Northern Blot

Use this protocol if you have hybridization bags.

Hybridization of DIG labeled (non-radioactive) DNA probe to a nylon membrane blotted with RNA.

Detection of the probe with Anti-DIG-AP antibody and chemiluminescent AP-substrate.

Stripping the DNA probes off the membrane.

IMPORTANT: *Once you have started this set of protocols, never let the membrane dry at any point (not even after it has been stripped). If the membrane is allowed to dry, it cannot be stripped. If handled properly, the membrane can be stripped and reprobed up to 14 times.*

Hybridization

Determine how much *DIG Easy Hyb* you will need for the procedures below. You will need enough buffer to completely cover the membrane during incubations. For $100cm^2$ of membrane, you will need:

a. 10 – 15 ml for prehybridization

b. 3.5 ml for hybridization

I. Prehybridization

1. Prewarm buffer: Place the correct amount of *DIG Easy Hyb* in a sterile tube. Place the tube in a 50^0C water bath.
2. Place the blot membrane into a hybridization bag.
3. Add the appropriate amount of prewarmed *DIG Easy Hyb* to the bag.

4. Remove air bubbles and seal the bag.
5. Submerge a test tube rack in a 50^0C water bath. Place the hybridization bag on top of the rack and weigh the bag down at the corners. *Make sure the weights do not set on the membrane.
6. Incubate the blot for _ to 3 hours at 50^0C. Agitate the membrane gently during the prehybridization.

During the prehybridization (step 6), prepare the hybridization solution:

1. Determine how much DIG-labeled DNA probe you need (20-50ng probe/ml buffer).
2. Place the appropriate amount of probe in a microfuge tube with 50μl of TE.
3. Place the tube in a boiling water bath for 5 minutes.
4. Immediately transfer the tube to an ice bath. Chill for 2 minutes.
5. Centrifuge the tube briefly.
6. Immediately add the denatured probe to a tube containing the appropriate amount of prewarmed *DIG Easy Hyb* (3.5ml per $100cm^2$) and mix by inversion.

II. Hybridization

1. Add the hybridization solution to the bag as follows: Cut open the sealed hybridization bag and pour out the prehybridization solution. Immediately replace with prewarmed hybridization solution (with probe). Remove air bubbles and seal the bag.
2. Submerge a test tube rack in a 50^0C water bath. Place the hybridization bag on top of the rack and weigh the bag down at the corners. *Make sure the weights do not set on the membrane.
3. Incubate the blot for 6 to16 hours at 50^0C. Agitate the membrane gently during the hybridization.

III. Stringent Wash

1. Fill a small plastic tray with enough Low Stringency Buffer to completely cover the membrane.
2. Cut open the bag and pour off the hybridization solution from the blot.

3. Immediately submerge the membrane in the tray containing the Low Stringency Buffer.
4. Incubate the tray at room temperature for 5 minutes with shaking.
5. Pour off the used buffer and immediately cover the membrane with fresh low Stringency Buffer.
6. Continue shaking at room temp for an additional 5 minutes.
7. During steps 4 – 6, preheat High Stringency Buffer to 50^0C.
8. Pour off the Low Stringency Buffer.
9. Immediately add the preheated High Stringency Buffer to the tray with the blot. Incubate the blot twice (2x 15 min, with shaking) in High Stringency Buffer at 50^0C. * If the probe is <80% homologous to the target, you need to perform these washes at a lower temp.

***S*olutions for Hybridization**

Low Stringency Buffer

2X SSC

0.1% SDS

High Stringency Buffer

0.1X SSC

0.1% SDS

20x SSC (1L)	[final]
175.3g NaCl	3M
88.2g Sodium Citrate	0.3M

Dissolve in 800ml H_2O.

Adjust pH to 7.0

Bring up to 1L

Autoclave if needed. Store at room temp.

Detection

Notes:

Unless indicated otherwise, all of the incubations in this section are performed at room temp with shaking.

The volumes suggested are for a 100 cm^2 blot. Volumes can be adjusted according to the size of the blot and container. Be sure each solution completely covers the membrane and keeps it from sticking to the container as it shakes.

The transparency technique, which conserves chemiluminescent substrate, can be used as an alternative to steps 11 through 13. You can find the directions at the end of this section.

Always wear gloves when handling CSPD or CDP-*Star*.

1. Transfer the membrane to a plastic tray containing 100ml of Washing Buffer.
2. Incubate 2 min at room temp with shaking.
3. Discard the Washing Buffer and add 100 ml of Blocking Solution.
4. Incubate membrane for 30 min (up to 3 hrs is ok) with shaking.
5. Discard Blocking Solution and add 20ml of Antibody Solution.
6. Incubate membrane for 30 min with shaking.
7. Discard the antibody solution.
8. Wash membrane twice (2 x 15 min) with 100ml portions of Washing Buffer.
9. Equilibrate membrane 3 min in 20ml Detection Buffer.
10. Place the membrane (RNA side up) inside a development folder (acetate sheets), hybridization bag, or other tightly sealable envelope-like container. (Do not use plastic wrap)
11. **Wear gloves.** For every 100cm^2 of membrane, apply 1ml (20-30 drops) of Ready-to-use CSPD or CDP-Star (chemiluminescent substrate for alkaline phosphatase), dropwise, over the surface of the blot until the entire surface is evenly soaked. **As you are applying the substrate, immediately** cover the dampened part of the membrane with the second side of the container so the substrate is spread evenly

over the membrane. Do not let air bubbles get trapped between the membrane and the upper surface of the container.

12. Incubate the membrane for 5 min at room temp.
13. Squeeze excess liquid out of the container and seal the sides of the container close to the membrane.
14. **If using CSPD**, incubate for 10 min at 37^{0}C to enhance the luminescence reaction. **If using CDP-Star**, skip this step.
15. Expose the sealed envelope (containing membrane) to luminescence optimized X-ray film (15-25 min).
16. Based on the results in step 15, adjust the exposure time to get a darker or lighter band pattern. Repeat exposures can be made up to two days after adding the substrate.

Solutions for detection

Maleic Acid Buffer

0.1 M Maleic acid

0.15 M NaCl

pH to 7.5 with solid NaOH

Store at room temp

Blocking Solution

Dilute 10x Blocking Solution 1:10 with Maleic Acid Buffer

Prepare fresh

Washing Buffer

0.1 M Maleic acid

0.15 M NaCl

pH to 7.5

0.3% Tween 20

Store at room temp

Antibody Solution

Centrifuge Anti-Digoxygenin-AP for 5 min at 10krpm in its original vial prior to each use. Pipet the necessary amount carefully from the top surface.

Dilute Anti-Digoxygenin-AP 1:10 000 (75mU/ml) in Blocking Solution.

Prepare fresh (2hrs, 4^0C)

Detection Buffer

0.1 M Tris-HCl

0.1 M NaCl

pH to 9.5 (20^0C)

Store at room temp

Transparency Technique: an alternative to steps 11-14.

1. Place the membrane on a sheet of transparency film.
2. Cover the membrane with dilute chemiluminescent substrate in Detection Buffer. For each 100cm^2 of membrane, use 500 µl of one of the following:

   1:100 dilution of 25 mM stock CSPD

   undiluted 0.25 CSPD ready-to use

   1:200 – 1:500 dilution of 25 mM stock CDP-Star

   1:2 – 1:5 dilution of 0.25 mM CDP-Star ready-to-use
3. Cover the damp membrane with a second sheet of transparency film.
4. Incubate 5 min.
5. Let excess liquid drip off membrane and heat seal the transparency sandwich around the damp membrane.

Stripping

* Always prepare Stripping Buffer just before use.

1. Rinse membrane thoroughly with double distilled water.
2. Incubate membrane twice (2 x 60 min) at 80^{0}C in Stripping Buffer (50% deionized formamide, 5% SDS, 50mM Tris-HCl, pH 7.5) in a sealed bag.
3. Rinse membrane 5 minutes in 2x SSC at room temp.
4. Membrane can be reprobed immediately or stored wet in 2x SSC.