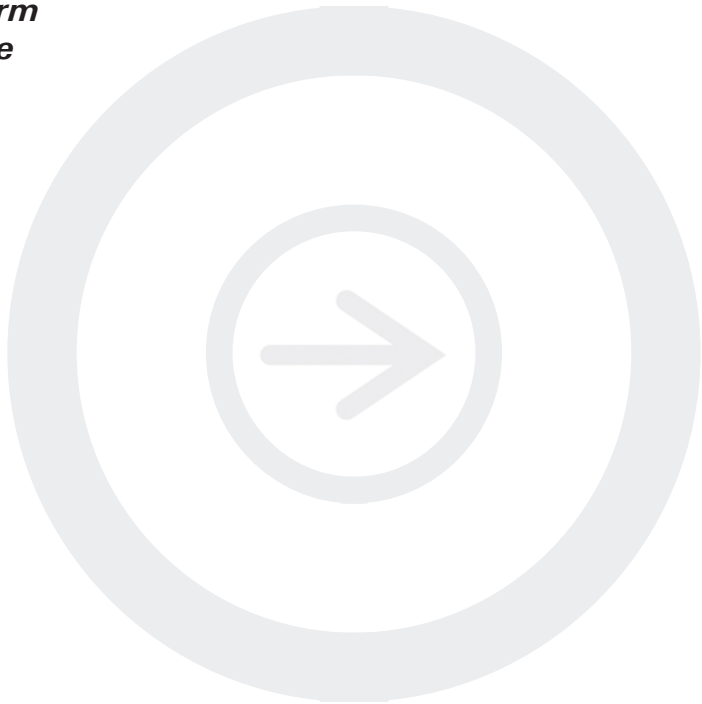
Odyssey Western Blot Blocker Optimization for Near-Infrared (NIR) Detection

Developed for:

Aerius and Odyssey® Family of Imagers

Please refer to your manual to confirm that this protocol and application are compatible with your Odyssey Imager model.

Part Numbers: 927-40040



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Published February 2009. Revised March 2017. The most recent version of this document is posted at licor.com/bio/support.

Visit us on protocols.io! Explore an interactive version of this protocol at bit.ly/BlockerOptimization_Nitro or bit.ly/BlockerOptimization_PVDF.

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I. Introduction

This document describes a method to determine optimal blocking conditions for NIR Western blot detection with the Odyssey family of Imagers. The specific lysate and antibodies used in your system will be evaluated in four different blocking buffer systems. The buffering system will also be evaluated in this experiment. Odyssey Blocking Buffer is provided in either TBS (TRIS Buffered Saline) or PBS (Phosphate Buffered Saline) formulation. In general, the washing step should include the same buffering system as the blocking step (e.g., 1X TBS wash when using Odyssey Blocking Buffer (TBS)).

II. Required Reagents

	LI-COR P/N
■ Odyssey Protein Molecular Weight Marker	928-40000
■ IRDye® Secondary Antibodies (LI-COR)	
■ Blocking Buffer	
• Blocking Buffer Optimization Kit	927-40040
– Odyssey Blocking Buffer (TBS)	927-50000; 927-50100
– Odyssey Blocking Buffer (PBS)	927-40000; 927-40100
– Casein Blocking Buffer (PBS)	927-40200; 927-40300
• Blocking buffer of your choice (milk, BSA, etc.)	
■ Membrane	
• Odyssey Nitrocellulose (0.22 µm)	926-31090; 926-31092
• 4X Protein Sample Loading Buffer and PVDF Membrane Kit	926-31097

■ Primary antibodies (primary antibodies must be from host species compatible with the secondary antibodies being used. If using subclass-specific antibodies, please refer to Technical Note "Western Blot and In-Cell Western™ Assay Detection Using IRDye® Subclass Specific Antibodies").

LI-COR P/N

- Tween® 20
- PBS Buffer (1X)
- TBS Buffer (1X)
- Methanol (when using Immobilon®-FL PVDF membrane)
- SDS (when using Immobilon-FL PVDF membrane)
- Western Blot Incubation Box 929-97201; 929-97205; 929-97210

  Aerius or Odyssey Family Imager

III. Gel Preparation for Blocker Optimization

Standard protein electrophoresis conditions and reagents can be used for gel and sample preparation. Following is a suggested template for sample electrophoresis to maximize blocker optimization and efficiently choose the best blocking conditions for a given primary antibody.

Using two 15-well gels, load the following samples in the order indicated:

Lane	Sample	Amount
1	Primary Antibody (as positive control)	5 µL of a 1:1000* dilution in PBS
2	Sample Lysate	313 ng
3	Sample Lysate	625 ng
4	Sample Lysate	1.25 µg
5	Sample Lysate	2.5 µg
6	Sample Lysate	5 µg
7	Sample Lysate	10 µg
8	Protein Marker	1-3 µL
9	Primary Antibody (as positive control)	5 µL of a 1:1000* dilution in PBS
10	Sample Lysate	313 ng
11	Sample Lysate	625 ng
12	Sample Lysate	1.25 µg
13	Sample Lysate	2.5 µg
14	Sample Lysate	5 µg
15	Sample Lysate	10 µg

** Suggested starting point; may need to be altered, depending on concentration of primary antibody.*

IV. Western Blocker Optimization Method

Western blots should be prepared using standard blotting procedures and Millipore Immobilon®-FL PVDF or Odyssey Nitrocellulose Membrane. Allow blots to dry for at least 1 hour before proceeding with detection. Dry blots can be stored between filter paper overnight at room temperature.

NOTE: Membranes should be handled only by their edges, with clean forceps. Take great care to never touch the membrane with bare or gloved hands.

NOTE: Do not write on membranes with an ink pen or marker, as the ink will fluoresce on the Odyssey Imager. Mark with pencil or Odyssey Pen (P/N 926-71804) to avoid this problem. Use pencil only for PVDF membrane, because wetting in methanol will cause ink to run.

If using the gel configuration described in Section III (Gel Preparation for Blocker Optimization), cut each membrane along the protein marker in lane 8 as shown in Figure 1. Be careful not to touch the membranes with bare or gloved hands. Label appropriately with pencil.

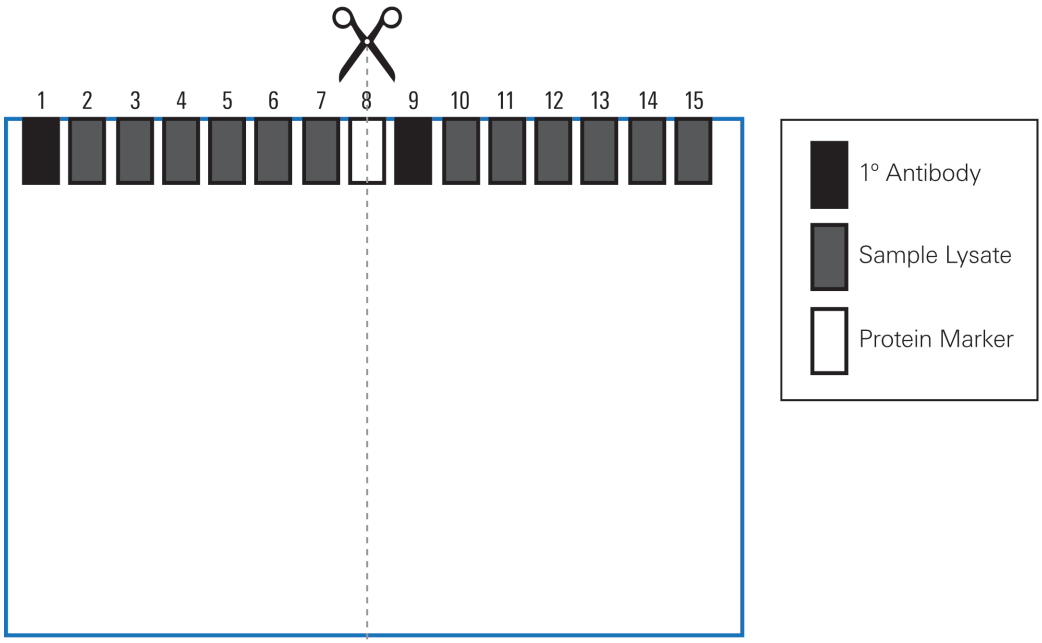

Figure 1. Cut both Western blot membranes along the Marker lanes to generate four individual optimization blots.

After cutting membranes, perform the following steps:

1. For Immobilon-FL PVDF membranes:
   - Pre-wet 1 minute in 100% methanol
   - Rinse with ultrapure water
   - Wet one piece in 1X TBS for 2 minutes
     - Wet remaining pieces in 1X PBS for 2 minutes

For Odyssey Nitrocellulose Membranes:

- Wet in one piece in 1X TBS for 2 minutes
  - Wet remaining pieces in 1X PBS for 2 minutes

2. Place cut membranes into 4 different Western Blot Incubation Boxes and block with 10 mL Blocking Buffer for 1 hour at room temperature while gently shaking.
   - Box 1 – Odyssey Blocking Buffer (TBS)
   - Box 2 – Odyssey Blocking Buffer (PBS)
   - Box 3 – Casein Blocking Buffer
   - Box 4 – Blocking buffer of your choice (milk, BSA, etc.)

3. Dilute primary antibody* in 10 mL of appropriate diluent listed below:
   - Box 1 – Odyssey Blocking Buffer (TBS)+ 0.2% Tween® 20 + Primary Antibody
   - Box 2 – Odyssey Blocking Buffer (PBS) + 0.2% Tween 20 + Primary Antibody
   - Box 3 – Casein Blocking Buffer + 0.2% Tween 20 + Primary Antibody
   - Box 4 – Blocking Buffer of your choice + 0.2% Tween 20 + Primary Antibody

   **** The correct working range for antibody dilution depends on the characteristics of your primary antibody. Start with the dilution recommended by the primary antibody vendor for Western blot applications.***

4. Incubate blots in diluted primary antibody for 1 to 4 hours* at room temperature, or overnight at 4 °C while gently shaking.

   ** Incubation times vary for different primary antibodies.*

5. Wash membranes:
   - Pour off primary antibody solution.
     - **Rinse and wash each blot with a buffer that matches the buffer system used for blocking.**
     - Box 1: Rinse with 1X TBS-T (0.1% Tween 20).
       Cover blot with 1X TBS-T for washing.
     - Box 2: Rinse with 1X PBS-T (0.1% Tween 20).
       Cover blot with 1X PBS-T for washing.
     - Box 3: Rinse with 1X PBS-T (0.1% Tween 20).
       Cover blot with 1X PBS-T for washing.
     - Box 4: Use 1X TBS-T or PBS-T (0.1% Tween 20) as appropriate.

   - Wash blots by shaking vigorously on platform shaker at room temperature for 5 minutes.
     - Pour off wash solution.
     - Repeat 3 additional times.

6. Dilute secondary antibody** in 10 mL of appropriate diluent listed below:

   Secondary antibody diluent for Immobilon®-FL PVDF membrane
   - Box 1 – Odyssey Blocking Buffer (TBS) + 0.2% Tween® 20 + 0.01% SDS + Secondary Antibody
   - Box 2 – Odyssey Blocking Buffer (PBS) + 0.2% Tween 20 + 0.01% SDS + Secondary Antibody
   - Box 3 – Casein Blocking Buffer + 0.2% Tween 20 + 0.01% SDS + Secondary Antibody
   - Box 4 – Blocking Buffer of your choice + 0.2% Tween 20 + 0.01% SDS + Secondary Antibody

   Secondary antibody diluent for Odyssey Nitrocellulose Membrane
   - Box 1 – Odyssey Blocking Buffer (TBS) + 0.2% Tween 20 + Secondary Antibody
   - Box 2 – Odyssey Blocking Buffer (PBS) + 0.2% Tween 20 + Secondary Antibody
   - Box 3 – Casein Blocking Buffer + 0.2% Tween 20 + Secondary Antibody
   - Box 4 – Blocking Buffer of your choice + 0.2% Tween 20 + Secondary Antibody

   ** *Dilution factors for secondary antibodies*
   - For IRDye® 800CW and IRDye 680RD conjugates, suggested dilution range is 1:5,000 to 1:25,000 and may require optimization.
   - For IRDye 680LT conjugates, suggested dilution range is 1:20,000 to 1:50,000. Please consult pack insert.

7. Incubate blots in diluted secondary antibody for 60 minutes at room temperature with gentle shaking.
   Protect membranes from light during incubation.

8. ***Protect from light during washes.***
   Wash membranes:
   - Pour off secondary antibody solution.
   - **Rinse and wash each blot with a buffer that matches the buffer system used for blocking, as described in Step 5.**
   - Box 1: 1X TBS-T (0.1% Tween® 20)
   - Box 2: 1X PBS-T (0.1% Tween 20)
   - Box 3: 1X PBS-T (0.1% Tween 20)
   - Box 4: Use 1X TBS-T or PBS-T (0.1% Tween 20), as appropriate

   - Shake vigorously on platform shaker at room temperature for 5 minutes.
   - Pour off wash solution.
   - Repeat 3 additional times.

9. Rinse each membrane with 1X TBS or 1X PBS (as appropriate) to remove residual Tween® 20. Membranes can be imaged wet or dry.

10. Image all four blots side-by-side.

11. Visual inspection of images with Image Studio™ software or Odyssey application software can be used to determine which blocking buffer works best for the primary antibody you are testing.

    - View all blots together in a single image, with uniform image display settings, to compare membrane background levels and band intensity (Fig. 2A).

    - Individually adjust the image display settings for each blot to get the "best" image (Fig. 2B).

    - Evaluate non-specific banding in each blocking buffer condition.

    - Look for blocking buffer conditions that provide strong signals for the expected band(s), low membrane background, and few non-specific background bands from the primary antibody.

    - Trade-offs may be necessary. Blocking conditions that yield very strong bands might also have higher membrane background or non-specific banding.

    - The "best" blocking conditions depend on the antigen-antibody pair you are using. Some primary antibodies are dramatically affected by blocking conditions. An inappropriate blocker can alter binding specificity, affecting the intensity of target bands and increasing non-specific banding. The pattern of non-specific bands may also be affected.

    - Choose the blocking conditions that are most appropriate for the context and goals of your experiment.

12. Quantitative analysis of specific bands on each blot (Section V; Fig. 2) can be used to determine if signal intensity (after background subtraction) is significantly different between blocker types.

V. Example: Analysis of Optimization Blots

1. In this optimization experiment, ERK1/2 was detected in serial dilutions of A431 cell lysates.

2. After imaging, blocking buffer performance was evaluated. In addition to visual comparison, quantitative analysis was used to examine signal intensity of the ERK1/2 doublet under the conditions tested.

Figure 2A. All blots were viewed together in a single image, with identical image display settings. Signal intensity values are shown in each lane. Higher membrane background was observed on the blot blocked with 5% Milk in TBS.

Figure 2B. After cropping, image display settings were adjusted individually to display the "best" image for each blot. Signal intensity values are shown in each lane.

Figure 2C. Quantification data from all four blots were plotted. Band intensity was highest in Odyssey Blocking Buffer (PBS). Intensity was lowest in 5% Milk and Odyssey Blocking Buffer (TBS).

Signal intensity values in Figures 2A and 2B were not affected by changes to the image display settings. *Signal intensity measurements are derived from the raw image data. Image display settings adjust the visual mapping of raw data to the pixels on the computer monitor, but do not affect the raw data.*

3. Analysis and interpretation of optimization data

   - Blocking with 5% Milk in TBS resulted in higher membrane background than other blocking conditions (Fig. 2A). For comparison of membrane background levels, blots were viewed together as a single image, with uniform image display settings.

   - The strongest signals were observed with Odyssey Blocking Buffer (PBS) (OBB-PBS, Fig. 2C). Signals observed with casein blocker were also good.

   - Weaker signals were observed with Odyssey Blocking Buffer (TBS) (OBB-TBS) and 5% Milk.

   - Non-specific background bands were seen with OBB (TBS), OBB (PBS), and Casein blocking buffers (Fig 2B, arrows). Non-specific banding was reduced with 5% Milk (TBS).

   - OBB (PBS) and Casein may be appropriate choices for this antibody-antigen pair.

   - If non-specific banding is a concern, 5% Milk (TBS) could be used with this primary antibody. Band intensity would be somewhat reduced, but strong bands would still be visible. However, increased membrane background may make it difficult to detect fainter bands. Optimization of other factors, such as antibody dilution, may also reduce non-specific banding.

   - **The "best" choice of blocking buffer depends on the antigen-antibody pair you are using. Choose the blocking conditions that are most appropriate for the context and goals of your experiment.**

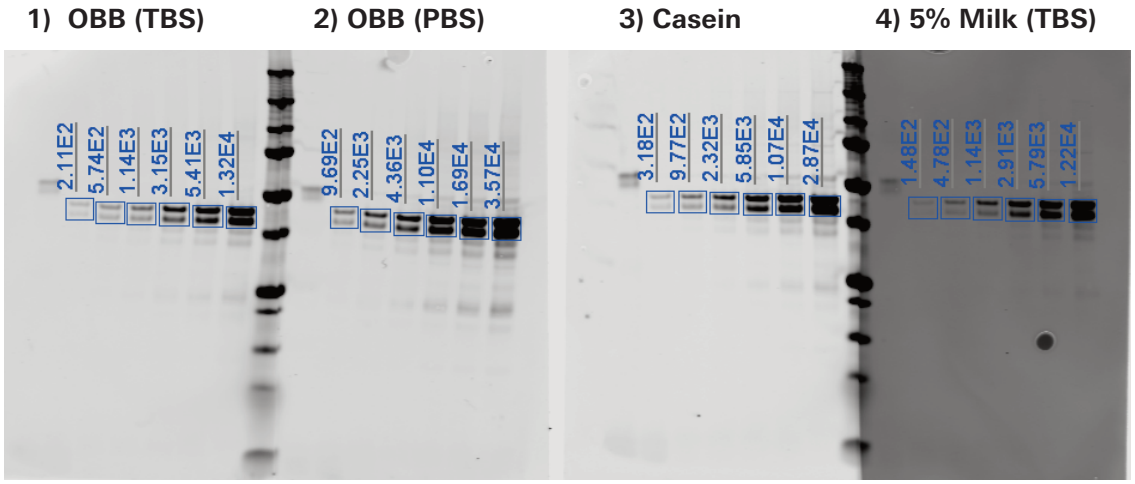

Figure 2A. Blots viewed as a single image, with uniform image display settings.

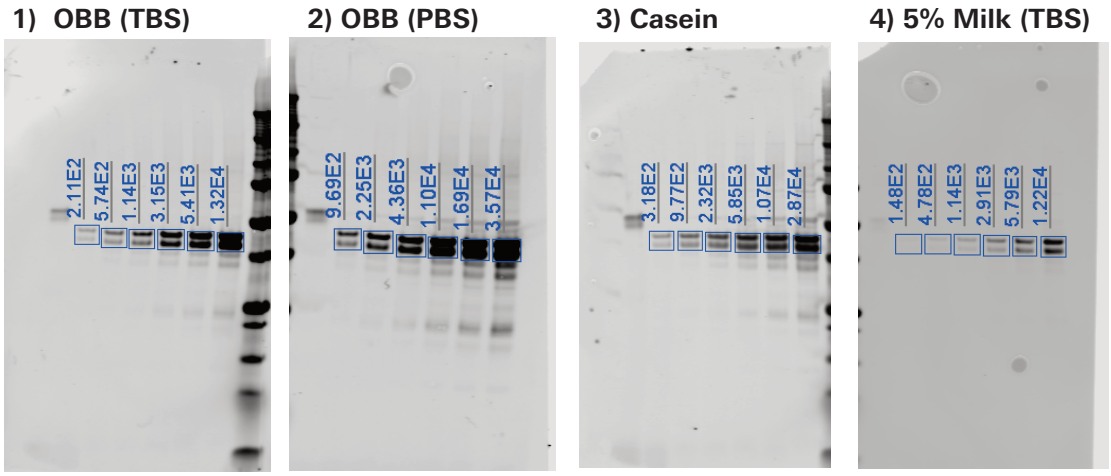

Figure 2B. Image display settings individually adjusted for each blot

Figure 2. Two-fold serial dilutions of A431 cell lysate were separated by electrophoresis (sample loads ranged from 313 ng to 10 µg, left to right). Primary antibody was loaded in the first lane of each gel as a positive control. Gels were transferred to nitrocellulose membrane and incubated in the designated buffer for blocking. Membranes were probed with rabbit anti-ERK1/2 (Santa Cruz Biotechnology; sc-94), and then IRDye® 680RD goat anti-rabbit IgG (LI-COR; P/N 926-68071) prior to imaging on Odyssey CLx.

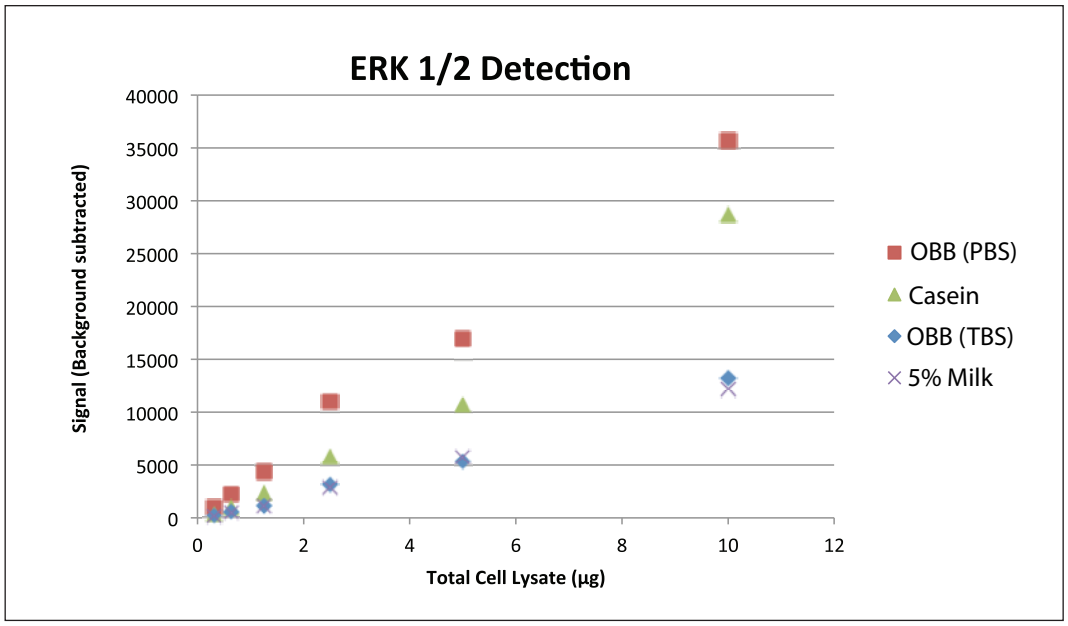

Figure 2C. Quantification data of images shown in Figures 2A and 2B.

Tips

- Follow the protocol carefully.
- For additional Odyssey Western detection tips, visit www.licor.com/WesternBlotTips

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