Odyssey®

Infrared Imaging System

Northern Blot Analysis

Using Biotin PCR Labeled Probes

Published January, 2006. Last updated October, 2007. The most recent version of this protocol is posted at http://biosupport.licor.com/support

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Biosciences

Doc# 988-09394

Contents

I. Required Reagents

Northern Blotting and Hybridization

- Biodyne® B Nylon Membranes (Pall, Cat. #60200).
- LI-COR recommends the NorthernMax® Kit (Ambion, Cat. #1940) for formaldehyde gels.
- RNA Loading Buffer (Sigma, Cat. #R-4268).
- ULTRAhyb™–Oligo (Ambion, Cat. #8663). This solution has been found to have best performance with infrared probes. Other hybridization solutions may cause high background.
- Sterile DEPC treated water.

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 conjugate (LI-COR, Cat. #926-32230) or Streptavidin IRDye® 680 conjugate (LI-COR, Cat. #926-32231)
- 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.

II. Northern Blotting Methods

This is a modified version of the NorthernMax® protocol for Northern blotting. LI-COR's system has been optimized using the NorthernMax Kit. Other transfer systems should work provided:

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.

IMPORTANT: All equipment must be RNase free and dilutions should be made with RNase-free water to prevent RNA degradation.

Gel Electrophoresis

1.	Separate RNA on a denaturing formaldehyde MOPS gel, as described in the Ambion NorthernMax protocol. ■ **Tip:** Ethidium bromide in large amounts increases background. If ethidium bromide is required, use Sigma loading buffer (Cat. #R-4268) since it does not cause excess background but contains the ethidium bromide necessary for sample visualization. This loading buffer works well for RNA markers.

Transfer

2.	Transfer for 2 hours or more using the NorthernMax transfer buffer. ⚠ **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.

Cross-link

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

III. Biotin Probe Labeling Using PCR Amplification

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

PCR Probe Amplification and Biotin-16-dUTP Incorporation

⚠ **Important:** For Northern detection to be a success, it is essential to optimize probe amplification and Biotin-16-dUTP incorporation. Each users' 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 probe purification, 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 probe purification or Northern blot hybridization.** 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 nuclease-free water warmed to 65°C directly to the center of the column to elute. Let stand at room temperature 5 minutes. Centrifuge as above. Repeat elution twice (total of three times).

IV. Northern Blot Hybridization

Pre-hybridization

⚠ **Important: Do not use the ULTRAhyb buffer provided in the NorthernMax Kit.** UlTRAhyb buffer is designed for RNA probes. Use only ULTRAhyb–Oligo hybridization solution (Ambion, Cat. #8663) for Odyssey Northern Blots. ULTRAhyb–Oligo has been found to have best performance with infrared probes. Other hybridization solutions may cause high background.

1.	Place blot in hybridization bottle or bag.
2.	Pre-hybridize Northern blot for a minimum of 1 hour at 42°C in Ambion ULTRAhyb™–Oligo Buffer, 5 ml per 10 x 10 cm blot. ■ **Tip:** Hybridization stringency can be increased or decreased by the hybridization temperature; however, we recommend 42°C as a starting point.

Denature Probe

3.	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 should be used initially.
4.	Denature probe for 5-10 minutes at 95°C and place immediately on ice.

Hybridization

5.	Pour pre-hybridization solution off of blot. ⚠ **Important:** Always remove the prehybridization solution and replace with fresh hybridization solution. Bromophenol blue from the loading buffer washes off into the pre-hybridization solution and may cause excess background if the pre-hybridization solution is not replaced before overnight hybridization.
6.	Add freshly denatured probe directly into fresh ULTRAhyb™–Oligo hybridization solution. Do not use more than 3-5 ml per 10 x 10 cm blot of hybridization solution for each 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 usually needs to be optimized for each user's system.
7.	Add hybridization solution containing probe to the bottle or bag containing blot. ⚠ **Important:** Do not to touch the pipette tip or probe directly onto the blot.
8.	Hybridize overnight at 42°C. Time can vary for each sample. Shorter times are possible.

Stringency Washes

9.	Remove hybridization solution and wash at room temperature in NorthernMax Low-Stringency Wash Solution for 5 minutes. Repeat.
10.	Wash 15 minutes at 50°C in NorthernMax High Stringency Wash Solution. Repeat. ■ **Tip:** Start with 50°C, then increase temperature in small increments if necessary.

V. Biotin Detection for Northern Blots

Blocking

1.	Add 5 ml of 20% SDS to 95 ml Odyssey Blocking Buffer for a final concentration of 1% SDS. ⚠ **Important:** 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 680 conjugate or Streptavidin- IRDye 800CW conjugate with Odyssey Blocking Buffer plus 1% SDS to a concentration of 1:10,000.
4.	Remove old blocking buffer and lightly cover the blot with the 1:10,000 streptavidin-IRDye 680 or Streptavidin-IRDye 800CW buffer, approximately 5 ml/10 cm^2. Incubate 30 minutes at room temperature while gently shaking. ⚠ **Important:** This reaction is light-sensitive; it is essential to incubate in darkness.

Wash

5.	Wash the blot 3 times in 1X PBST (0.1% Tween-20) for 5 minutes each, shaking at room temperature. Follow with a rinse in 1X PBS for 5 minutes at room temperature. Wash steps must be performed in darkness. Use a black dish or cover container with aluminum foil.

Scan Blot On Odyssey

6.	Scan blot on Odyssey. Start with the **Intensity** parameter set to 5 for Northern blots. If necessary, scan again and adjust intensity.

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.

VI. 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 RNA transfer to membrane, view the gel with UV transilluminator to see if any RNA has remained in the gel.
	RNA degraded.	Use DEPC or Nuclease free water for all dilutions.
		Keep all plastics and glassware RNase free.
	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 RNA.
		Verify that RNA was not degraded by visualizing on gel before transfer.
	Too high stringency.	Decrease time or temperature of stringency washes.
	RNA on membrane inaccessible to probe.	Place membrane in tube or bag with RNA side exposed to the hybridization solution.

Problem	Possible Cause	Solution / Prevention
Low sensitivity (continued).	Intensity set too low when the scan is started.	Increase the intensity settings on the Scanner Console window by increments of 0.5 in one or both channels. Re-scan membrane.
		If the intensity was only slightly low during scanning, images can be visually modified using the "alter intensity" dialog box. Sensitivity, brightness and contrast can be adjusted.
	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.
		Ensure that hybridization solution is pre-warmed and completely in solution before using.
		Make sure to use ULTRAhyb™–Oligo (Ambion, Cat. #8663). It has been optimized for the Odyssey system.
	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 RNA Loading Buffer (Sigma, Cat. #R-4268). 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; make 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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