Odyssey®

Infrared Imaging System

Southern Blot Analysis

Using Biotin-Labeled Probes

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LI-COR® Biosciences

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Contents

I. Required Reagents

Southern Blotting and Hybridization

- Biodyne® B Nylon Membranes (Pall, Cat. #60200)
- DNA hybridization solution (see ***II. Making Solutions for Southern Blot Analysis***)
- Wash Solutions #1, #2, and #3 (see ***II. Making Solutions for Southern Blot Analysis***)
- Sheared and denatured salmon sperm DNA
- 100X Denhardt's solution

Biotin Probe Labeling and Detection

- PCR Amplification Reagents
- Biotin-16-dUTP (Roche, Cat. #1 093 070)*
- Odyssey® Blocking Buffer (LI-COR, Cat. #927-40000)
- Streptavidin IRDye® 800CW, 0.5 mg (LI-COR, Cat. #926-32230**)
- QIAquick® PCR Purification Kit, 50 reactions (Qiagen, Cat. #28104)
- 20% SDS
- 1X PBST (0.1% Tween®-20)
- 1X PBS

* LI-COR Biosciences recommends that all restrictions placed on product labels and product inserts for biotin-16-dUTP be followed. Applications other than those recommended on the product insert may require a license under certain patents owned by third parties. LI-COR Biosciences does not grant any additional license to make, use or sell this product.

** Streptavidin IRDye® 800CW can also be purchased from Rockland Immunochemicals (Cat. #S000-31).

II. Making Solutions for Southern Blot Analysis

Solutions referred to in this protocol can be made as indicated below. Storage conditions for excess solution are listed.

DNA Hybridization Solution (200 ml; store at 4°C): 10% (w/v) Dextran Sulfate; 5X SSPE; 2% (w/v) SDS

Wash Solution #1 (1L; store at room temperature): 2X SSPE

Wash Solution #2 (1L; store at room temperture): 2X SSPE; 1% (w/v) SDS

Wash Solution #3 (1L; store at room temperature): 0.1X SSPE

III. Southern Blotting Methods

Most Southern blotting systems should work as long as the following guidelines are observed:

a. Biodyne B Nylon Membranes are used;
b. The loading buffer contains only small amounts of bromophenol blue.

Biodyne B Nylon Membranes work well because they have been tested for reduced infrared background using both IRDye and Biotin labeling methods. Bromophenol blue is detected by Odyssey and can cause high background. Small amounts of the dye can be removed during prehybridization. Ideally, use a loading buffer that does NOT contain bromophenol blue.

Southern Blotting

1.	Prepare membranes for hybridization using Southern or dot/spot/slot blot methods. ⚠ **Important:** Do not touch the membrane; always handle by the corners and only with clean forceps. Fingerprints, even from a glove, will clearly show on the scanned image of the membrane. ■ **Tip:** For best performance, use Odyssey reagents for blotting. Hybridization solution and wash solutions should be made according to ***II. Making Solutions for Southern Blot Analysis***. Any additional reagents used should be of the highest grade available to reduce background on the membrane. Filter all reagents prior to blotting. ■ **Tip:** High concentrations of ethidium bromide in the agarose gel can increase background. If ethidium bromide is necessary, soak the gel for 30 minutes to de-stain prior to transfer. ■ **Tip:** Use a 6X xylene cyanol loading buffer only (0.1% xylene cyanol/30% glycenol). Dyes such as bromophenol blue fluoresce and cause high background on the membrane. Note that xylene cyanol runs at approximately 700-800 bp. Do not run the dye front past halfway.

Cross-link

2.	Cross-link RNA onto nylon using a UV crosslinker, or bake at 80°C for 30 minutes.

IV. Biotin Probe Labeling Using PCR Amplification

This modified biotin labeling protocol is designed to fit directly into any Southern protocol; however, system optimization may be necessary.

PCR Probe Amplification and Biotin-16-dUTP Incorporation

⚠ **Important:** For Southern detection to be a success, it is essential to optimize probe amplification and Biotin-16-dUTP incorporation. Each user's system will be different.

1. In the PCR reaction, replace the dTTP with 60% unmodified dTTP and 40% biotin-16-dUTP as illustrated below in an example PCR reaction using M13 primers:

Component		
DNA	10 ng	
M13F (50 pM)	0.5 µl	
M13R (50 pM)	0.5 µl	
10X Buffer	2.5 µl	
$MgCl_2$ (25 mM)	5.0 µl	
dATP (10 mM)	0.625 µl	
dCTP (10 mM)	0.625 µl	
dGTP (10 mM)	0.625 µl	
dTTP (10 mM)	0.375 µl	(60%) ←
Biotin-16-dUTP (1 mM)	2.5 µl	(40%) ←
Taq® Polymerase (5µ/µl)	0.25 µl	
H_2O	__ µl	
TOTAL VOLUME	25.0 µl	

2. Amplify the probe using the standard PCR protocol for your specific product. An example program for M13 primers is given below.

Program:

Cycles	Temperature (°C)	Time
1	94	6 minutes
30	95 45 72	1 minute 2 minutes 3 minutes
1	72	10 minutes
1	4	hold

3.	Before proceeding with purification, it is highly recommended that you run 5 µl of the PCR amplified product on an 0.8% agarose gel and visualize using a UV transilluminator.

Visualization on an agarose gel will confirm adequate probe amplification. **If no product can be visualized, do NOT proceed with purification or Southern blot hybridization.** The PCR reaction must be optimized before continuing. If the visualized PCR is not a clean fragment or multiple fragments are present, gel extraction and purification of the appropriate size fraction is advised.

Probe Purification

We recommend using QIAquick® PCR Purification Kit (Qiagen, Cat # 28106).

4.	Add 125 µl of Buffer PB to sample tube. Mix well and add to column. Centrifuge at 12,000 xg for 1 minute. Discard flow through.
5.	Add 750 µl of Buffer PE to column. Make sure ethanol is added to the PE buffer before it is used. Centrifuge as in step 4 and discard flow through. Centrifuge again to remove excess PE buffer. Place column into a clean RNase-free centrifuge tube.
6.	Add 20 µl of Buffer EB, warmed to 65°C, directly to the center of the column to elute. Let stand at room temperature for 5 minutes. Centrifuge as in step 4. Repeat elution step 2 more times.

V. Southern Blot Hybridization

Pre-hybridization

1.	Add 50 µl of 100X Denhardt's Solution to 5 ml of DNA hybridization solution.
2.	Add 10 µg denatured and sheared salmon sperm DNA per 1 ml DNA hybridization solution containing 1X Denhardt's Solution.
3.	Pre-warm hybridization solution to 65°C. ■ **Tip:** Pre-warmed hybridization solution should be completely dissolved. Mix well before using.
4.	Place blot in hybridization bottle or bag.
5.	Pre-wet the membrane in Wash Solution #1.
6.	Pre-hybridize Southern blot for a minimum of 1 hour at 65°C in pre-warmed DNA hybridization solution containing 1X Denhardt's and salmon sperm DNA (0.1 ml hybridization solution per cm^2 nylon membrane). ■ **Tip:** Membranes may be prehybridized longer to decrease background. ■ **Tip:** When using larger sized blots, increase the amount of hybridization solution per cm^2. Use only enough solution to cover the membrane.

Denature Probe

7.	Denature probe for 5-10 minutes at 95°C and place immediately on ice.

Hybridization

The first time a probe is used, hybridize with the entire PCR product. Optimization can be done to reduce the amount of probe per hybridization. No less than 500 ng of PCR product should be used initially.

8.	Pour pre-hybridization solution off of blot. ⚠ **Important:** Always remove the prehybridization solution and replace with fresh hybridization solution (step 9).
9.	Add freshly denatured probe directly into fresh hybridization solution containing both 1X Denhardt's and salmon sperm DNA. Do not use more than 3-5 ml of hybridization solution per 10 x 10 cm blot. ⚠ **Important:** Do not touch the blot with the pipette tip or probe. ■ **Tip:** The correct probe concentration is essential in obtaining optimal results. If larger volumes are used, the amount of probe must be adjusted accordingly. ***This step will need to be optimized for your system.*** Start by adding the entire volume of probe.
10.	Add hybridization solution containing probe to the bottle or bag containing blot.
11.	Hybridize overnight at 65°C. ■ **Tip:** Time can vary for each sample. Shorter times are possible; however, there may be a reduction in signal intensity sensitivity. Temperature may be lowered for less stringent conditions and must be optimized for some applications.

Stringency Washes

Use clean containers and forceps to avoid cross-contamination and reduce background.

12.	Carefully remove membrane from the hybridization solution and place membrane in a clean container for washing. Washing may also be performed in the hybridization bottles. ■ **Tip:** Multiple membranes can be washed together, provided there is ample volume for each membrane to move freely.
13.	Remove hybridization solution and wash twice at room temperature in Wash Solution #1 for 5 minutes. ■ **Tip:** Start with 50°C, then increase temperature in small increments if necessary.
14.	Wash twice for 15 minutes at 60°C with Wash Solution #2. ■ **Tip:** If hybridization was done at a temperature lower than 65°C, the wash temperature should also be lowered to reduce stringency.
15.	Wash twice for 15 minutes at 60°C with Wash Solution #3.

VI. Biotin Detection for Southern Blots

⚠ **Important:** When using this labeling method on Odyssey, it is important that recommended reagents be used for detection.

Blocking

1.	Add 5 ml of 20% SDS to 95 ml Odyssey Blocking Buffer for a final concentration of 1% SDS. ⚠ **Important:** This step is essential. Failure to add SDS will result in very high background on blots.
2.	In a container, cover blot with Odyssey Blocking Buffer plus SDS and gently shake at room temperature for a minimum of 30 minutes. For more sensitive detection, blocking for a longer time may reduce background.

Streptavidin Incubation

3.	Dilute streptavidin-IRDye 800CW conjugate with Odyssey Blocking Buffer plus 1% SDS to a concentration of 1:10,000.
4.	Remove old blocking buffer and cover the blot with a thin layer of diluted streptavidin-IRDye 800CW solution. Use approximately 5 ml of buffer per 10 cm^2 of membrane. Incubate 30 minutes at room temperature while shaking. ⚠ **Important:** This IRDye 800CW conjugate is light-sensitive; protect from light during incubation.

Wash

Protect from light during wash steps.

5.	Wash the blot 3 times in 1X PBST (0.1% Tween-20) with shaking, for 5 minutes each, at room temperature. Follow with a rinse in 1X PBS, with shaking, for 5 minutes at room temperature.

Scan Blot On Odyssey

6.	Start with intensity setting of 7.

Chapter 3 of the Odyssey Operator's Manual describes how to place the blot on the Odyssey scanning surface. Chapter 2 of the Odyssey User Guide describes how to start scans and set the scanning parameters.

VII. Troubleshooting Guide

Problem	Possible Cause	Solution / Prevention
Low Sensitivity (faint bands or no bands).	Insufficient hybridization time.	For most applications, hybridize overnight.
	Incomplete transfer.	Following DNA transfer to membrane, view the gel with UV transilluminator to see if any DNA has remained in the gel.
	Target DNA not effectively fixed on membrane.	Check UV lamp or oven temperature.
	Poorly labeled probe.	Visualize PCR incorporated probe on an agarose gel to verify adequate amplification and incorporation of biotin-16-dUTP.
	Low probe concentration.	Probe concentrations vary. Quantify PCR product to verify probe concentration.
		Make sure you added the ethanol to the wash buffer in the cleanup kit.
		Increase the amount of probe used in the hybridization reaction.
	Low hybridization efficiency.	Increase hybridization time or probe concentration.
	Low target concentration.	Increase amount of target DNA used.
		Verify that DNA was not degraded on agarose gel before digestion.
	Too high stringency.	Decrease time or temperature of stringency washes.
	Membrane with hybridized target DNA inaccessible to the probe.	Place membrane in tube or bag with DNA side exposed to the hybridization solution.

Problem	Possible Cause	Solution / Prevention
Low Sensitivity (continued).	Intensity set too low on Odyssey when the scan is started.	Increase the intensity settings in the Scanner Console window by increments of 0.5 in one or both channels. Re-scan membrane.
		Images with weak signal can be enhanced by selecting Alter Intensity from the View menu and adjusting the sensitivity, brightness or contrast.
	Not enough streptavidin.	Increase the amount of streptavidin used in the detection steps.
Uneven, blotchy, speckled, or high background.	Membrane contamination.	Always handle the membranes by the edges and only with forceps. Fingerprints, even from a glove, cause increased background.
	Insufficient pre-hybridization of nylon.	Use adequate hybridization buffer to cover membranes and possibly extend the prehybridization time.
		Make sure that hybridization solution is pre-warmed and completely in solution before using.
	Contaminated forceps or dishes.	Always clean forceps after they are used with hybridization solutions containing labeled probe. Dirty forceps may deposit dye on membrane that will not wash away.
		Use clean dishes, bags, or bottles for incubations.
	Hybridizing or washing multiple membranes together in a small volume.	When hybridizing multiple membranes, make sure they do not overlap and there is enough hybridization solution to cover the membranes.
		When washing membranes together, provide enough wash solution to allow the membranes to move freely in the dish.

Problem	Possible Cause	Solution / Prevention
Uneven, blotchy, speckled, or high background (continued).	Too low of stringency or not long enough wash time.	Increase time of stringency wash to remove background signal.
		Increase temperature of stringency wash.
	Membrane not fully wetted or has become partially dry.	Keep membrane completely wet after hybridizing. This is particularly crucial if blot will be stripped and re-used.
		Do not allow the membrane to dry between pre-hybridization and hybridization.
	Probe added onto membrane.	Add the labeled probe to the hybridization solution. Avoid touching the membrane with the pipette tip containing the labeled probe.
	Too much labeled probe.	Decrease the amount of labeled probe added to the hybridization. This reduces background while retaining sensitivity.
	Incorrect loading buffer used.	Use 6X loading buffer (0.1% Xylene Cyanol + 30% glycerol). Bromophenol Blue and other dyes cause background fluorescence.
	Inadequate PCR amplification.	Visualize the PCR incorporated probe on an agarose gel. If the fragment is not a sharp band or there are multiple fragments present, gel extract the appropriate fragment, purify, and use that as the probe instead of the entire PCR reaction.
		Use sequence or gene specific primers for PCR amplification rather than vector related primers (example: M13). If this is not possible, digest PCR reaction to cleave off the vector sequence and gel purify insert.
	PCR amplified probe was not purified.	Purify PCR reaction.
	SDS was NOT added to the Odyssey Blocking Buffer.	Add 1% SDS to the Odyssey Blocking Buffer before using in blocking and detection steps of protocol.

Problem	Possible Cause	Solution / Prevention
Uneven, blotchy, speckled, or high background (continued).	Inadequate washing following streptavidin conjugation.	Increase PBST wash time following streptavidin conjugation.
	Inadequate blocking time before addition of streptavidin.	Increase blocking time before streptavidin conjugation making sure to use fresh blocking reagent in the streptavidin conjugation step.
	Too much streptavidin in conjugation step.	Reduce the amount of streptavidin used in conjugation step.

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