Technical Note

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Title: Colony Lifts Using Immobilon™-NY+ Membrane

Introduction

Positively charged nylon membrane can be used as a replacement for nitrocellulose when performing colony lifts (1). In this technical note, data are presented that demonstrate Immobilon-Ny+ (2, 3, 4, 5, 6) outperforms both nitrocellulose and a competitor's positively charged nylon membrane. Further, it is determined that UV cross-linking, rather than baking, generally generates membranes that exhibit higher sensitivity. Different experimental conditions have been analyzed for the optimal UV energy required. This technical note concludes with a protocol for colony lifts using Immobilon-Ny+.

Summary

- Immobilon-Ny+ performs better than traditional nitrocellulose and a competitor's positively charged nylon membrane on colony lifts.
- UV cross-linking enhances signal intensity and exhibits higher sensitivity than baking.
- Optimal UV cross-linking energy varies depending on colony size. 5,000 μJoules/cm^2 for direct spotting of bacterial suspension on a membrane without further culture, 60,000 mJoules/cm^2 for colonies of <1-2 mm in diameter, 120,000 mJoules/cm^2 for colonies of 3-5 mm in diameter are recommended.
- Determination of optimal UV energy is required for different experimental conditions for the maximum sensitivity.
- If maximum sensitivity and re-probing are not required, baking is an acceptable fixation method for colony lifts on Ny+ because reasonable detection sensitivity can be obtained without optimizing UV energy.

Materials and Methods

Colony Transfer and Lysis. A positive clone of JM109 transformed with a plasmid pLH2 (a 2.0 kbp fragment of phage DNA Hind III cloned into pUC19) and a negative clone of JM109 with pUC19 having no insert were utilized in this study. Colonies were grown on an LB/ampicillin agar plate with an X-gal/IPTG solution applied onto the surface (1) at 37C for 13 to 17 h. After chilling the agar plates at 4C for 30 to 60 min, an 82 mm disc membrane was placed on the surface of the agar plate until completely wet. The membrane was placed colony side up on filter paper saturated with Denaturing solution (0.5 N NaOH, 1.5 M NaCl) for 5 min. The membrane was transferred to filter paper saturated with Neutralization solution (0.5 M Tris-Cl, pH7.4, 1.5 M NaCl) for 5 min. then placed on filter paper saturated with 2X SSC for 5 min.

UV Fixation of DNA. Membranes were air-dried then dried completely at 80C for 15 to 20 min. DNA was fixed to the membranes by UV irradiation at 254 nm with 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 control of UV irradiation at lower energy levels. The side of the membrane with the bound DNA faced the bulbs. Drift in power output of the UV bulbs was compensated for by using the energy mode on the Stratalinker. This was considered essential for low energy exposures since the energy output of the UV bulbs was approximately 1500 Joules/cm^2 per second.

Probe preparation. /Hind III DNA fragments were heated to 65C for 10 minutes and then chilled on ice to denature the cos sites. The DNA was resolved in a 1.0 % agarose gel using modified TAE (40 mM Tris-acetate, pH 8.0, 0.1 mM EDTA) at 120 volts. After electrophoresis, the gels were stained in ethidium bromide (0.5 mg/mL). Using a long-wavelength (365 nm) UV light, the slice of agarose containing the 2.0 kbp band was excised with a razor blade. The slice of agarose was placed into Ultrafree-DA (Millipore, Bedford, MA) and spun at 5,000 x g for 10 min (7). The DNA recovery was 73%. The filtrate containing DNA was used to probe template without further purification. The DNA was labeled by random primer method (8) with a 32P dCTP (3,000 Ci/mmol).

Pre-hybridization and Hybridization (1). Membranes were wet with 2X SSC, then incubated in Pre-wash solution (5X SSC, 0.5% (w/v) SDS, 1 mM EDTA, pH 8.0) at 50C for 30 min. with gentle agitation. The bacterial debris was gently removed from the surface of the membrane using Kimwipes soaked in Pre-wash solution. Membranes were placed in glass hybridization tubes. For pre-hybridization, 10 mL of hybridization solution (5X SSPE, 5X Denhardt's, 0.5% (w/v) SDS, 100mg/mL sheared DNA) was added to each tube. The tubes were placed in a rolling bottle hybridization oven for 1.5 to 2 h at 68°C. The pre-hybridization solution was decanted and replaced with 3 mL of hybridization solution containing 32P-labeled probe DNA at a ratio of 1.0 x 105 cpm per cm^2 of membrane surface area. The tubes were returned to the rolling bottle hybridization oven and incubated for 16 to 20 h at 68°C. Membranes were then washed twice for 5 min each in 2 x SSC, 0.1% SDS, at room temperature, and twice for 15 min each in 0.2 x SSC, 0.1% SDS, at 68°C.

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

Comparison of Immobilon-Ny+ with a nitrocellulose membrane

Immobilon-Ny+ was compared with a traditional nitrocellulose membrane (Immobilon-NC HATF, Millipore). Immobilon-Ny+ showed higher signal intensity than nitrocellulose and UV cross-linking with 60,000 mJoules/cm^2 performed best in this experiment. However, there was no significant difference in hybridization signal when comparing 15,000 and 120,000 mJoules/cm^2 (Fig 2). For consistent results, UV cross-linking with 60,000 mJoules/cm^2 is recommended for lifts with colonies less than 1-2 mm in diameter.

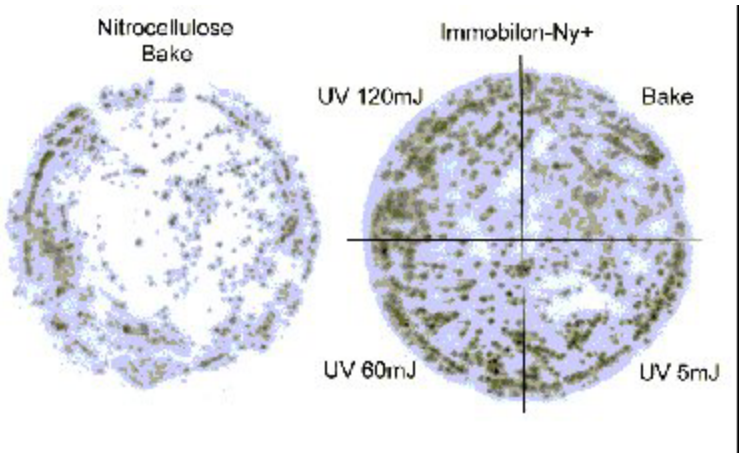


Fig 1. Comparison of Immobilon-Ny+ and Immobilon-HATF
A mixed bacterial suspension of JM109 carrying pUC19 or pLH2 (2.0 kbp fragment of phage DNA Hind III cloned into pUC19) was plated on LB agar plates and incubated at 37C overnight. After chilling the plates at 4C for 30 min., the colonies (approx. 1-2 mm diameter) were transferred to an 82 mm disc membrane of nitrocellulose or Immobilon-Ny+. Each membrane was processed through colony lysis and membrane drying step according to the protocol described below. The DNA on the membrane was fixed either by baking at 80C for 1 hr. under vacuum or UV cross-linking with different UV energy at 254nm. Then, the membranes were hybridized with a 32P labeled DNA probe and visualized using a phosphor imaging system.

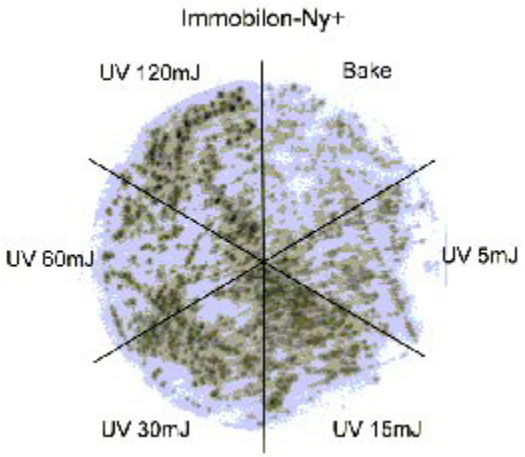


Fig 2. UV energy on colony lifts with small colonies
A mixed bacterial suspension of JM109 carrying pUC19 or pLH2 (2.0 kbp fragment of phage DNA Hind III cloned into pUC19) was plated on LB agar plates and incubated at 37C overnight. After chilling the plates at 4C, the colonies (<0.5 mm diameter) were transferred to an 82 mm disc membrane of Immobilon-Ny+. The membrane was processed through colony lysis and membrane drying step according to the protocol described below. The DNA on the membrane was fixed either by baking at 80C for 1 hr. under vacuum or UV cross-linking with different UV energy at 254nm. Then, the membranes were hybridized with a 32P labeled DNA probe and visualized using a phosphor imaging system.

Comparison with Competitor's Membrane

Immobilon-Ny+ exhibited better hybridization signals than competitor A.

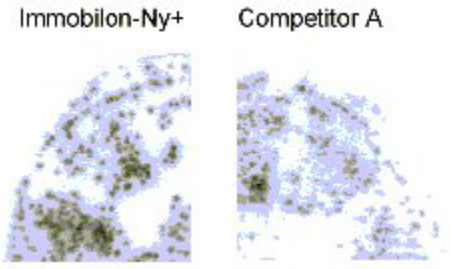


Fig 3. Comparison of Immobilon-Ny+ and Competitor A

A mixed bacterial suspension of JM109 carrying pUC19 or pLH2 (2.0 kbp fragment of phage DNA Hind III cloned into pUC19) was plated on LB agar plates and incubated at 37C overnight. After chilling the plates at 4C for 30 min., the colonies (approx. 1-2 mm diameter) were transferred to an 82 mm disc membrane of Immobilon-Ny+ or a competitor's positively charged nylon. Each membrane was processed according to the protocol described below. The DNA on the membrane was UV cross-linked with 60,000 mJoules/cm^2 at 254nm. Then, the membranes were hybridized with a 32P labeled DNA probe and visualized using a phosphor imaging system.

Optimal UV energy varies depending on bacterial loading (Graph 1 and 2)

To compare the hybridization signal by quantification, an equivalent amount of the bacterial suspension was directly applied to a membrane surface and processed immediately. The maximum hybridization signal was obtained using 5,000 mJoules/cm^2. This was an equivalent UV cross-linking energy when performing Southern hybridization using pure phage DNA Hind III digested fragments (2). The intensity profile was similar for a dilute bacterial suspension of 8.6 x 103 (data not shown). Although maximum hybridization signal was obtained at 5,000 mJoules/cm^2, above 60,000 mJoules/cm^2, the signals were less than no UV (0mJ). Baking performed better than cross-linking with improper UV energy.

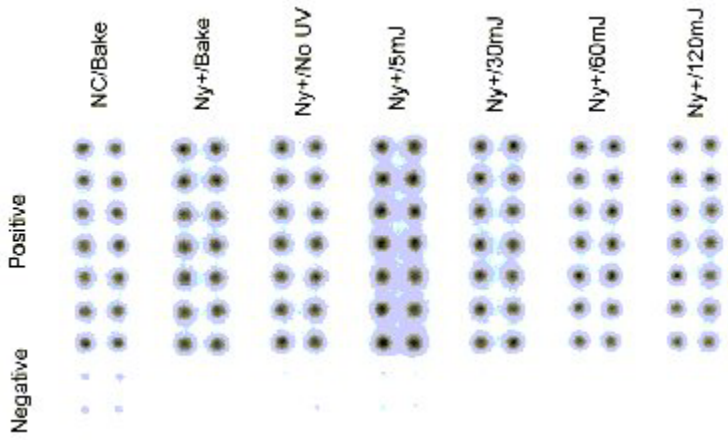
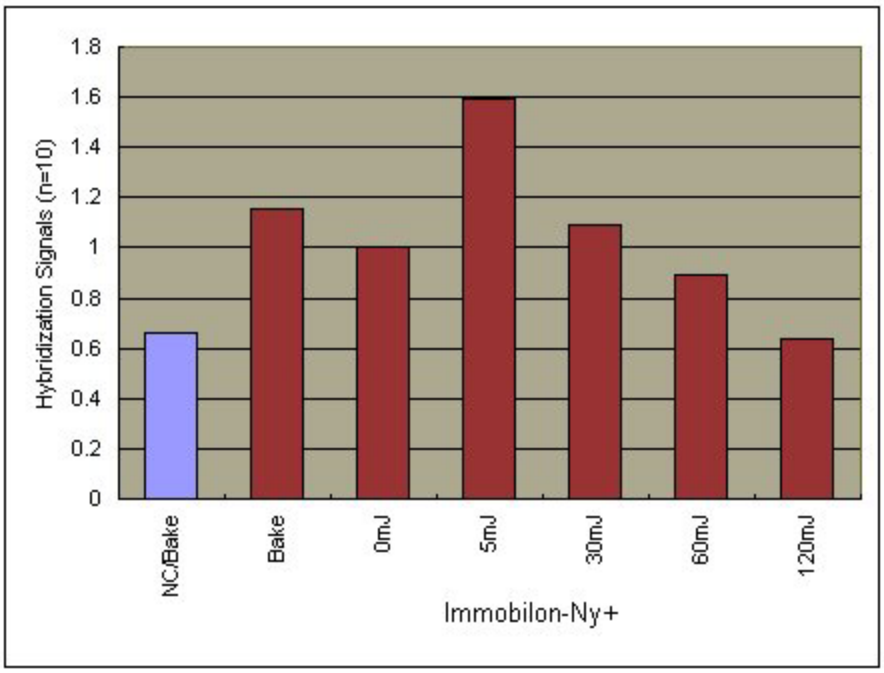
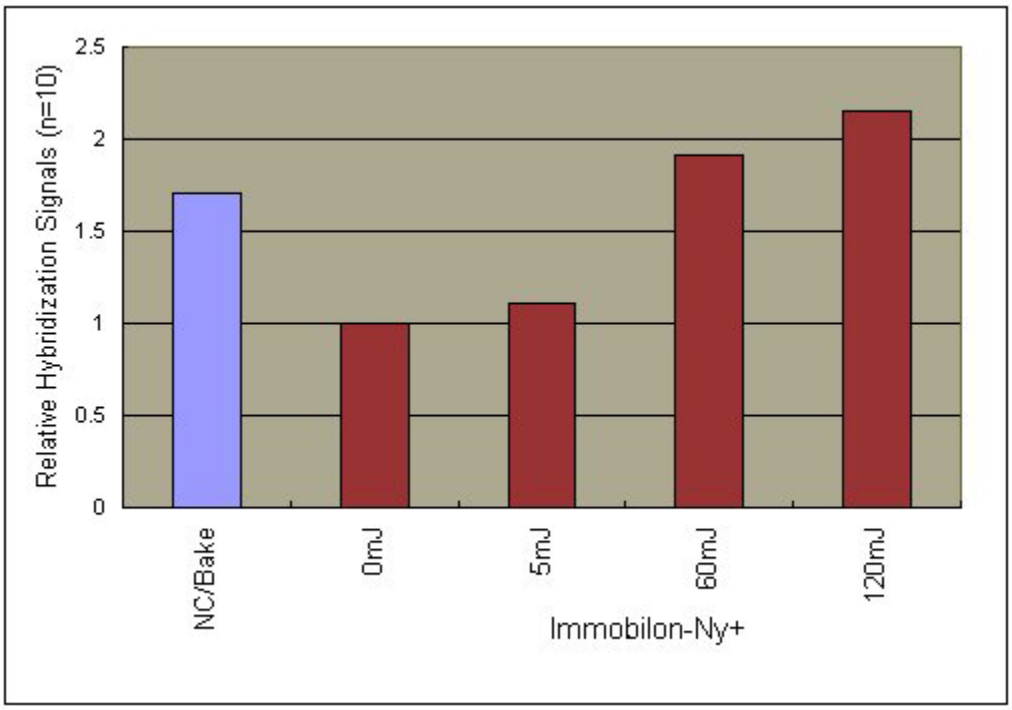


Fig 4. Direct spotting of bacterial suspension onto a membrane.

Aliquots of 1 mL bacterial suspension (8.6 x 105 cells/mL) of JM109 carrying pUC19 (negative) or pLH2 (2.0 kbp fragment of phage DNA Hind III cloned into pUC19) (positive) were spotted onto dry nitrocellulose or Immobilon-Ny+ membrane. Each membrane was processed through colony lysis and membrane drying step according to the protocol described below. The DNA on the membrane was fixed either by baking or UV cross-linking with different UV energy at 254nm. Then, the membranes were hybridized with a 32P labeled DNA probe and visualized using a phosphor imaging system.



Graph 1. Quantification of hybridization signals.
The hybridization signals obtained in Fig. 4 were quantified by phosphor imaging. Each signal was normalized to the signal of no UV cross-linking at 0 mJoules/cm^2.



Graph 2. Quantification of hybridization signals.
Aliquots of 0.3 mL bacterial suspension (1.2 x 102 cells/mL) of JM109 carrying pUC19 (negative) or pLH2 (positive) were spotted onto nitrocellulose or Immobilon-Ny+ membrane placed on an LB agar plate. After overnight incubation at 37C (with bacterial colonies of approx. 3 - 5 mm in diameter), the membrane was removed from the agar plate and processed through colony lysis and membrane drying step according to the protocol described below. The DNA on the

membrane was fixed either by baking or UV cross-linking with different UV energy at 254nm. Then, the membranes were hybridized with a 32P labeled DNA probe and visualized using a phosphor imaging system. The hybridization signals were quantified by phosphor imaging. Each signal was normalized to the signal of no UV cross-linking at 0 mJoules/cm^2. Maximum hybridization signal was obtained at 120,000 mJoules/cm^2. This suggests that a large colony needs high UV dosage because the excessive bacterial debris stacked up on the membrane surface attenuates UV energy.

Helpful Hints for Better Results on Colony Lifts

- A soft lead pencil with dull tip is recommended to label membranes. Sharp pencils can tear a membrane and ink can cause problematic background. Be aware that writing on the membrane crushes the microporous structure.
- Any fine particulates in solutions used for hybridization should be removed using an appropriate filter unit with 0.22 to 0.45 mm pore size (e.g., Stericup GP vacuum-driven filter unit, Millipore) to prevent splotchy background.
- Small colonies (< 0.5 mm in diameter) are preferable because they produce sharp signal and less smearing.
- Agar plates must be chilled at 4C for at least 30 minutes before lift.
- Do not leave a membrane on the plate longer than necessary to prevent smearing of the colonies.
- Do not wet the side of the membrane carrying the colonies during denaturation and neutralization, otherwise hybridization signals may become blurry.
- Optimal UV cross-linking energy varies depending on size of colony. If you intend to optimize the UV energy for maximum hybridization signal, refer to following examples:
  - 5,000 mJoules/cm2 at 254 nm for bacterial suspensions.
  - 60,000 mJoules/cm2 at 254 nm for < 1-2 mm diameter colonies.
  - 120,000 mJoules/cm2 at 254 nm for 3-5 mm diameter colonies.

References

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6. Optimization of Northern Blotting on ImmobilonTM-Ny+. Technical Note TN110, Millipore Corporation, Bedford, Massachusetts, USA.

7. Ultrafree-DA For DNA Extraction from Agarose gels. PF228, Millipore Corporation, Bedford, Massachusetts, USA.

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Colony Lift Protocol

This section describes how to grow bacterial colonies, prepare the membrane and replicate colonies onto Immobilon-Ny+. 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.

4. Wrap the pile of Immobilon-Ny+ nylon membranes in aluminum foil. Then autoclave them at 15 lb./sq. in. on a liquid cycle for sterilization.

5. See the next section to replicate colonies onto the membranes.

Replicate Colonies onto Immobilon-Ny+

1. Prepare LB agar plates that contain the appropriate antibiotic.

2. Place the bacterial suspension on the 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 37C.

5. Place the plates at 4C 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+” further in these instructions.

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 37C. Then seal the plate with Parafilm and store it in an inverted position at 4C.

Colony Lysis Protocol and DNA Fixation

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. 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.

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. Then follow the steps in the “DNA Fixation” section.

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+ with UV cross-linking or baking. This section provides steps on both methods.
The traditional method is baking under vacuum and requires a vacuum oven. The UV cross-linking method gives better sensitivity. **The optimal UV energy will vary depending on colony size. Thus, optimization of UV cross-linking may be required in advance for the maximum sensitivity.**

DNA Fixation with UV Cross-linking

1. Allow the blot to dry completely at 80 C for 15 min.

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.

3. Expose the side of the blot with the bound DNA to a UV light source (254 nm).s Optimal UV cross-linking energy varies depending on size of colony. If you intend to optimize the UV energy for maximum hybridization signal, refer to following examples, then see the "Hybridization to Colony Lifts on Immobilon-Ny+" section:
• 5,000 mJoules/cm^2 at 254 nm for direct spotting of bacterial suspensions on a membrane surface without further culture.
• 60,000 mJoules/cm^2 at 254 nm for < 1-2 mm diameter colonies.
• 120,000 mJoules/cm^2 at 254 nm for 3-5 mm diameter colonies.

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 clean filter paper.

2. Bake the blot and filter papers for 1 to 2 hours at 80C in a vacuum oven.

3. Remove the blot from the oven and let cool to room temperature. Then see the "Hybridization to Colony Lifts" section.

Hybridization to Colony Lifts on Immobilon-Ny+

1. Prepare the buffer and wash solutions as follows:
• 2 x SSC
• Prewash Solution: 5 x SSC, 0.5% SDS, 1 mM EDTA (pH 8.0)
• Hybridization Buffer: 5 x SSPE, 5 x Denhardt's, 0.1% SDS, 100 g/mL sheared DNA, 0.5% SDS
• Wash Solution I: 2 x SSC, 0.1% SDS
• Wash Solution II: 0.2 x SSC, 0.1% 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.

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

4. Soak Kimwipes 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.)

6. Add the recommended amount of Hybridization buffer to pre-hybridize the Immobilon-Ny+ membranes. Incubate at 68C for 2 hours.

7. Pour off the Hybridization Buffer and add fresh Hybridization buffer containing labeled probe. Incubate the membranes for 16 -18 hours at 68C. 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. Pour off the Wash solution. Repeat this step once.

9. Fill the tube halfway with Wash solution II, pre-heated to 68C. Incubate for 15 minutes at 68C. Pour off the Wash solution. Repeat this step once.

10. Dry the membranes at room temperature on 3MM filter paper. 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.

Immobilon-Ny+ Transfer Membranes
For highest sensitivity with superior reprobing

Filter Diameter, mm	Qty/Pk	Catalog Number / Order
N/A	1	INYC00010 add to order
N/A	10	INYC26260 add to order
N/A	100	INYC2222C add to order
N/A	10	INYC22220 add to order
N/A	10	INYC20200 add to order
N/A	10	INYC15150 add to order
N/A	10	INYC10100 add to order
N/A	10	INYC09120 add to order
N/A	50	INYC61150 add to order
137	50	INYC13750 add to order
132	50	INYC13250 add to order
85	50	INYC08550 add to order
82	50	INYC08250 add to order