. Then mount the membrane on a piece of 3MM filter paper, wrap it in plastic wrap, and expose it to autoradiographic film.
11. Identify the positive colonies by aligning the colonies on the film with those on the agar plate using the needle marks on the blot and the agar plate as orientation marks.

DOMINIQUE DUTSCHER SA

Product Ordering Information

Membrane Dimensions (0.45 µm pore size)	*Qty./Pk.*	*Immobilon-Ny+ Catalogue Number*
30 cm × 3.3 m roll	1	INYC 000 10
26 cm × 26 cm sheets	10	INYC 262 60
22.2 cm × 22.2 cm sheets	10	INYC 222 20
22.2 cm × 22.2 cm sheets	100	INYC 222 2C
20 cm × 20 cm sheets	10	INYC 202 00
15 cm × 15 cm sheets	10	INYC 151 50
10 cm × 10 cm sheets	10	INYC 101 00
9 cm × 12 cm sheets	10	INYC 091 20
6 cm × 11 cm sheets	50	INYC 611 50
82 mm discs	50	INYC 082 50
85 mm discs	50	INYC 085 50
132 mm discs	50	INYC 132 50
137 mm discs	50	INYC 137 50

DOMINIQUE DUTSCHER SA

Technical Assistance

For more information, contact the Millipore office nearest you. In the U.S., call **1-800-MILLIPORE** (1-800-645-5476). Outside the U.S., see your Millipore catalogue for the phone number of the office nearest you or go to our web site at www.millipore.com/offices for up-to-date worldwide contact information. You can also visit the tech service page on our web site at www.millipore.com/techservice.

Standard Warranty

Millipore Corporation ("Millipore") warrants its products will meet their applicable published specifications when used in accordance with their applicable instructions for a period of one year from shipment of the products. **MILLIPORE MAKES NO OTHER WARRANTY, EXPRESSED OR IMPLIED. THERE IS NO WARRANTY OF MERCHANTABILITY OR FITNESS FOR A PARTICULAR PURPOSE.** The warranty provided herein and the data, specifications and descriptions of Millipore products appearing in Millipore's published catalogues and product literature may not be altered except by express written agreement signed by an officer of Millipore. Representations, oral or written, which are inconsistent with this warranty or such publications are not authorized and if given, should not be relied upon.

In the event of a breach of the foregoing warranty, Millipore's sole obligation shall be to repair or replace, at its option, the applicable product or part thereof, provided the customer notifies Millipore promptly of any such breach. If after exercising reasonable efforts, Millipore is unable to repair or replace the product or part, then Millipore shall refund to the customer all monies paid for such applicable product or part. **MILLIPORE SHALL NOT BE LIABLE FOR CONSEQUENTIAL, INCIDENTAL, SPECIAL OR ANY OTHER INDIRECT DAMAGES RESULTING FROM ECONOMIC LOSS OR PROPERTY DAMAGE SUSTAINED BY ANY CUSTOMER FROM THE USE OF ITS PRODUCTS.**

COMMUNIQUE DNTSCHER SA

DOMINIQUE DUTSCHER SA

MILLIPORE