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Good Westerns Gone Bad:
Tips to Make Your NIR Western Blot Great

Developed for:
Odyssey Family of Imagers

Please refer to your manual to confirm that this protocol is appropriate for the applications compatible with your model of Odyssey Imager.
I. Introduction to Western Blotting

Western blotting is used to positively identify a protein from a complex mixture. It was first introduced by Towbin, et al. in 1979 as a simple method of electrophoretic blotting of proteins to nitrocellulose sheets. Since then, Western blotting methods for immobilizing proteins onto a membrane have become a common laboratory technique. Although many alterations to the original protocol have also been made, the general premise still exists. Macromolecules are separated using gel electrophoresis and transferred to a membrane, typically nitrocellulose or polyvinylidene fluoride (PVDF). The membrane is blocked to prevent non-specific binding of antibodies and probed with some form of detection antibody or conjugate.

Infrared fluorescence detection on the Odyssey® Imaging System provides a quantitative two-color detection method for Western Blots. This document will discuss some of the factors that may alter the performance of a near-infrared (IR) Western blot, resulting in “good Westerns, gone bad.”

II. Factors That Alter the Performance of a Western Blot

A. Membrane

A low background membrane is essential for IR Western blot success. Background can be attributed to membrane autofluorescence or to detection of antibody non-specifically binding to the membrane. Polyvinylidene fluoride (PVDF) and nitrocellulose are typically used for Western blotting applications. There are many brands and vendors for both types of membrane. Before any Western blot is performed on an Odyssey System, the membrane of choice should be imaged “out of the box” on an Odyssey System to determine the level of autofluorescence. LI-COR has evaluated many different membranes for Western blotting and examples of membrane performance can be seen in Figure 1. There is typically more variability in PVDF performance than nitrocellulose.

NOTE: Not all sources of PVDF and nitrocellulose have been evaluated by LI-COR; therefore, it is important to evaluate the membrane before use. Membranes can be quickly evaluated by imaging them both wet and dry on the Odyssey.

B. Blocking Reagent

There are many different sources and types of blocking reagents sold for Western blot applications. Antibody performance can sometimes be compromised by the blocking reagent chosen. Milk-based blockers may contain IgG that can cross-react with anti-goat antibodies. This can significantly increase background and reduce sensitivity. Milk-based blockers may also contain endogenous biotin or phospho-epitopes that can cause higher background.
If an antibody fails with one blocking condition, it may be advantageous to try another. Figure 2 is an example of the behavior of the anti-PKCα antibody in 5% BSA, 5% Milk, and Odyssey® blocking reagents on a nitrocellulose membrane. Figure 3 is a similar example using Odyssey blocking reagent, I-Block™, and 5% BSA for detection of anti-pAkt and β-tubulin in 293T Cells stimulated with TGF-β.

We tested the PathScan PDGFR Tyrosine Kinase Activity Assay (Cell Signaling Technology, P/N 7180), using five different blocking/diluent solutions. Figure 4 shows results from this experiment. The five phosphoproteins could be clearly visualized with each of the blocking solutions, with the exception of 5% Milk, which had very high background. The S6 Ribosomal protein (total protein loading control) was almost completely absent in blots where Odyssey Blocking Buffer (P/N 927-40010, 927-40003, 927-40000, 927-40100) was used. This data clearly suggests that there is not a universal blocker that is best for all antibodies.

![Image](image-url)

**Figure 1.** Western blot detection of transferrin using various vendors and brands of PVDF membrane on the Odyssey Infrared Imaging System in both 700 and 800 nm channels.
C. Detergents

Addition of detergents to diluted antibodies can significantly reduce background on the blot. Optimal detergent concentration will vary, depending on the antibodies, membrane type, and blocker used. Keep in mind that some primaries do not bind as tightly as others and may be washed away by too much detergent. Never expose the membrane to detergent until blocking is complete, as this may cause high membrane background.

Figure 2. Western blots detected with anti-PKCα and IRDye® 800CW Goat anti-mouse. All blots were treated equally, with the exception of blocking reagent. All images were generated on the Odyssey® Infrared Imager with scan intensity setting of 5, sensitivity of 5.

Figure 3. Western blots of 293T Cells stimulated with TGF-β (0, 2.5, and 5 minutes) detected with anti-pAkt and β-tubulin. All blots were treated equally, with the exception of blocking reagent. All images were generated on the Odyssey Infrared Imager with scan intensity setting of 3.5/5 (700/800 nm), sensitivity of 5.
1. Tween 20
   a. Blocker – do not put Tween 20 into the blocking reagent during blocking.
   b. Primary and secondary antibody diluents should have a final concentration of 0.1 - 0.2% Tween® 20 for nitrocellulose membranes, and a final concentration of 0.1% for PVDF membranes. A higher concentration of Tween 20 may increase background on PVDF.
   c. Wash solutions should contain 0.1% Tween 20.

2. SDS
   a. Blocker - do not put SDS into the blocking reagent during blocking.
   b. When using PVDF membrane, secondary antibody diluents should have a final concentration of 0.01 - 0.02% SDS. SDS can be added to the antibody diluents when using nitrocellulose to dramatically reduce overall membrane background and also reduce or eliminate non-specific binding. It is critical to use only a very small amount. SDS is an ionic detergent and can disrupt antigen-antibody interactions if too much is present at any time during the detection process.
   c. Wash solutions should not contain SDS.
D. Primary Antibody

An antibody produced to detect a specific antigen is called the primary antibody. It binds directly to the molecule of interest. Primary antibodies can be produced in a wide variety of species such as mouse, rabbit, goat, chicken, rat, guinea pig, human, and many others. Primary antibodies for the same antigen can perform very differently. It may be necessary to test multiple primary antibodies for the best performance in your Western blot system. Figure 5 is an example of how different primary antibodies may react.

E. Secondary Antibody Quality

One of the primary benefits of using an Odyssey® System for Western blot detection is the ability to detect two targets simultaneously. Two-color detection requires careful selection of primary and secondary antibodies. The two primary antibodies must be derived from different host species so they can be discriminated by secondary antibodies of different specificities (for example, primaries from rabbit and mouse will be discriminated by anti-rabbit and anti-mouse secondary antibodies). One secondary antibody must be labeled with IRDye® 680LT or IRDye 680 and the other with IRDye 800CW.

The exception to this is when using IRDye Subclass Specific Antibodies. IRDye Goat anti-Mouse IgG1, Goat anti-Mouse IgG2a, and Goat anti-Mouse IgG2b, allow for two-color detection using primary antibodies derived from the same species (mouse). IRDye Subclass Specific antibodies react with the heavy (gamma) chain only of the primary antibody. In mice, there are five unique subclasses of IgG; IgG1, IgG2a, IgG2b, IgG2c, and IgG3. Each subclass is based on small differences in amino acid sequences in the constant region of the heavy chains, so antibodies directed against a particular subclass will not recognize antibodies directed against other subclasses. For example, IRDye goat anti-mouse IgG1 recognizes mouse gamma 1, but will not recognize mouse gamma 2a, 2b, 2c or gamma 3. For details, refer to Western Blot and In-Cell Western™ Assay Detection Using IRDye Subclass Specific Antibodies, for a complete description.

A. 1 2 3 4 5 6 7 8

B. | Antibody | Host | Manufacturer | Part # |
<table>
<thead>
<tr>
<th></th>
<th></th>
<th></th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>α-GAPDH</td>
<td>Mouse</td>
<td>Ambion</td>
</tr>
<tr>
<td>2</td>
<td>GAPDH</td>
<td>Sheep</td>
<td>AbCam</td>
</tr>
<tr>
<td>3</td>
<td>GAPDH</td>
<td>Rabbit</td>
<td>Rockland</td>
</tr>
<tr>
<td>4</td>
<td>GAPDH</td>
<td>Mouse</td>
<td>AbCam</td>
</tr>
<tr>
<td>5</td>
<td>GAPDH</td>
<td>Chicken</td>
<td>ProSci Inc.</td>
</tr>
<tr>
<td>6</td>
<td>GAPDH (N-14)</td>
<td>Goat</td>
<td>Santa Cruz Bio</td>
</tr>
<tr>
<td>7</td>
<td>GAPDH (V-18)</td>
<td>Goat</td>
<td>Santa Cruz Bio</td>
</tr>
<tr>
<td>8</td>
<td>α-GAPDH</td>
<td>Mouse</td>
<td>Sigma</td>
</tr>
</tbody>
</table>

Figure 5. MPX™ screening of eight different GAPDH primary antibodies on a HeLa cell lysate sample. Primary antibodies were diluted in Odyssey Blocking Buffer according to manufacturer’s recommendations.
Always use highly cross-adsorbed secondary antibodies for two-color detection. Failure to use cross-adsorbed antibodies may result in increased cross-reactivity as shown in Figure 6. LI-COR® IRDye® conjugated secondary antibodies are optimized for two-color Western blot detection. They are highly cross-adsorbed with a dye-to-protein ratio maximized for optimal signal-to-noise ratio in both Western blot and In-Cell Western™ assay detection. Figure 7 shows a comparison of LI-COR highly cross-adsorbed IRDye goat anti-mouse to a non-cross-adsorbed goat anti-mouse secondary antibody and their reactivity to the different mouse IgG sub-classes.

There are many choices in secondary antibodies for Western blot detection. LI-COR offers IRDye whole IgG (H + L) secondary antibodies and IRDye Sub-class Specific secondary antibodies. Figure 8 demonstrates the performance of LI-COR IRDye goat anti-mouse compared to various other secondary antibody options for detection of a mouse IgG primary antibody. Figure 9 demonstrates the differences between IRDye Subclass Specific detection and IRDye whole anti-mouse IgG detection.

**F. Secondary Antibody Dilution**

The amount of secondary antibody that is used for IR Western blots can vary a great deal. When using LI-COR IRDye 800CW and IRDye 680 conjugated secondary antibodies, the recommended dilution range is 1:5,000 to 1:25,000. When using LI-COR IRDye 680LT secondary antibodies, the recommended dilution range is 1:10,000 to 1:50,000. The dilution should be optimized for the primary antibody being used and the preferred appearance of the Western blot. The Odyssey® imaging software can be used to maximize the appearance of the image using a wide range of secondary antibody dilutions (Figure 10).
Secondary Antibodies used at a 1:5000 dilution unless otherwise indicated

1) Goat anti-Mouse IgA, IgG, IgM
2) Rabbit anti-Mouse IgG
3) Goat anti-Mouse IgG Fcγ (heavy chain specific)
4) Goat anti-Mouse IgG F(ab)2
5) Goat anti-Mouse IgG, IgM
6) F(ab)2 Goat anti-Mouse IgG
7) F(ab)2 Goat anti-Mouse IgG, IgM
8) F(ab)2 Goat anti-Mouse IgG Fab
9) F(ab)2 Goat anti-Mouse IgG Fcγ (heavy chain specific)
10) Donkey anti-Mouse (LI-COR®)
11) Goat anti-Mouse IgM 1:5000
12) Goat anti-Mouse IgM 1:7500
13) Goat anti-Mouse IgG (LI-COR) 1:2500
14) Goat anti-Mouse IgG (LI-COR) 1:5000

Figure 8. IRDye® 800CW labeled anti-mouse antibodies against purified mouse IgG.

Figure 9. Western blot detection of various purified subclasses. Each lane was loaded with 50 ng of antibody. Blots were detected with IRDye labeled Sub-class Specific antibodies or IRDye labeled whole IgG.

Figure 10. Secondary Antibody Concentration of IRDye 800CW goat anti-mouse with maximized Odyssey imaging capabilities.
G. Miscellaneous Contamination

There are many things that can cause contamination of an infrared Western blot. Contamination can appear as a global increase in background, large smears of signal, or speckled blots. Common sources of contamination are listed in Table 1. Some example images are shown in Figure 11 on the following page.

<table>
<thead>
<tr>
<th>Contamination Source</th>
<th>Appearance</th>
<th>Solution</th>
</tr>
</thead>
<tbody>
<tr>
<td>Blue loading buffer used during gel electrophoresis</td>
<td>Smeared signal in the 700 nm channel</td>
<td>Use LI-COR® 4X Protein Sample Loading Buffer (Part #928-40004).</td>
</tr>
<tr>
<td>Dirty transfer pads</td>
<td>Blotches can be seen on the blot that align with the transfer cassette holes</td>
<td>Replace transfer pads.</td>
</tr>
<tr>
<td>Acrylamide residue on membrane after transfer</td>
<td>Speckles and blotches can be seen in 700/800 nm channel</td>
<td>Carefully rinse off membrane in 1X PBS before it dries.</td>
</tr>
<tr>
<td>Blue pen used on membrane</td>
<td>Smeared signal in the 700 nm channel</td>
<td>Use pencil to mark blots.</td>
</tr>
<tr>
<td>Dirty processing containers:</td>
<td></td>
<td></td>
</tr>
<tr>
<td>1. Coomassie Stain/gel stain/anything blue</td>
<td>1. In the 700 nm channel, entire membrane dark, smeared signal, or speckles, depending on the amount of stain residue in container.</td>
<td>1. Use different containers for gel staining and Western blot detection.</td>
</tr>
<tr>
<td>2. Bacterial Growth</td>
<td>2. Speckles and blotches can be seen in 700/800 nm channel.</td>
<td>2. Wash containers with detergent, rinse thoroughly with distilled water and a final rinse with methanol.</td>
</tr>
<tr>
<td>3. Acrylamide Residue</td>
<td>3. Speckles and blotches can be seen in 700/800 nm channel.</td>
<td>3. Wash containers as indicated above.</td>
</tr>
<tr>
<td>Fingerprints</td>
<td>Blotches can be seen in 700/800 nm channel where gloved/ungloved hands have touched the membrane.</td>
<td>Handle Western membrane with clean forceps only.</td>
</tr>
<tr>
<td>Dirty Forceps</td>
<td>Blotches can be seen in 700/800 nm channel where forceps have touched the membrane.</td>
<td>Do not use rusty forceps. Forceps can be washed with detergent, rinsed with water, and a final rinse with methanol.</td>
</tr>
<tr>
<td>Bacterial growth in Antibodies (primary or secondary)</td>
<td>Speckles and blotches can be seen in 700/800 nm channel.</td>
<td>Replace antibodies.</td>
</tr>
</tbody>
</table>
III. Imaging Issues That Can Alter the Performance of a Western Blot

There are adjustments that can be made during the process of imaging a Western on an Odyssey System that can greatly influence the data acquired from the instrument.

A. Starting with a clean scan bed or imaging tray is critical. If you acquire an image and the area that doesn’t have a membrane appears to have signal in either channel, the scan bed or imaging tray is contaminated. The contamination source may be as simple as dust or as complex as dye.

B. Air bubbles can result in reduced signal detection during imaging. Flatten the membrane with a roller to remove bubbles and excess liquid. See Figure 12.

C. A Western blot can be imaged either wet or dry on an Odyssey System. Typically, the signal is higher when a dry blot is imaged; however, the background also will increase. **Note:** Once a blot is dry, or partially dried, stripping of the membrane for reuse is ineffective. See Figure 13.

The Odyssey Fc Imaging System is optimized for acquiring Western blot images without saturated pixels or further adjustment by the operator. The following two items apply only to the Odyssey Infrared Imaging System.

D. Improper adjustment of the Odyssey Focus Offset can result in reduced signal collection from the Odyssey Infrared Imaging System. The focus offset should be set at 0 mm for scanning a Western blot. This can be done in the “Scan Console” Window of the Odyssey software. For more details see Chapter 2: Starting Scans, in the Odyssey User Guide.

E. Improper optimization of the Odyssey Scan Intensity can result in saturation of signal and reduced linear dynamic range. Figure 14 shows the quantification variation that can occur by changing the intensity settings in which the image is acquired. Intensity optimization can be done in the Scan Console Window of the Odyssey software. For more details, see Chapter 2: Starting Scans, in the Odyssey®
User Guide. It is important to note that saturated pixels (pixels that appear white in the image) cannot be accurately quantified. Signal saturation can also result in signal transfer to the alternate channel in the Odyssey® Infrared Imaging System. For example, saturated signal in the 800 nm channel of the Odyssey can be seen as 700 nm signal in the 700 channel scan (see Figure 15). This can easily be eliminated by scanning at a lower intensity.

There are two common problems that can be corrected with a few adjustments of the Odyssey Infrared Imaging System software or the Image Studio software on the Odyssey Fc Imaging System. These include blots that exhibit: • No Fluorescence • Dim Bands. Keep in mind that these software enhancements will only work on blots that are not experiencing binding chemistry problems.

**For the Odyssey Infrared Imaging System – No Fluorescence**

Blots that unexpectedly exhibit no fluorescence can be enhanced by changing the sensitivity setting of the image from Linear Auto to Linear Manual. These settings can be changed from the Alter Image Display menu. To enhance the image, simply click the Linear Manual radio button and adjust the slider. By manually managing the sensitivity settings, the most desirable image can be chosen. For more details, see Chapter 11: Changing the Appearance of Scanned Images, in the Odyssey User Guide.

**Dim Bands**

Improving the appearance of dim bands is as simple as adjusting the Brightness and Contrast of the image. The default software setting is 50. Adjust the Brightness and Contrast sliders to brighten and darken the pixels until the image is optimal. Each channel can be adjusted independently. Image adjustments can also be made in grayscale; very faint bands can be visualized better in gray. For more details, see Chapter 11: Changing the Appearance of Scanned Images, in the Odyssey User Guide. Additional enhancement of images can also be done using “Adjust Image Display Curves”.

**For the Odyssey Fc Imaging System – No Fluorescence**

Click on the Auto Adjust button in the Image LUTs tab. For more details, see Chapter 5: Manipulating an Image in the Odyssey Fc Tutorial (Doc #984-11074).

**Dim Bands**

Click and drag the min, max, and K value dots on the histogram in the Image LUTs tab to adjust the intensity of the image. For more details, see Chapter 5: Manipulating an Image in the Odyssey Fc Tutorial.

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**Figure 14.** The same Western blot scanned on the Odyssey Infrared Imaging System at 5 different intensity settings. The top row of images are displayed using the auto sensitivity setting in the Odyssey Software. The bottom images were optimized using the manual sensitivity option for display. Quantification is shown in the chart. Note that the saturated signal at the Intensity setting of 10 cannot be quantified.

**Figure 15.** Saturated signal in the 800 nm channel (A) of the Odyssey Infrared Imaging System can be visualized in the 700 nm channel (B). The only detection that should be seen in the 700 nm channel is the ladder on the far left of the image. Optimizing scan intensity can eliminate this.
IV. Data Analysis Using the Odyssey® Infrared Imaging System

Background
For accurate Western blot quantification, the background setting in the Odyssey software must be applied effectively. The Background method sets the background calculation method for use in quantification, by averaging the intensity of the pixels selected as the background region. There are several different methods for background subtraction, each unique to a specific need.

i. **No Background** selection uses zero for the background calculations. This is the best choice for assays with their own background calculation methods, such as concentration standards used with In-Cell Western™ Assays. The No Background method is rarely used for Western blotting purposes.

ii. **Average Background** takes the average value of the pixels on all four sides of the feature. It is possible to choose the number of pixels to include in the calculation by changing the Border Width.

iii. **Median** function sets the background level to the median value of the pixels outside the feature. The sides (All, Top/Bottom, or Right/Left) of the feature can be selected to optimize quantification. This feature is also available with the Average Background method.

iv. **User-Defined** background selection averages the intensity of pixels enclosed by a selected feature. To implement this method, display both image channels, draw a feature over an area of typical background (be sure not to include any hot pixels), select the feature, choose the Background icon from the toolbar, and change the background method to User Defined. Click Save, and OK to the message. Notice that the ‘regular feature’ has now changed to a ‘background feature.’ Multiple features can be selected for User Defined Background. This method is not preferred over Average or Median due to possible inconsistencies in noise across the image.

V. Data Analysis using the Odyssey Fc Imaging System

Background
The same background settings used in the Odyssey software are available in the Image Studio software on the Odyssey Fc Imaging System. They can be found by clicking on ‘Define Type’ in the Background group on the Analyze ribbon. To implement the User-Defined background selection in the Image Studio software, draw one or more shapes over an area of typical background. Select the shape(s) and click on ‘Assign Shape’ in the Background group in the Analyze ribbon. The background setting will change to User-Defined.

With the Western Key, the Background group on the Western and MPX Western Analysis ribbons also includes the option of Lane background subtraction. This setting subtracts the background of the Lane from each Band. The same background settings as above can also be used in the Western and MPX Western Analysis ribbons by clicking on ‘Other’ and ‘Western Define Type’.

VI. Summary
There are many ways to maximize the performance of a Western blot. A fully optimized Western blot is the best place to start. LI-COR provides high quality reagents for optimal Western blot detection. For a more detailed protocol on how to do an Odyssey Western blot, see the Odyssey Western Blot Analysis protocol.

VII. References
Odyssey® Western Blotting Kits LT

Developed for:

Odyssey® Family of Imagers

Please refer to your manual to confirm that this protocol is appropriate for the applications compatible with your Odyssey Imager model.

Part Numbers:

926-31062
926-31064
926-31066
926-31068

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I. Required Reagents

■ One of the following IRDye® Western Kits:
  • **Odyssey Western Blotting Kit I LT (LI-COR®, P/N 926-31062)**
    - IRDye 800CW Goat Anti-Mouse Secondary Antibody (25 µL, 1 mg/mL)
    - IRDye 680LT Goat Anti-Rabbit Secondary Antibody (25 µL, 1 mg/mL)
    - Odyssey Blocking Buffer (500 mL)
    - Millipore Immobilon®-FL PVDF Membrane (0.45 µm, 10 x 10 cm)
  • **Odyssey Western Blotting Kit II LT (LI-COR, P/N 926-31064)**
    - IRDye 800CW Goat Anti-Rabbit Secondary Antibody (25 µL, 1 mg/mL)
    - IRDye 680LT Goat Anti-Mouse Secondary Antibody (25 µL, 1 mg/mL)
    - Odyssey Blocking Buffer (500 mL)
    - Millipore Immobilon-FL PVDF Membrane (0.45 µm, 10 x 10 cm)
  • **Odyssey Western Blotting Kit III LT (LI-COR, P/N 926-31066)**
    - IRDye 800CW Goat Anti-Mouse Secondary Antibody (25 µL, 1 mg/mL)
    - IRDye 680LT Goat Anti-Rabbit Secondary Antibody (25 µL, 1 mg/mL)
    - Odyssey Blocking Buffer (500 mL)
    - Odyssey Nitrocellulose Membrane (0.22 µm, 7 x 8.5 cm)
  • **Odyssey Western Blotting Kit IV LT (LI-COR, P/N 926-31068)**
    - IRDye 800CW Goat Anti-Rabbit Secondary Antibody (25 µL, 1 mg/mL)
    - IRDye 680LT Goat Anti-Mouse Secondary Antibody (25 µL, 1 mg/mL)
    - Odyssey Blocking Buffer (500 mL)
    - Odyssey Nitrocellulose Membrane (0.22 µm, 7 x 8.5 cm)
  - Primary antibodies (when using the above IRDye Western Kits, the primary antibodies must be from Rabbit or Mouse host species)
  - Tween® 20
  - PBS buffer (LI-COR, P/N 928-40018 or 928-40020)
  - Methanol (when using Western Blotting Kit I or II)
  - SDS (when using Western Blotting Kit I or II)
  - Western Blot Incubation Box (appropriate for blot size)
    - Small (7.3 x 5.1 x 3.0 cm) (LI-COR, P/N 929-97101, 929-97105, 929-97110)
    - Medium (8.9 x 6.6 x 2.9 cm) (LI-COR, P/N 929-97201, 929-97205, 929-97210)
    - Large (11.6 x 8.9 x 2.9 cm (LI-COR, P/N 929-97301, 929-97305, 929-97310)
    - X-Large (15.2 x 10.2 x 3.2 cm) (LI-COR, P/N 929-97401, 929-97405, 929-97410)
  - Odyssey Pen (LI-COR, P/N 926-71804) (optional)
II. Western Detection Method

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

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

*Note:* Do not write on any membranes with a regular ink pen or marker as the ink will fluoresce on the Odyssey Imaging Systems. Mark the nitrocellulose membrane with pencil or the Odyssey Pen (P/N 926-71804). Use only pencil on the PVDF membrane, as the ink from the Odyssey Pen will dissolve in the methanol used to pre-wet the PVDF membrane.

After transfer, perform the following steps:

1. For Immobilon-FL PVDF membrane:
   - Pre-wet 1 minute in 100% methanol
   - Rinse with ultra pure water
   - Wet in 1X PBS for 2 minutes
   
   For Odyssey nitrocellulose membrane:
   - Wet in 1X PBS for 2 minutes
   
   *Note:* Leave the membrane immersed in 1X PBS until the next step.

2. Place the membrane in a Western Blot Incubation Box and block the membrane in Odyssey Blocking Buffer for 1 hour with gentle shaking.

   Use the amount of liquid per box indicated below for this and the following steps.
   - 5 ml Odyssey Blocking Buffer for Small Box
   - 10 ml Odyssey Blocking Buffer for Medium Box
   - 15 ml Odyssey Blocking Buffer for Large Box
   - 20 ml Odyssey Blocking Buffer for X-Large Box

3. Prepare primary antibody dilution:
   - Prepare the primary antibody diluent by adding Tween® 20 to Odyssey Blocking Buffer for a final concentration of 0.2%.
   - Dilute the primary antibody in the diluent (Odyssey Blocking Buffer with 0.2% Tween 20) using the vendor’s recommended dilution for Western blot applications for that primary antibody. Dilutions may range from 1:200 – 1:5000, depending on the primary antibody.

4. Incubate the blot in the diluted primary antibody (see step 2 for volume) for 1 – 4 hours* at room temperature or overnight at 4°C with gentle shaking.

   *incubation times vary for different primary antibodies

5. Wash membranes:
   - Carefully pour off the primary antibody solution.
   - Rinse the membrane with 1X PBS-T (0.1% Tween 20).
   - Cover the blot with 1X PBS-T (see step 2 for volume).
   - Shake on a platform shaker at room temperature for 5 minutes.
   - Carefully pour off the wash solution.
   - Repeat 3 additional times.
6. Dilute secondary antibody provided in Western Blotting Kit in the appropriate diluent listed below:
   - For IRDye® 800CW secondaries, suggested dilution range is 1:5,000 to 1:25,000.
   - For IRDye 680LT secondaries, suggested dilution range is 1:20,000 to 1:50,000.

   **Secondary antibody diluent for Immobilon®-FL PVDF membrane**
   - Add Tween® 20 to a final concentration of 0.2% and SDS to a final concentration of 0.01 – 0.02% in Odyssey Blocking buffer

   **Secondary antibody diluent for Odyssey nitrocellulose membrane**
   - Add Tween 20 to a final concentration of 0.2% in Odyssey Blocking buffer

7. **Protect the membrane from light during incubation.** Incubate the blot in the diluted secondary antibody for 30-60 minutes at room temperature with gentle shaking.

   **Note:** Do not incubate for longer than 60 minutes, as the background may increase.

8. **Protect the membrane from light during washes.**
   To wash the membranes:
   - Carefully pour off the secondary antibody solution.
   - Rinse the membrane with 1X PBS-T.
   - Cover the blot with 1X PBS-T (see step 2 for volume).
   - Shake vigorously on a platform shaker at room temperature for 5 minutes.
   - Carefully pour off the wash solution.
   - Repeat 3 additional times.

9. Rinse the membrane with 1X PBS to remove residual Tween 20. The membrane can be scanned wet or dry.

10. Image on an instrument from the Aerius® or Odyssey family of imagers. Protect from light prior to imaging.

    Store the membranes at 4°C protected from light. Store dry for several months or in PBS buffer for a few weeks.

    **Note:** If you plan to strip a Western blot, do not allow the membrane to dry. Once a membrane has dried, or partially dried, stripping is ineffective.

**Optimization Tips**
- **Follow the protocol carefully.**
- No single blocking reagent will be optimal for every antigen-antibody pair. Some primary antibodies may exhibit greatly reduced signal or non-specific binding in different blocking solutions. If you have difficulty detecting your target protein, changing the blocking solution may dramatically improve performance. If the primary antibody has worked well in the past using chemiluminescent detection, try that same blocking solution for Odyssey near infrared detection.
- To avoid background speckles on blots, use high-quality ultra pure water for buffers. Rinsing previously-used incubation boxes with methanol can reduce background contamination of future blots.
Never perform Western incubations or washes in dishes that have been used for Coomassie staining. Membranes should be handled only by their edges, with clean forceps. Always pour off antibody solution and washes from the same corner of the box to ensure complete removal of previous solutions. After handling membranes that have been incubating in antibody solutions, clean forceps thoroughly with methanol, then rinse with distilled water.

### III. Guidelines for Two-Color Detection

Two different antigens can be detected simultaneously on the same blot using IRDye® labeled antibodies. When performing a two-color blot, use the standard Western blot protocol with the following modifications:

- Combine the two primary antibodies in the diluted antibody solution in step 3. Incubate simultaneously with the membrane (step 4). The primary antibodies must be from two different host species.
- Combine the two IRDye secondary antibodies in the diluted antibody solution in step 6. Incubate simultaneously with the membrane (step 7).

Two-color detection requires careful selection of primary and secondary antibodies. The following guidelines provide further information that will help you successfully design two-color experiments:

- **The two primary antibodies must be derived from different host species** so that they can be discriminated by secondary antibodies of different specificities (for example, primaries from rabbit and mouse will be discriminated by anti-rabbit and anti-mouse secondary antibodies, respectively).
- Before combining primary antibodies in a two-color experiment, always perform preliminary blots with each primary antibody alone to determine the expected banding pattern and possible background bands. Slight cross-reactivity may occur and can complicate interpretation of your blot, particularly if the antigen is very abundant. If cross-reactivity is a problem, load less protein or reduce the amount of antibody.
- One secondary antibody must be labeled with IRDye 680LT and the other with IRDye 800CW.
- Always use highly cross-adsorbed secondary antibodies for two-color detection. Failure to use cross-adsorbed antibodies may result in increased cross-reactivity.
- For best results, avoid using primary antibodies from mouse and rat together in a two-color experiment. The two species are so closely related it is not possible to completely adsorb away all cross-reactivity. If there is no other option but to use mouse and rat together, it is crucial to run single-color blots first with each individual antibody to be certain of expected band sizes.
- If possible, the two secondary antibodies should be derived from the same host species (for example, goat anti-mouse and goat anti-rabbit) to eliminate the chance of the secondary antibodies reacting against one another.
IV. General Tips

- Store the IRDye® secondary antibody vials at 4°C in the dark. Do not freeze antibodies, as this will affect performance. Minimize exposure to light and take care not to introduce contamination into the vial. Dilute immediately prior to use. If particulates are seen in the antibody solution, mix gently by inversion and centrifuge before use.
- Protect membrane from light during IRDye secondary antibody incubations and washes.
- Use gels that contain the narrowest well size possible to minimize load volume and concentrate the target protein.
- The best transfer conditions, membrane, and blocking agent for each experiment will vary, depending on the antigen, sample type, and antibody.
- For proteins <100 kDa, the recommended transfer buffer is 1X Tris-glycine buffer (LI-COR®, P/N 928-40010 or 928-40012) with 20% methanol and no SDS. Addition of SDS to the transfer buffer can greatly reduce binding of transferred proteins to the membrane (for both PVDF and nitrocellulose). For proteins >100 kDa, decrease the methanol concentration in the transfer buffer to 10%. For transfers to PVDF membrane, methanol can be eliminated from the transfer buffer.
- Soak the gel in transfer buffer for 5-10 minutes before setting up the transfer. Soaking equilibrates the gel and removes SDS so that it will not be carried over into the transfer tank.
- To maximize retention of transferred proteins on the membrane, allow the membrane to air-dry completely after transfer (approximately 1-2 hours).
- Do not over-block. Extended blocking times, particularly when using nonfat dry milk at 2% or higher, can cause loss of target protein from the membrane (J. Immunol. Meth. 122:129-135, 1989).

V. Troubleshooting Guide

<table>
<thead>
<tr>
<th>Problem</th>
<th>Possible Cause</th>
<th>Solution / Prevention</th>
</tr>
</thead>
<tbody>
<tr>
<td>High background, uniformly distributed</td>
<td>Inefficient or ineffective blocking.</td>
<td>Blocking solutions containing BSA may cause high membrane background. Try switching to a different blocker. LI-COR® offers Odyssey Blocking Buffer (P/N 927-40000) and Casein Blocking Buffer (927-40200).</td>
</tr>
<tr>
<td>Background on nitrocellulose.</td>
<td>Increase amount of Tween® 20 added to the diluted antibodies, staying in the range of 0.1-0.2%. Add SDS to diluted secondary antibody, staying in the range of 0.01-0.02%.</td>
<td></td>
</tr>
<tr>
<td>Background on PVDF.</td>
<td>Reduce Tween 20 in diluted antibodies to 0.1%. Add 0.01-0.02% SDS to diluted secondary antibody.</td>
<td></td>
</tr>
<tr>
<td>Membrane autofluorescence.</td>
<td>Scan an unused dry membrane to check for autofluorescence using the same image acquisition parameters as the high background Western blot.</td>
<td></td>
</tr>
<tr>
<td>Streptavidin conjugate.</td>
<td>Add SDS to diluted streptavidin conjugate, staying in the range of 0.02-0.1% (v/v) for nitrocellulose and PVDF membranes.</td>
<td></td>
</tr>
<tr>
<td>Problem</td>
<td>Possible Cause</td>
<td>Solution / Prevention</td>
</tr>
<tr>
<td>---------</td>
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</tr>
<tr>
<td>Antibody concentrations too high.</td>
<td>Increase number of washes and buffer volume. Make sure that 0.1% Tween® 20 is present in wash buffer and increase if needed. Note that excess Tween 20 (0.5-1%) may decrease signal.</td>
<td>Optimize primary and secondary antibody dilutions.</td>
</tr>
<tr>
<td>Insufficient washing.</td>
<td>Increase number of washes and buffer volume. Make sure that 0.1% Tween® 20 is present in wash buffer and increase if needed. Note that excess Tween 20 (0.5-1%) may decrease signal.</td>
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</tr>
<tr>
<td>Cross-reactivity of antibody with contaminants in blocking buffer.</td>
<td>Use Odyssey Blocking Buffer instead of milk. Milk-based blockers may contain IgG that can cross-react with anti-goat antibodies. This can significantly increase background and reduce antibody titer. Milk-based blockers may also contain endogenous biotin or phospho-epitopes that can cause higher background.</td>
<td>Use Odyssey Blocking Buffer instead of milk. Milk-based blockers may contain IgG that can cross-react with anti-goat antibodies. This can significantly increase background and reduce antibody titer. Milk-based blockers may also contain endogenous biotin or phospho-epitopes that can cause higher background.</td>
</tr>
<tr>
<td>Inadequate antibody volume used.</td>
<td>Increase antibody volume so entire membrane surface is sufficiently covered with liquid at all times (use heat-seal bags if volume is limiting). Do not allow any area of membrane to dry out. Use agitation for all antibody incubations.</td>
<td>Increase antibody volume so entire membrane surface is sufficiently covered with liquid at all times (use heat-seal bags if volume is limiting). Do not allow any area of membrane to dry out. Use agitation for all antibody incubations.</td>
</tr>
<tr>
<td>Membrane contamination.</td>
<td>Always handle membranes carefully and with forceps. Do not allow membrane to dry. Use clean dishes, bags, or trays for incubations.</td>
<td>Always handle membranes carefully and with forceps. Do not allow membrane to dry. Use clean dishes, bags, or trays for incubations.</td>
</tr>
<tr>
<td>Uneven, blotchy or speckled background</td>
<td>Membrane not fully wetted or allowed to partially dry.</td>
<td>Keep membrane completely wet at all times. This is particularly crucial if blot will be stripped and re-probed. If using PVDF, remember to first pre-wet in 100% methanol.</td>
</tr>
<tr>
<td>Blocking multiple membranes together in small volume.</td>
<td>If multiple membranes are being blocked together in the same dish, ensure that blocker volume is adequate for all membranes to move freely and make contact with liquid.</td>
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</tr>
<tr>
<td>Contaminated forceps, dishes, or transfer equipment.</td>
<td>Always carefully clean forceps after they are dipped into an antibody solution, particularly dye-labeled secondary antibody. Dirty forceps can deposit dye on the membrane that will not wash away. Use clean dishes, bags or trays for incubations.</td>
<td>Always carefully clean forceps after they are dipped into an antibody solution, particularly dye-labeled secondary antibody. Dirty forceps can deposit dye on the membrane that will not wash away. Use clean dishes, bags or trays for incubations.</td>
</tr>
<tr>
<td>Dirty scanning surface, silicone mat, or Odyssey Fc Imaging tray.</td>
<td>Clean imaging surface, mat or tray carefully before each use with methanol. Dust, lint, and residue will cause speckles.</td>
<td>Clean imaging surface, mat or tray carefully before each use with methanol. Dust, lint, and residue will cause speckles.</td>
</tr>
<tr>
<td>Incompatible marker or pen used to mark membranes.</td>
<td>Use only pencil to mark membrane. (Odyssey Pen can also be used on nitrocellulose.)</td>
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</tr>
<tr>
<td>Problem</td>
<td>Possible Cause</td>
<td>Solution / Prevention</td>
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<tr>
<td>-------------------------------------------</td>
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</tr>
<tr>
<td>Weak or no signal</td>
<td>Not using optimal blocking reagent.</td>
<td>Primary antibody may perform substantially better with a different blocker. LI-COR® offers Odyssey Blocking Buffer (P/N 927-40000) and Casein Blocking Buffer (P/N 927-40200).</td>
</tr>
<tr>
<td></td>
<td></td>
<td>LI-COR® offers Odyssey Blocking Buffer (P/N 927-40000) and Casein Blocking Buffer (P/N 927-40200).</td>
</tr>
<tr>
<td></td>
<td></td>
<td>INSUFFICIENT AMOUNT OF ANTIBODY USED.</td>
</tr>
<tr>
<td></td>
<td></td>
<td>PRIMARY ANTIBODY MAY BE OF LOW AFFINITY. INCREASE AMOUNT OF ANTIBODY OR TRY A DIFFERENT SOURCE.</td>
</tr>
<tr>
<td></td>
<td></td>
<td>EXTEND PRIMARY ANTIBODY INCUBATION TIME (TRY 4-8 HRS AT ROOM TEMPERATURE, OR OVERNIGHT AT 4°C).</td>
</tr>
<tr>
<td></td>
<td></td>
<td>INCREASE AMOUNT OF PRIMARY OR SECONDARY ANTIBODY, OPTIMIZING FOR BEST PERFORMANCE.</td>
</tr>
<tr>
<td></td>
<td></td>
<td>PRIMARY OR SECONDARY ANTIBODY MAY HAVE LOST REACTIVITY DUE TO AGE OR STORAGE CONDITIONS.</td>
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<tr>
<td></td>
<td></td>
<td>USE FRESH OR UNEXPIRED ANTIBODIES.</td>
</tr>
<tr>
<td></td>
<td></td>
<td>TOO MUCH DETERGENT PRESENT; SIGNAL BEING WASHED AWAY.</td>
</tr>
<tr>
<td></td>
<td></td>
<td>DECREASE TWEEN® 20 AND/OR SDS IN DILUTED ANTIBOIES. RECOMMENDED SDS CONCENTRATION IS 0.01-0.02%, BUT SOME ANTIBODIES MAY REQUIRE AN EVEN LOWER CONCENTRATION.</td>
</tr>
<tr>
<td></td>
<td></td>
<td>INSUFFICIENT ANTIGEN LOADED. LOAD MORE PROTEIN ON THE GEL. USE THE NARROWEST POSSIBLE WELL SIZE TO CONCENTRATE ANTIGEN.</td>
</tr>
<tr>
<td></td>
<td></td>
<td>PROTEIN DID NOT TRANSFER WELL. CHECK TRANSFER BUFFER CHOICE AND BLOTTING PROCEDURE. USE PRE-STAINED MOLECULAR WEIGHT MARKER TO MONITOR TRANSFER, AND STAIN GEL AFTER TRANSFER TO MAKE SURE PROTEINS ARE NOT RETAINED IN GEL.</td>
</tr>
<tr>
<td></td>
<td></td>
<td>PROTEIN LOST FROM MEMBRANE DURING INCUBATIONS.</td>
</tr>
<tr>
<td></td>
<td></td>
<td>REDUCE BLOCKING TIMES OR DECREASE HIGH CONCENTRATIONS OF DETERGENT IN DILUTED ANTIBODIES.</td>
</tr>
<tr>
<td></td>
<td></td>
<td>PROTEINS NOT RETAINED ON MEMBRANE DURING TRANSFER.</td>
</tr>
<tr>
<td></td>
<td></td>
<td>ALLOW MEMBRANE TO AIR DRY COMPLETELY (1 - 2 HR) AFTER TRANSFER. THIS HELPS MAKE THE BINDING IRREVERSIBLE.</td>
</tr>
<tr>
<td></td>
<td></td>
<td>ADDITION OF 20% METHANOL TO TRANSFER BUFFER MAY IMPROVE ANTIGEN BINDING, ESPECIALLY ON NITROCELLULOSE.</td>
</tr>
<tr>
<td></td>
<td></td>
<td>NOTE: METHANOL DECREASES THE PORE SIZE OF THE GEL AND CAN HAMPER TRANSFER OF LARGE PROTEINS.</td>
</tr>
<tr>
<td></td>
<td></td>
<td>SDS IN TRANSFER BUFFER MAY INTERFERE WITH BINDING OF TRANSFERRED PROTEINS, ESPECIALLY FOR LOW MOLECULAR WEIGHT PROTEINS. TRY REDUCING OR ELIMINATING SDS.</td>
</tr>
<tr>
<td></td>
<td></td>
<td>NOTE: THE PRESENCE OF UP TO 0.05% SDS IMPROVES TRANSFER EFFICIENCY OF SOME PROTEINS.</td>
</tr>
<tr>
<td>Problem</td>
<td>Possible Cause</td>
<td>Solution / Prevention</td>
</tr>
<tr>
<td>----------------------------------------------</td>
<td>---------------------------------------------------------------</td>
<td>--------------------------------------------------------------------------------------------------------</td>
</tr>
<tr>
<td>Weak or no signal (Continued)</td>
<td>Proteins not retained on membrane during transfer.</td>
<td>Small proteins may pass through membrane during transfer (“blow-through”). Use a membrane with a smaller pore size or reduce the transfer time.</td>
</tr>
<tr>
<td>Non-specific or unexpected bands.</td>
<td>Antibody concentrations too high.</td>
<td>Reduce the amount of antibody used. Reduce the antibody incubation times.\nIncrease Tween® 20 in diluted antibodies.\nAdd or increase SDS in diluted secondary antibodies.</td>
</tr>
<tr>
<td>Not using optimal blocking reagent.</td>
<td></td>
<td>Choice of blocker may affect background bands. Try a different blocker.</td>
</tr>
<tr>
<td>Cross-reactivity between primary and secondary antibodies in a two-color experiment.</td>
<td></td>
<td>Double-check the sources and specificities of the primary and secondary antibodies used (See III. Guidelines for Two-Color Detection).</td>
</tr>
<tr>
<td>Bleed through of signal from one channel into another channel.</td>
<td></td>
<td>Reduce signal in future experiments by reducing the amount of protein loaded or antibody used.</td>
</tr>
</tbody>
</table>
VI. Kit Replacement Reagents and More

<table>
<thead>
<tr>
<th>Kit Replacement Reagents</th>
<th>LI-COR® P/N</th>
</tr>
</thead>
<tbody>
<tr>
<td>IRDye® 800CW Goat Anti-Mouse Secondary Antibody, 0.5 mg</td>
<td>926-32210</td>
</tr>
<tr>
<td>IRDye 680LT Goat Anti-Rabbit Secondary Antibody, 0.5 mg</td>
<td>926-68021</td>
</tr>
<tr>
<td>IRDye 800CW Goat Anti-Rabbit Secondary Antibody, 0.5 mg</td>
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</tr>
<tr>
<td>IRDye 680LT Goat Anti-Mouse Secondary Antibody, 0.5 mg</td>
<td>926-68020</td>
</tr>
<tr>
<td>Odyssey Blocking Buffer</td>
<td>927-40000</td>
</tr>
<tr>
<td>Casein Blocking Buffer</td>
<td>927-40200</td>
</tr>
<tr>
<td>Odyssey Nitrocellulose Membrane</td>
<td>926-31090</td>
</tr>
<tr>
<td>Blocking Buffer &amp; Membrane Kit: 10 bottles of Odyssey Blocking Buffer, and 1 roll of Millipore Immobilon®-FL PVDF membrane</td>
<td>829-31080</td>
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</tbody>
</table>

Other Western Products .......................................................... .LI-COR® P/N

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<thead>
<tr>
<th>Other Western Products</th>
<th>LI-COR® P/N</th>
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</thead>
<tbody>
<tr>
<td>IRDye 800CW Goat Anti-Human Secondary Antibody, 0.5 mg</td>
<td>926-32232</td>
</tr>
<tr>
<td>IRDye 800CW Goat Anti-Mouse Secondary Antibody, IgG1 Specific, 0.5 mg</td>
<td>926-32350</td>
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<tr>
<td>IRDye 800CW Goat Anti-Mouse Secondary Antibody, IgG2a Specific, 0.5 mg</td>
<td>926-32351</td>
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<tr>
<td>IRDye 800CW Goat Anti-Mouse Secondary Antibody, IgG2b Specific, 0.5 mg</td>
<td>926-32352</td>
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<tr>
<td>IRDye 800CW Donkey Anti-Mouse Secondary Antibody, 0.5 mg</td>
<td>926-32212</td>
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<tr>
<td>IRDye 800CW Donkey Anti-Rabbit Secondary Antibody, 0.5 mg</td>
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<tr>
<td>IRDye 800CW Donkey Anti-Goat Secondary Antibody, 0.5 mg</td>
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<tr>
<td>IRDye 800CW Goat Anti-Mouse Secondary Antibody, 25 µL Liquid</td>
<td>827-08364</td>
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<tr>
<td>IRDye 800CW Goat Anti-Rabbit Secondary Antibody, 25 µL Liquid</td>
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<tr>
<td>IRDye 800CW Goat Anti-Rat Secondary Antibody, 0.5 mg</td>
<td>926-32219</td>
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<tr>
<td>IRDye 800CW Donkey Anti-Chicken Secondary Antibody, 0.5 mg</td>
<td>926-32218</td>
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<tr>
<td>IRDye 800CW Donkey Anti-Guinea Pig Secondary Antibody, 0.5 mg</td>
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<tr>
<td>IRDye 800CW Streptavidin, 0.5 mg</td>
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<tr>
<td>IRDye 680LT Donkey Anti-Mouse Secondary Antibody, 0.5 mg</td>
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<td>IRDye 680LT Donkey Anti-Chicken Secondary Antibody, 0.5 mg</td>
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<td>IRDye 680LT Goat Anti-Rat Secondary Antibody, 0.5 mg</td>
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<td>IRDye 680LT Donkey Anti-Guinea Pig Secondary Antibody, 0.5 mg</td>
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<td>IRDye 680LT Goat Anti-Mouse Secondary Antibody, IgG1 Specific, 0.5 mg</td>
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<td>IRDye 680LT Goat Anti-Mouse Secondary Antibody, IgG2a Specific, 0.5 mg</td>
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</tr>
<tr>
<td>IRDye 680LT Goat Anti-Mouse Secondary Antibody, IgG2b Specific, 0.5 mg</td>
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<td>IRDye 680LT Goat Anti-Mouse Secondary Antibody, 25 µL</td>
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<td>IRDye 680LTR Goat Anti-Rabbit Secondary Antibody, 25 µL</td>
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<td>IRDye 680Goat Anti-Mouse Secondary Antibody, 0.5 mg</td>
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<tr>
<td>IRDye 680 Donkey Anti-Guinea Pig Secondary Antibody, 0.5 mg</td>
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<tr>
<td>Beta-Actin Rabbit Monoclonal Antibody, 100 µL</td>
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<td>Beta-Actin Mouse Monoclonal Antibody, 100 µL</td>
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<td>Beta-Tubulin Rabbit Polyclonal Antibody, 100 µL</td>
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<td>10X Tris-Glycine Buffer</td>
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<td>10X Tris-Glycine-SDS Buffer</td>
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<tr>
<td>10X PBS Buffer</td>
<td>928-40018</td>
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<tr>
<td>4X Protein Sample Loading Buffer</td>
<td>928-40004</td>
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<tr>
<td>Odyssey One-Color Molecular Weight Markers (10-250 kDa)</td>
<td>928-40000</td>
</tr>
<tr>
<td>Odyssey Two-Color Molecular Weight Markers (10-250 kDa)</td>
<td>928-40001</td>
</tr>
<tr>
<td>IRDye® Blue Protein Stain</td>
<td>928-40002</td>
</tr>
<tr>
<td>Odyssey Blocking Buffer &amp; PVDF Membrane Kit</td>
<td>926-31098</td>
</tr>
<tr>
<td>Odyssey Blocking Buffer, Qty 3 x 500 mL</td>
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<tr>
<td>Odyssey Blocking Buffer, Qty 10 x 500 mL</td>
<td>927-40010</td>
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<tr>
<td>Blocking Buffer Sample Pack</td>
<td>927-40050</td>
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<tr>
<td>5X NewBlot™ Nitro Stripping Buffer</td>
<td>928-40030</td>
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<tr>
<td>5X NewBlot PVDF Stripping buffer</td>
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<tr>
<td>Odyssey Pens (Pkg of 4)</td>
<td>926-71804</td>
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Western Blot Analysis

Developed for:

**Odyssey Family of Imagers**

*Please refer to your manual to confirm that this protocol is appropriate for the applications compatible with your Odyssey Imager model.*
I. Required Reagents

- Blotted nitrocellulose (LI-COR, P/N 926-31090)
  or low-fluorescent PVDF membrane (LI-COR, P/N 926-31098)
- Odyssey Blocking Buffer (LI-COR, P/N 927-40000)
- Primary antibodies
- Infrared IRDye® secondary antibodies (LI-COR)
- Tween® 20
- PBS wash buffer (LI-COR, P/N 928-40018 or 928-40020)
- Ultrapure water
- Methanol for wetting of PVDF
- SDS (if desired)
- Other blocking buffers (if desired)
- NewBlot™ Stripping Buffer, if desired, for nitrocellulose (LI-COR, P/N 928-40030)
  or PVDF (LI-COR, P/N 928-40032) membranes

### Fluorescent Dyes Appropriate for Use with the Odyssey System

<table>
<thead>
<tr>
<th>Dye</th>
<th>Sensitivity</th>
<th>Odyssey Channel</th>
</tr>
</thead>
<tbody>
<tr>
<td>IRDye 800CW</td>
<td>+ + +</td>
<td>800</td>
</tr>
<tr>
<td>IRDye 680LT</td>
<td>+ + +</td>
<td>700</td>
</tr>
<tr>
<td>IRDye 680</td>
<td>+ + +</td>
<td>700</td>
</tr>
<tr>
<td>IRDye 700DX</td>
<td>+ +</td>
<td>700</td>
</tr>
<tr>
<td>Alexa Fluor® 680</td>
<td>+ + +</td>
<td>700</td>
</tr>
<tr>
<td>Alexa Fluor 700</td>
<td>+ +</td>
<td>700</td>
</tr>
<tr>
<td>Alexa Fluor 750</td>
<td>+ +</td>
<td>700/800 (not recommended; signal appears in both channels)</td>
</tr>
<tr>
<td>Alexa Fluor 647</td>
<td>+</td>
<td>700</td>
</tr>
<tr>
<td>Cy®5.5</td>
<td>+ +</td>
<td>700</td>
</tr>
<tr>
<td>Cy5</td>
<td>+</td>
<td>700</td>
</tr>
</tbody>
</table>

The most current information on dye compatibility can be found on the LI-COR website (www.licor.com).
II. Western Detection Methods

Nitrocellulose or PVDF membranes may be used for protein blotting. Pure cast nitrocellulose is generally preferable to supported nitrocellulose. Protein should be transferred from gel to membrane by standard procedures. Membranes should be handled only by their edges, with clean forceps.

After transfer, perform the following steps:

<p>| | |</p>
<table>
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</thead>
</table>
| 1. | Wet the membrane in PBS for several minutes. If using a PVDF membrane that has been allowed to dry, pre-wet briefly in 100% methanol and rinse with ultrapure water before incubating in PBS.  
   **Notes:**  
   • Ink from most pens and markers will fluoresce on the Odyssey® Imagers. The ink may wash off and re-deposit elsewhere on the membrane, creating blotches and streaks. Pencil should be used to mark membranes. (The Odyssey pen doesn’t fluoresce and can be used with nitrocellulose membranes, since the membrane will not be soaked in methanol causing the ink to run.) |
| 2. | Block the membrane in Odyssey Blocking Buffer for 1 hour. Be sure to use sufficient blocking buffer to cover the membrane (a minimum of 0.4 mL/cm² is suggested).  
   **Notes:**  
   • Membranes can be blocked overnight at 4°C if desired.  
   • Odyssey Blocking Buffer often yields higher and more consistent sensitivity and performance than other blockers. Nonfat dry milk or casein dissolved in PBS can also be used for blocking and antibody dilution, but be aware that milk may cause higher background on PVDF membranes. If using casein, a 0.1% solution in 0.2X PBS buffer is recommended (Hammersten-grade casein is not required).  
   • Milk-based reagents can interfere with detection when using anti-goat antibodies. They also deteriorate rapidly at 4°C, so diluted antibodies cannot be kept and re-used for more than a few days.  
   • Blocking solutions containing BSA can be used, but in some cases they may cause high membrane background. **BSA-containing blockers are not generally recommended** and should be used only when the primary antibody requires BSA as blocker. |
| 3. | Dilute primary antibody in Odyssey Blocking Buffer. Optimum dilution depends on the antibody and should be determined empirically. A suggested starting range can usually be found in the product information from the vendor. To lower background, add 0.1 - 0.2% Tween® 20 to the diluted antibody before incubation. The optimum Tween 20 concentration will depend on the antibody.  
   **Notes:**  
   • Two-color detection requires careful selection of primary and secondary antibodies. For details, see **III. Guidelines for Two Color Western Detection**.  
   • The MPX™Blotting System can be used to efficiently determine the optimum antibody concentration. For details, see **One Blot Western Optimization Using the MPX Blotting System (979-10184)** at http://biosupport.licor.com. |
| 4. | Incubate blot in primary antibody for 60 minutes or longer at room temperature with gentle shaking. Optimum incubation times vary for different primary antibodies. Use enough antibody solution to completely cover the membrane. |
| 5. | Wash membrane 4 times for 5 minutes each at room temperature in PBS + 0.1% Tween 20 with gentle shaking, using a generous amount of buffer. |
6. Dilute the fluorescently labeled secondary antibody in Odyssey® Blocking Buffer. Avoid prolonged exposure of the antibody vial to light. Recommended dilution can be found in the pack insert for the IRDye® conjugate. Add the same amount of Tween® 20 to the diluted secondary antibody as was added to the primary antibody.

**Notes:**
- For detection of small amounts of protein, try using more secondary antibody (1:5000-1:10,000 dilution).
- Be careful not to introduce contamination into the antibody vial.
- Diluted secondary antibody can be saved and re-used. Store at 4°C and protect from light. However, for best sensitivity and performance, use freshly diluted antibody solution.
- Adding 0.01% - 0.02% SDS to the diluted secondary antibody (in addition to Tween 20) will substantially reduce membrane background, particularly when using PVDF. However, DO NOT add SDS during blocking or to the diluted primary antibody. See V. Adapting Western Blotting Protocols for Odyssey Detection for more information about how and why to use SDS in the secondary antibody incubation.
- The MPX™ Blotting System can be used to efficiently determine the optimum antibody concentration. For details, see One Blot Western Optimization Using the MPX Blotting System (979-10184) at http://biosupport.licor.com.

7. Incubate blot in secondary antibody for 30-60 minutes at room temperature with gentle shaking. Protect from light during incubation.

**Notes:**
- Incubating more than 60 minutes may increase background.

8. Wash membrane 4 times for 5 minutes each at room temperature in PBS + 0.1% Tween 20 with gentle shaking. Protect from light.

9. Rinse membrane with PBS to remove residual Tween 20. The membrane is now ready to scan.

**Notes:**
- Scan in the appropriate channels (see I. Required Reagents for details).
- Protect the membrane from light until it has been scanned.
- Keep the membrane wet to strip and re-use it. Once a membrane has dried, stripping is ineffective.
- Blots can be allowed to dry before scanning if desired. Signal strength may be enhanced on a dry membrane. The membrane can also be re-wetted for scanning.
- The fluorescent signal on the membrane will remain stable for several months, or longer, if protected from light. Membranes may be stored dry or in PBS buffer at 4°C.
- If signal on membrane is too strong or too weak, re-scan the membrane at a lower or higher scan intensity setting, respectively.

**Molecular Weight Marker**

If you loaded the Odyssey Two-Color Molecular Weight Marker (LI-COR, P/N 928-40001) on your gel before transfer, it will be visible in both 700 and 800 nm channels. If you loaded the Odyssey Prestained Molecular Weight Marker (LI-COR, P/N 928-40000), it will be visible in the 700 nm channel and also faintly visible in the 800 nm channel. If the marker is subjected to numerous
freeze/thaw cycles, it may degrade. This is observed as multiple, high-molecular weight bands appearing in the 800 nm channel. If this occurs, discard the aliquot and use a fresh one.

Prestained blue molecular weight markers from other sources can also be used. Load 1/3 to 1/5 of the amount you would normally use for Western transfer. Too much marker can result in very strong marker bands that may interfere with visualization of sample lanes. If using multicolored markers, some bands may not be visualized.

**Optimization Tips**
- Follow the protocol carefully.
- No single blocking reagent will be optimal for every antigen-antibody pair. Some primary antibodies may exhibit greatly reduced signal or different non-specific binding in different blocking solutions. If it is difficult to detect the target protein, changing the blocking solution may dramatically improve performance. If the primary antibody has worked well in the past using chemiluminescent detection, try that blocking solution for infrared detection.
- Addition of detergent such as Tween® 20 can reduce membrane background and non-specific binding. Refer to V. Adapting Western Blotting Protocols for Odyssey® Detection for details.
- To avoid background speckles on blots, use ultrapure water for buffers and rinse plastic dishes well before and after use. Never perform Western incubations or washes in dishes that have been used for Coomassie staining.
- Membranes should be handled only by their edges, with clean forceps.
- After handling membranes that have been incubating in antibody solutions, clean forceps thoroughly with distilled water and/or ethanol. If forceps are not cleaned after being dipped in antibody solutions, they can cause spots or streaks of fluorescence on the membrane that are difficult to wash away.
- Do not wrap the membrane in plastic when scanning.
- If a Western blot will be stripped, do not allow the membrane to dry. Stripping is ineffective once a membrane has dried, or even partially dried.

**III. Guidelines for Two-Color Detection**

Two different antigens can be detected simultaneously on the same blot using antibodies labeled with near-infrared dyes that are visualized in different fluorescence channels (700 and 800 nm). Two-color detection requires careful selection of primary and secondary antibodies.

The following guidelines will help design two-color experiments:
- If the two primary antibodies are derived from different host species (for example, primary antibodies from mouse and chicken), IRDye® whole IgG secondary antibodies derived from the same host and labeled with different IRDye fluorophores must be used (for example, IRDye 800CW Donkey anti-Mouse and IRDye 680LT Donkey anti-Chicken).
- If the two primary antibodies are monoclonals (mouse) and are IgG1, IgG2a, or IgG2b, IRDye Subclass Specific secondary antibodies must be used. The same subclasses cannot be combined in a two-color Western blot (for example, two IgG1 primary antibodies). For details refer to Western Blot and In-Cell Western™ Assay Detection Using IRDye Subclass Specific Antibodies).
- Before combining primary antibodies in a two-color experiment, always perform preliminary blots with each primary antibody alone to determine the expected banding pattern and possible
background bands. Slight cross-reactivity may occur and can complicate interpretation of a blot, particularly if the antigen is very abundant. If cross-reactivity is a problem, load less protein or reduce the amount of antibody.

- One secondary antibody must be labeled with a 700 channel dye, and the other with an 800 channel dye. For a list of fluorescent dyes and the channels where they can be visualized, see I. Required Reagents.

- Always use highly cross-adsorbed secondary antibodies for two-color detection. Failure to use highly cross-adsorbed antibodies may result in cross-reactivity.

- For best results, avoid using primary antibodies from mouse and rat together for a two-color experiment. It is not possible to completely adsorb away cross-reactivity because the species are so closely related. If using mouse and rat together, it is crucial to run single-color blots first with each individual antibody to be certain of expected band sizes.

When performing a two-color blot, use the standard Western blot protocol with the following modifications:

- Combine the two primary antibodies in the diluted antibody solution in step 3. Incubate simultaneously with the membrane (step 4).

- Combine the two dye-labeled secondary antibodies in the diluted antibody solution in step 6. Incubate simultaneously with the membrane (step 7).

IV. Stripping the Membrane

Typically, both PVDF and nitrocellulose membranes can be stripped up to three times. LI-COR® NewBlot™ Stripping Buffer is available under P/N 928-40030 for nitrocellulose or 928-40032 for PVDF. If a blot is to be stripped, DO NOT allow it to dry before, during, or after imaging (keep the blot as wet as possible). Complete usage instructions are given in the NewBlot Stripping Buffer pack insert that is shipped with the product. Before proceeding, read the instructions in the pack insert, including the frequently asked questions.

V. Adapting Western Blotting Protocols for Odyssey Detection

When adapting Western blotting protocols for Odyssey® detection or using a new primary antibody, it is important to determine the optimal antibody concentrations. Optimization will help achieve maximum sensitivity and consistency. Three parameters should be optimized: primary antibody concentration, dye-labeled secondary antibody concentration, and detergent concentration in the diluted antibodies.

Primary Antibody Concentration

Primary antibodies vary widely in quality, affinity, and concentration. The correct working range for antibody dilution depends on the characteristics of the primary antibody and the amount of target antigen to detect. Suggested dilutions are 1:500, 1:1500, 1:5000, and 1:10,000 (start with the dilution factor normally used for chemiluminescent detection, and also refer to the product information from the vendor). Use the MPX™ Blotting System to optimize the primary dilution to achieve maximum performance and conserve antibody (refer to One Blot Western Optimization Using the MPX Blotting System) at http://biosupport.licor.com).
Secondary Antibody Concentration

Optimal dilutions of dye-conjugated secondary antibodies should also be determined. Suggested starting dilutions to test are 1:5000, 1:10,000, and 1:20,000 (refer to the IRDye® conjugate pack insert for recommendations). The amount of secondary required varies depending on how much antigen is being detected – abundant proteins with strong signals require less secondary antibody. Use the MPX™ Blotting system to optimize (refer to One Blot Western Optimization Using the MPX Blotting System at http://biosupport.licor.com).

Detergent Concentration

Addition of detergents to diluted antibodies can significantly reduce background on the blot. Optimal detergent concentration will vary, depending on the antibodies, membrane type, and blocker used. Keep in mind that some primaries do not bind as tightly as others and may be washed away by too much detergent. Never expose the membrane to detergent until blocking is complete, as this may cause high membrane background.

Tween® 20:

- Add Tween 20 to both the primary antibody and secondary antibody solutions when the antibodies are diluted in blocking buffer. A final concentration of 0.1 - 0.2% is recommended for nitrocellulose membranes, and a final concentration of 0.1% is recommended for PVDF membranes (higher concentrations of Tween 20 may actually cause increased background on PVDF).
- Wash solutions should contain 0.1% Tween 20.

SDS:

- Adding 0.01 - 0.02% SDS to the diluted secondary antibody can dramatically reduce overall membrane background and also reduce or eliminate non-specific binding. It is critical to use only a very small amount, because SDS is an ionic detergent and can disrupt antigen-antibody interactions if too much is present at any time during the detection process.
- Addition of SDS is particularly helpful for reducing the higher overall background that is seen with PVDF membrane. When working with IRDye 680LT conjugates on PVDF membranes, SDS (final concentration of 0.01 - 0.02%) and Tween 20 (final concentration of 0.1 - 0.2%) must be added during detection incubation step to avoid non-specific background staining.
- **DO NOT add SDS during the blocking step or to the diluted primary antibody.** Presence of SDS during binding of the primary antibody to its antigen may greatly reduce signal. **Add SDS only to the diluted secondary antibody.**
- When diluting the dye-labeled secondary antibody in blocking buffer, add both 0.1 - 0.2% Tween 20 and 0.01 - 0.02% SDS to the antibody solution.
- Wash solutions should contain 0.1% Tween 20, but no SDS.
- Some antibody-antigen pairs may be more sensitive to the presence of SDS and may require even lower concentrations of this detergent (less than 0.01%) for best performance. Titrate the amount of SDS to find the best level for the antibodies used.
- If high background is seen when using BSA-containing blocking buffers, adding SDS to the secondary antibody may alleviate the problem.
VI. General Tips

- Milk-based blockers may contain IgG that can cross-react with anti-goat antibodies. This can significantly increase background and reduce antibody titer. Milk may also contain endogenous biotin or phospho-epitopes that can cause higher background.

- Store the IRDye® secondary antibody vial at 4°C in the dark. Do not thaw and refreeze the vial, as this will affect antibody performance. Minimize exposure to light and take care not to introduce contamination into the vial. Dilute immediately prior to use. If particulates are seen in the antibody solution, centrifuge before use.

- Protect membrane from light during secondary antibody incubations and washes.

- Use the narrowest well size possible for the loading volume to concentrate the target protein.

- The best transfer conditions, membrane, and blocking agent for experiments will vary, depending on the antigen and antibody. If there is high background or low signal level, a good first step is to try a different blocking solution.

- Small amounts of purified protein may not transfer well. Adding non-specific proteins of similar molecular weight can have a “carrier” effect and substantially increase transfer efficiency.

- For proteins <100 kDa, try blotting in standard Tris-glycine buffer with 20% methanol and no SDS. Addition of SDS to the transfer buffer can greatly reduce binding of transferred proteins to the membrane (for both PVDF and nitrocellulose).

- Soak the gel in transfer buffer for 10-20 minutes before setting up the transfer. Soaking equilibrates the gel and removes buffer salts that will be carried over into the transfer tank.

- To maximize retention of transferred proteins on the membrane, allow the membrane to air-dry completely after transfer (approximately 1-2 hours).

- Do not over-block. Long blocking incubations, particularly with nonfat dry milk at 2% or higher, can cause loss of target protein from the membrane (J. Immunol. Meth. 122:129-135, 1989).

- To enhance signal, try extended primary antibody incubation at room temperature or overnight incubation at 4°C. Avoid extended incubations in secondary antibody.

VII. Imaging of Coomassie-Stained Protein Gels

IRDye® Blue Protein Stain is a convenient, safe alternative for gel staining to provide confirmation of protein transfer to the membrane. Unlike traditional Coomassie Blue stains, which require methanol and acetic acid for staining and destaining, IRDye Blue Protein Stain is water-based and requires no hazardous solvents. This stain offers excellent detection sensitivity in the 700 nm channel of the Odyssey® imaging systems (< 5 ng of BSA can be detected). IRDye Blue Protein Stain is Coomassie-based and is provided as a ready-to-use 1X solution. Prewashing and destaining steps are performed in water.

1. Wash gels with ultrapure water for 15 minutes.

2. Submerge gel in IRDye Blue Stain for 1 hour.

3. Destain with ultrapure water for 30 minutes or overnight if needed.

4. Scan on an Odyssey imaging system in the 700 nm channel only. If using the Odyssey software, select the Protein Gel scan preset. If using the Odyssey Sa software, set the focus offset to 3.0 plus one-half the thickness of the gel. In Image Studio, select Western.
# VIII. Troubleshooting Guide

<table>
<thead>
<tr>
<th>Problem</th>
<th>Possible Cause</th>
<th>Solution / Prevention</th>
</tr>
</thead>
<tbody>
<tr>
<td>High background, uniformly distributed.</td>
<td>BSA used for blocking.</td>
<td>Blocking solutions containing BSA may cause high membrane background. Try adding SDS to reduce background, or switch to a different blocker.</td>
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<tr>
<td></td>
<td></td>
<td><strong>Not using optimal blocking reagent.</strong></td>
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<tr>
<td></td>
<td></td>
<td><strong>Background on nitrocellulose.</strong></td>
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<tr>
<td></td>
<td></td>
<td><strong>Background on PVDF:</strong></td>
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<tr>
<td></td>
<td></td>
<td><strong>Antibody concentrations too high.</strong></td>
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<tr>
<td></td>
<td></td>
<td><strong>Insufficient washing.</strong></td>
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<tr>
<td></td>
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<td><strong>Cross-reactivity of antibody with contaminants in blocking buffer.</strong></td>
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<td></td>
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<td><strong>Inadequate antibody volume used.</strong></td>
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<td></td>
<td><strong>Membrane contamination.</strong></td>
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<tr>
<td>Problem</td>
<td>Possible Cause</td>
<td>Solution / Prevention</td>
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<tr>
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<tr>
<td>Uneven blotchy or speckled background.</td>
<td>Blocking multiple membranes together in small volume.</td>
<td>If multiple membranes are being blocked in the same dish, ensure that blocker volume is adequate for all membranes to move freely and make contact with liquid.</td>
</tr>
<tr>
<td></td>
<td>Membrane not fully wetted or allowed to partially dry.</td>
<td>Keep membrane completely wet at all times. This is particularly crucial if blot will be stripped and re-used. If using PVDF, remember to first pre-wet in 100% methanol.</td>
</tr>
<tr>
<td>Contaminated forceps or dishes.</td>
<td>Always carefully clean forceps after they are dipped into an antibody solution, particularly dye-labeled secondary antibody. Dirty forceps can deposit dye on the membrane that will not wash away.</td>
<td>Use clean dishes, bags or trays for incubations.</td>
</tr>
<tr>
<td>Dirty scanning surface or silicone mat.</td>
<td>Clean scanning surface and mat carefully before each use. Dust, lint, and residue will cause speckles.</td>
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<tr>
<td>Incompatible marker or pen used to mark membrane.</td>
<td>Use only pencil or Odyssey® pen (nitrocellulose only) to mark membranes.</td>
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<tr>
<td>Weak or no signal.</td>
<td>Not using optimal blocking reagent.</td>
<td>Primary antibody may perform substantially better with a different blocker.</td>
</tr>
<tr>
<td></td>
<td>Insufficient antibody used.</td>
<td>Primary antibody may be of low affinity. Increase amount of antibody or try a different source. Extend primary antibody incubation time (try 4 - 8 hr at room temperature, or overnight at 4°C). Increase amount of primary or secondary antibody, optimizing for best performance. Try substituting a different dye-labeled secondary antibody.</td>
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<tr>
<td></td>
<td></td>
<td>Primary or secondary antibody may have lost reactivity due to age or storage conditions.</td>
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<tr>
<td>Problem</td>
<td>Possible Cause</td>
<td>Solution / Prevention</td>
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<tr>
<td>Weak or no signal (continued)</td>
<td>Too much detergent present; signal being washed away.</td>
<td>Decrease Tween® 20 and/or SDS in diluted antibodies. Recommended SDS concentration is 0.01 - 0.02%, but some antibodies may require an even lower concentration.</td>
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<tr>
<td></td>
<td>Insufficient antigen loaded.</td>
<td>Load more protein on the gel. Try using the narrowest possible well size to concentrate antigen.</td>
</tr>
<tr>
<td></td>
<td>Protein did not transfer well.</td>
<td>Check transfer buffer choice and blotting procedure. Use pre-stained molecular weight marker to monitor transfer, and stain gel after transfer to make sure proteins are not retained in gel.</td>
</tr>
<tr>
<td></td>
<td>Protein lost from membrane during detection.</td>
<td>Extended blocking times or high concentrations of detergent in diluted antibodies may cause loss of antigen from the blotted membrane.</td>
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<tr>
<td></td>
<td>Proteins not retained on membrane during transfer.</td>
<td>Allow membrane to air dry completely (1 - 2 hr) after transfer. This helps make the binding irreversible. Addition of 20% methanol to transfer buffer may improve antigen binding. <em>Note: Methanol decreases pore size of gel and can hamper transfer of large proteins.</em> SDS in transfer buffer may interfere with binding of transferred proteins, especially for low molecular weight proteins. Try reducing or eliminating SDS. <em>Note: Presence of up to 0.05% SDS does improve transfer efficiency of some proteins.</em> Small proteins may pass through membrane during transfer (“blow-through”). Use membrane with smaller pore size or reduce transfer time.</td>
</tr>
<tr>
<td>Problem</td>
<td>Possible Cause</td>
<td>Solution / Prevention</td>
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<tr>
<td>Non-specific or unexpected bands.</td>
<td>Antibody concentrations too high.</td>
<td>Reduce the amount of antibody used.</td>
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<td></td>
<td></td>
<td>Reduce antibody incubation times.</td>
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<td>Increase Tween® 20 in diluted antibodies.</td>
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<td></td>
<td></td>
<td>Add or increase SDS in diluted secondary antibodies.</td>
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<tr>
<td></td>
<td>Not using optimal blocking reagent.</td>
<td>Choice of blocker may affect background bands. Try a different blocker.</td>
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<td></td>
<td>Cross-reactivity between antibodies in a two-color</td>
<td>Double-check the sources and specificities of the primary and secondary antibodies</td>
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<tr>
<td></td>
<td>experiment.</td>
<td>used (see III. Guidelines for Two-Color Detection).</td>
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<tr>
<td></td>
<td></td>
<td>Use only highly cross-adsorbed secondary antibodies.</td>
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<td></td>
<td></td>
<td>There is always potential for cross-reactivity in two-color experiments. Use less</td>
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<td>secondary antibody to minimize this.</td>
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<td></td>
<td>Always test the two colors on separate blots first so you know what bands to expect</td>
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<td></td>
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<td>and where.</td>
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<td></td>
<td>Avoid using mouse and rat antibodies together, if possible. Because the species are</td>
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<tr>
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<td></td>
<td>so closely related, anti-mouse will react with rat IgG to some extent, and anti-rat</td>
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<tr>
<td></td>
<td></td>
<td>with mouse IgG. Sheep and goat antibodies may exhibit the same behavior.</td>
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<tr>
<td></td>
<td>Bleedthrough of signal from one channel into other</td>
<td>Check the fluorescent dye used. Fluorophores such as Alexa Fluor® 750 may appear in</td>
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<tr>
<td></td>
<td>channel.</td>
<td>both channels and are not recommended for use with the Odyssey® Imaging Systems.</td>
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<td></td>
<td></td>
<td>If signal in one channel is very strong (near or at saturation), it may generate a</td>
</tr>
<tr>
<td></td>
<td></td>
<td>small amount of bleedthrough signal in the other channel. Minimize this by using a</td>
</tr>
<tr>
<td></td>
<td></td>
<td>lower scan intensity setting in the problem channel.</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Reduce signal by reducing the amount of protein loaded or antibody used.</td>
</tr>
</tbody>
</table>
Odyssey Western Blot Blocker
Optimization

Developed for:

Aerius and Odyssey® Family of Imagers

*Please refer to your manual to confirm that this protocol is appropriate for the applications compatible with your Odyssey Imager model.*

Part Numbers:  
927-40050  
927-40300  
927-40200  
927-40100  
927-40000  
927-40003  
927-40010

Published February 2009.  
Revised December 2010 and August 2011  
The most recent version of this pack insert is posted at http://biosupport.licor.com/support
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I. Required Reagents

- Odyssey Protein Marker

<table>
<thead>
<tr>
<th>Protein Marker</th>
<th>LI-COR P/N</th>
</tr>
</thead>
<tbody>
<tr>
<td>Two-Color Protein Molecular Weight Marker</td>
<td>928-40001</td>
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<tr>
<td>One-Color Protein Molecular Weight Marker</td>
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- IRDye® Secondary Antibodies

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<tr>
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<th>LI-COR P/N</th>
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</thead>
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<tr>
<td>IRDye 800CW</td>
<td>Goat anti-Mouse 926-32210</td>
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<tr>
<td>IRDye 800CW</td>
<td>Goat anti-Rabbit 926-32211</td>
</tr>
<tr>
<td>IRDye 800CW</td>
<td>Donkey anti-Mouse 926-32212</td>
</tr>
<tr>
<td>IRDye 800CW</td>
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<td>Donkey anti-Goat 926-32214</td>
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<td>IRDye 800CW</td>
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<td>IRDye 800CW</td>
<td>Goat anti-Rat 926-32219</td>
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<td>Donkey anti-Guinea Pig 926-32411</td>
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<td>IRDye 800CW</td>
<td>Goat anti-Human 926-32232</td>
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<td>Goat anti-Mouse IgG1 926-32350</td>
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<td>IRDye 800CW</td>
<td>Goat anti-Mouse IgG2a 926-32351</td>
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<tr>
<td>IRDye 800CW</td>
<td>Goat anti-Mouse IgG2b 926-32352</td>
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<td>IRDye 680LT</td>
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<td>IRDye 680LT</td>
<td>Goat anti-Mouse IgG2b 926-68052</td>
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• Blocking Buffer

<table>
<thead>
<tr>
<th>Blocking Buffer</th>
<th>Volume</th>
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<tr>
<td>Blocking Buffer Sample Pack:</td>
<td></td>
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<tr>
<td>Odyssey Blocking Buffer</td>
<td>125 mL</td>
<td>927-40100</td>
</tr>
<tr>
<td>Casein Blocking Buffer</td>
<td>500 mL</td>
<td>927-40000</td>
</tr>
<tr>
<td>Odyssey Blocking Buffer</td>
<td>125 mL</td>
<td>927-40100</td>
</tr>
<tr>
<td>Casein Blocking Buffer</td>
<td>500 mL</td>
<td>927-40200</td>
</tr>
</tbody>
</table>

• Membrane

<table>
<thead>
<tr>
<th>Membrane</th>
<th>Size</th>
<th>LI-COR P/N</th>
</tr>
</thead>
<tbody>
<tr>
<td>Odyssey Nitrocellulose (0.22 µm), 10 pack</td>
<td>7 x 8.5 cm</td>
<td>926-31090</td>
</tr>
<tr>
<td>Odyssey Nitrocellulose (0.22 µm), roll</td>
<td>30 cm x 3 m</td>
<td>926-31092</td>
</tr>
<tr>
<td>Millipore Immobilon®-FL (0.45 µm)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Western Blot Kits</td>
<td>10 x 10 cm (10 pack)</td>
<td>926-31050</td>
</tr>
<tr>
<td></td>
<td></td>
<td>926-31052</td>
</tr>
<tr>
<td>Blocking Buffer &amp; Membrane Kit</td>
<td>26.5 cm x 3.75 m</td>
<td>829-31080</td>
</tr>
</tbody>
</table>

• Primary antibodies (primary antibodies must be from host species compatible to 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”).

• Tween® 20

• PBS Buffer (LI-COR P/N 928-40018 or 928-40020)

• Methanol (when using Immobilon®-FL PVDF membrane)

• SDS (when using Immobilon-FL PVDF membrane)

• Western Blot Incubation Box
  – Medium (8.9 x 6.6 x 2.9 cm), LI-COR P/Ns 929-97201 (1 pack), 929-97205 (5 pack), and 929-97210 (10 pack)
II. Gel Preparation for Blocker Optimization

Standard protein electrophoresis conditions and reagents can be used for gel and sample preparation. The 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 a 15-well gel, load the following samples in order indicated:

<table>
<thead>
<tr>
<th>Lane</th>
<th>Sample</th>
<th>Amount</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>Protein Marker</td>
<td>2-10 µL</td>
</tr>
<tr>
<td>2</td>
<td>Primary Antibody</td>
<td>5 µL of a 1:1000* dilution in PBS</td>
</tr>
<tr>
<td>3</td>
<td>Sample Lysate</td>
<td>10 µg</td>
</tr>
<tr>
<td>4</td>
<td>Sample Lysate</td>
<td>5 µg</td>
</tr>
<tr>
<td>5</td>
<td>Sample Lysate</td>
<td>2.5 µg</td>
</tr>
<tr>
<td>6</td>
<td>Protein Marker</td>
<td>2-10 µL</td>
</tr>
<tr>
<td>7</td>
<td>Primary Antibody</td>
<td>5 µL of a 1:1000* dilution in PBS</td>
</tr>
<tr>
<td>8</td>
<td>Sample Lysate</td>
<td>10 µg</td>
</tr>
<tr>
<td>9</td>
<td>Sample Lysate</td>
<td>5 µg</td>
</tr>
<tr>
<td>10</td>
<td>Sample Lysate</td>
<td>2.5 µg</td>
</tr>
<tr>
<td>11</td>
<td>Protein Marker</td>
<td>2-10 µL</td>
</tr>
<tr>
<td>12</td>
<td>Primary Antibody</td>
<td>5 µL of a 1:1000* dilution in PBS</td>
</tr>
<tr>
<td>13</td>
<td>Sample Lysate</td>
<td>10 µg</td>
</tr>
<tr>
<td>14</td>
<td>Sample Lysate</td>
<td>5 µg</td>
</tr>
<tr>
<td>15</td>
<td>Sample Lysate</td>
<td>2.5 µg</td>
</tr>
</tbody>
</table>

* Suggested starting point; may need to be altered for concentration of primary antibody.

III. Western Blocker Optimization Method

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

**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 membrane 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, as wetting in methanol will cause ink to run.

If using the gel configuration outlined in the Gel Preparation for Blocker Optimization section above, cut the membrane, being careful not to touch the membrane along protein marker lanes 6 and 11 as shown in Figure 1. Label appropriately with pencil.
After cutting membrane, perform the following steps:

1. For Immobilon®-FL PVDF membranes:
   - Pre-wet 1 minute in 100% methanol
   - Rinse with ultra pure water
   - Wet in 1X PBS for 2 minutes

   For Odyssey Nitrocellulose Membranes:
   - Wet in 1X PBS for 2 minutes

2. Place membranes into 3 different Western Blot Incubation Boxes and block the membrane in 10 mL Blocking Buffer for 1 hour while gently shaking.
   - Box 1 – Odyssey Blocking Buffer
   - Box 2 – Casein Blocking Buffer
   - Box 3 – Blocking Buffer of your choice

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

   * 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 membrane with 1X PBS-T (0.1% Tween® 20).
   - Cover blot with 1X PBS-T (0.1% Tween 20).
   - Shake 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 + 0.2% Tween 20 + 0.01% SDS + Secondary Antibody
   - Box 2 – Casein Blocking Buffer + 0.2% Tween 20 + 0.01% SDS + Secondary Antibody
   - Box 3 – Blocking Buffer of your choice

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

   *For IRDye 800CW conjugates, suggested dilution range is 1:20,000 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 blot in diluted secondary antibody for 30-60 minutes at room temperature with gentle shaking.
   **Protect membrane from light during incubation.**

8. **Protect from light during washes.**
   Wash membranes:
   - Pour off secondary antibody solution.
   - Rinse membrane with 1X PBS-T (0.1% Tween 20).
   - Cover blot with 1X PBS-T (0.1% Tween 20) using same volumes indicated above for Western blot incubation boxes.
   - Shake vigorously on platform shaker at room temperature for 5 minutes.
   - Pour off wash solution.
   - Repeat 3 additional times.

9. Rinse membrane with 1X PBS to remove residual Tween 20. The membrane can be imaged wet or dry.

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

11. Visual inspection or data analysis with Odyssey application or Image Studio software can be used to determine which blocking buffer works best with the evaluated primary.

**Tips**
- Follow the protocol carefully.
- For additional Odyssey Western detection tips, see www.licor.com/bio
IV. Related Reagents and More

- Western Blotting Kits with PVDF Membrane, LI-COR P/Ns 926-31050, 926-31052
- Odyssey Blocking Buffer and PVDF Membrane Kit, LI-COR P/N 926-31098
- Western Blotting Kits with Nitrocellulose Membrane, LI-COR P/Ns 926-31058, 926-31060
- Odyssey Blocking Buffer, Qty 3 x 500 mL, LI-COR P/N 927-40003
- Odyssey Blocking Buffer, Qty 10 x 500 mL, LI-COR P/N 927-40010
- 10X Tris-Glycine Buffer, LI-COR P/Ns 928-40010, 928-40012
- 10X Tris-Glycine-SDS Buffer, LI-COR P/N 928-40014, 928-40016
- 10X PBS Buffer, LI-COR P/Ns 928-40018, 928-40020
- 4X Protein Sample Loading Buffer, LI-COR P/N 928-40004
- IRDye® Blue Protein Stain, LI-COR P/N 928-40002
- Western Blot Incubation Boxes:
  - Small (7.3 x 5.1 x 3.0 cm), LI-COR P/Ns 929-97101, 929-97105, 929-97110
  - Medium (8.9 x 6.6 x 2.9 cm), LI-COR P/Ns 929-97201, 929-97205, 929-97210
  - Large (11.6 x 8.9 x 2.9 cm), LI-COR P/Ns 929-97301, 929-97305, 929-97310
  - X-Large (15.2 x 10.2 x 3.2 cm), LI-COR P/Ns 929-97401, 929-97405, 929-97410
- NewBlot™ Stripping Buffer for Nitrocellulose, LI-COR P/N 928-40030
- NewBlot Stripping Buffer for PVDF, LI-COR P/N 928-40032
- MPX™ Blotting System, LI-COR P/N 921-00000
- IRDye 800CW Goat Anti-Mouse Secondary Antibody, 25 µL Liquid, LI-COR P/N 827-08364
- IRDye 800CW Goat Anti-Rabbit Secondary Antibody, 25 µL Liquid, LI-COR P/N 827-08365
- IRDye 800CW Streptavidin, 0.5 mg, LI-COR P/N 926-32230
- IRDye 680LT Goat Anti-Mouse Secondary Antibody, 25 µL, LI-COR P/N 827-11080
- IRDye 680LT Goat Anti-Rabbit Secondary Antibody, 25 µL, LI-COR P/N 827-11081
- IRDye 680LT Streptavidin, 0.5 mg, LI-COR P/N 926-68031
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Chemiluminescent Western Blots
Frequently Asked Questions

Developed for:
Odyssey® Fc Imaging System

May 2011. The most recent version of this protocol is posted at http://biosupport.licor.com
1. Blocking Buffer

1.1 Can I dilute the HRP-conjugated secondary antibodies in the Odyssey® blocking buffer?
No. Odyssey blocking buffer contains sodium azide as a preservative. Sodium azide binds irreversibly to the HRP enzyme, inhibiting the binding of the substrate and slowing the chemiluminescent reaction. This results in less light production that may affect the appearance of less intense bands or even the entire blot. For optimal results do not use any solutions containing sodium azide for chemiluminescent Western blotting.

1.2 Can I use the Odyssey blocking buffer to block my blot?
Yes. Use only for the blocking step and be aware that the sodium azide from the Odyssey blocking buffer may still be present on the membrane at the detection step and will bind to the HRP enzyme, resulting in reduced light production and less intense bands.

1.3 Can I use milk-based blockers?
Yes. Milk-based blockers can be used for chemiluminescent detection but should be avoided when detecting phosphoproteins or glycoproteins. Milk-based blockers may contain endogenous biotin and glycoproteins, resulting in higher background on the membrane.

1.4 What is the best blocker for chemiluminescent Western blots?
It is best to try several blockers to find the one that gives the most satisfying data for each antigen and antibody pair. There is not a best blocker for all conditions.

2. Primary and Secondary Antibodies

2.1 Why is the signal missing in the middle of the bands?
Too much secondary antibody on the membrane results in consumption of all the substrate in that area. Without substrate, there is no chemiluminescent signal and a white spot appears in the center of the band. Try different dilutions of the primary and secondary antibodies to find what gives the best results, or try changing the substrate.

2.2 Does it matter where I purchased the HRP-conjugated secondary antibodies?
The reactivity of secondary antibodies ranges widely between vendors. As well, the ratio of HRP enzyme to antibody varies, and may affect the detection of the target. If the secondary antibodies from one vendor are not working, trying antibodies from other vendors may be helpful.

2.3 Should the HRP-conjugated secondary antibodies be highly cross-adsorbed?
Although highly cross-adsorbed antibodies are essential for two-channel, multiplex detection, it is not always necessary with chemiluminescent blotting for a single target.

3. Washing Buffer

3.1 Does it matter how I wash the membranes after antibody incubation?
Yes. Adequately washing the membranes will greatly improve the appearance of the chemiluminescent Western blot. Wash the membranes with a saline-buffered solution con-
taining 0.05 to 0.1% of a non-ionic detergent such as Tween® 20. Wash four times for five minutes each time with ample wash solution on a shaker or rotator.

4. Substrate

4.1 Which substrate do I use?
There is a wide variety of chemiluminescent substrates for HRP detection and some are better suited for digital imaging than others. Generally, choose a substrate with a faster rate of reaction for use with the Odyssey® Fc Imaging system. (Some substrates that are designed for optimal performance on film may not be suitable for detection on an imaging system.)

4.2 How do I apply the substrate?
Make sure the substrate is at room temperature before use. Apply carefully and avoid pooling to prevent splotches and areas of high background. Carefully wick off any pools of substrate before imaging.

4.3 The membrane dried during imaging. Can I apply more substrate and image again?
No. Applying more substrate to a dried blot will likely result in high background.

4.4 How do I keep the membrane from drying out?
Place a clear, flat plastic covering on the chemiluminescent Western blot to keep the substrate in contact with the HRP enzyme and to prevent the blot from drying out. Image the plastic covering by itself first to determine if there is autofluorescence that will cause high background. You may need to try several types of plastic coverings before finding the best one.

5. Imaging

5.1 Can I use the Odyssey Fc Imaging Tray multiple times?
It is important to image with a clean tray to prevent unwanted background, so you may want to use a new tray. You can clean a previously used tray with ultrapure water or methanol to remove any traces of substrate or dye. If you have cleaned a used tray, image the tray by itself first to see if there is any contamination left. If there is still signal detected, clean the tray again with ultrapure water or methanol and re-image. If necessary, dispose of the contaminated tray and use a new tray.

5.2 Can I wrap the blot in plastic wrap before imaging?
Wrapping the blot in plastic wrap may cause unwanted background, especially if it is folded or handled roughly. If using plastic wrap it is important to avoid wrinkles as they scatter light, resulting in high background. You can also image the plastic wrap alone first to determine if the plastic itself scatters light. If it does, try different brands of plastic wrap to find the best one.

5.3 Why are the bands on my blot so light?
Use the Lookup Table (LUT) in Image Studio to adjust how the data is mapped to the display pixels of your computer screen.

continued
5.3 **Why are the bands on my blot so light? (continued)**

Overlaying the Lookup Table histogram is a curve with three adjustable points. Move the top Max Point to the left to map more of the higher intensity data to the brighter display pixels and the bands will appear darker. Move the lower Min Point to the right to map the lower intensity data to the background color, creating a visually cleaner background. The middle point smoothly adjusts the mapping from linear to logarithmic. Changing to a more logarithmic mapping reduces the contrast between the lower and higher intensity data, so the appearance of less intense bands is improved while avoiding overly dark bands.

For more information, refer to the Help system in the Image Studio software.
Optimizing Chemiluminescent Western Blots

Developed for:

**Odyssey® Fc Imaging System**

Published August 2011. The most recent version of this protocol is posted at: [http://biosupport.licor.com](http://biosupport.licor.com)
I. Introduction to Chemiluminescent Western Blotting on the Odyssey Fc Imager

Western blotting was first introduced by Towbin, et al. in 1979 as a simple method of electrophoretic blotting of proteins onto nitrocellulose sheets. It is now a common laboratory technique with many variations of the basic procedure. In the first step proteins are separated using gel electrophoresis, followed by transfer to a membrane that is then blocked to prevent non-specific binding of antibodies. The nitrocellulose or polyvinylidene fluoride (PVDF) membrane is then probed with a detection antibody or conjugate.

Chemiluminescent Western blots are probed with a primary antibody against the target protein, followed by a secondary antibody labeled with HRP (horseradish peroxidase) enzyme. A chemiluminescent substrate for the HRP enzyme is carefully applied to the blot, and light is emitted when the HRP enzyme modifies the substrate. Photographic film or an imaging system using a digital CCD camera captures the emitted light as an image.

The Odyssey Fc Imaging System is a CCD-based imager that detects chemiluminescent signal, visible signal at 600 nm, and near-infrared fluorescent signals at 700 nm and 800 nm wavelengths. This versatile, multichannel system can image both chemiluminescent and near-infrared Western blots. It can detect near-infrared fluorescent markers on a chemiluminescent blot as well, providing a less expensive alternative to HRP-labeled markers.
II. Chemiluminescent Western Blot Workflow

1. Prepare samples and determine protein concentrations
2. Separate protein samples by SDS-PAGE
3. Transfer to a membrane (LI-COR, *Protein Electrotransfer Methods*)
4. Block the membrane
5. Incubate with primary antibody
   - Wash membrane
6. Incubate with HRP-conjugated secondary antibody
   - Wash membrane
7. Add substrate
8. Detect with the Odyssey Fc Imager
III. Factors that Affect a Chemiluminescent Western Blot

A. Membrane

The selection of a membrane type is critical to the overall success of a chemiluminescent Western blot. Factors that affect the sensitivity include efficiency of protein transfer, protein binding capacity, and the autofluorescence of the membrane, particularly in the 700 channel. Panel A in Figure 1 highlights the background differences between two PVDF membranes in the 700 channel. These image overlays capture the molecular weight marker signal in the 700 channel and the target signal from the Western blot in the Chemi channel. Panel B in Figure 1 shows only the Chemi channel images and highlights protein binding capacity and detection sensitivity of these two membranes.

Figure 2 shows a thorough comparison of Western blot performance on a wide selection of PVDF membranes. Detection sensitivities can be greatly affected by the choice of membrane, and multiple membranes should be evaluated. Protein characteristics such as molecular weight, amino acid composition, and post-translational modifications (e.g., glycosylation or phosphorylation) can alter the transfer efficiency and ultimately, detection sensitivity. Protein Electrophoresis Methods and the Odyssey Infrared Imaging System (LI-COR Biosciences) provides additional information for optimizing protein transfer.

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**Figure 1.** Two-fold dilutions of C32 lysate (1.25 μg - 156 μg) were resolved on a 10% Bis Tris gel and transferred to the indicated PVDF membranes. The membranes were blocked with skim milk, probed with rabbit β-actin antibody (LI-COR, P/N 926-42211), and detected with Goat Anti-Rabbit-HRP. The blots were incubated with SuperSignal® West Dura (Thermo Scientific) and immediately imaged on the Odyssey Fc Imager for two minutes in both Chemi and 700 channels. Image overlays of the 700 and Chemi channel are shown in Panel A; Chemi channel only is shown in Panel B. All images shown with normalized image display settings.

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**Figure 2.** Odyssey Fc (2 minute) images shown in the Chemi channel. The settings for all of the images are normalized by linking the Lookup Tables in Image Studio software. Experimental details are identical to those provided in Figure 1 above.
B. Blocking Buffer

Incubating the membrane in blocking buffer after the transfer step will result in enhanced sensitivity of your blot. Blocking buffer contains proteins that stick to the membrane, promoting specific binding of the primary antibody and minimizing non-specific interactions. Various blocking buffers are available, and it is important to try several blockers to find the optimal solution for each antigen and antibody pair as there is not a best blocker for all conditions. Milk is a common blocking buffer; however, milk-based blockers that contain endogenous biotin and glycoproteins may result in higher background on the membrane when detecting with streptavidin. Milk may also contain active phosphatases that can de-phosphorylate phosphoproteins on the membrane.

![Figure 3](image)

**Figure 3.** Serial dilutions of NIH/3T3 cell lysate were probed with Alpha-Tubulin Mouse mAb (LI-COR, P/N 926-42213). Substrate applied was SuperSignal® West Dura (Thermo Scientific). Images were acquired on the Odyssey Fc Chemi channel for 2 minutes, shown with normalized image display settings. The blot on the left (A) was detected using a blocker that did not contain sodium azide. The blot on the right (B) was detected using Odyssey Blocking Buffer for the secondary antibody detection step, resulting in diminished chemiluminescent signal.

Blocking buffer is also often used as a diluent for the primary and secondary antibodies.  

**Note:** It is very important that you do not dilute the HRP-conjugated secondary antibodies in any solution containing sodium azide (including the Odyssey Blocking Buffer).

Sodium azide binds irreversibly to the HRP enzyme, inhibiting the binding of the substrate and slowing the chemiluminescent reaction. This results in less light production that may affect the appearance of less intense bands or even the entire blot. The Odyssey Blocking Buffer can be used to block the blot and to dilute the primary antibody. Figure 3 shows the impact of sodium azide on chemiluminescent Western blot detection.
C. Primary Antibody Selection

An antibody produced to detect a specific antigen is called a primary antibody. It binds directly to the molecule of interest. Primary antibodies can be produced in a wide variety of species such as mouse, rabbit, goat, chicken, rat, guinea pig, human, and many others. Primary antibodies for the same antigen can perform very differently. It may be necessary to test multiple primary antibodies for the best performance in your Western blot system. Figure 4 is an example of how different primary antibodies to the same target may react.

D. HRP-Conjugated Secondary Antibody Selection

The reactivity of secondary antibodies ranges widely between vendors, even within the same species and especially between host species. The ratio of HRP enzyme to antibody varies, and may affect the detection of the target. Try secondary antibodies from several vendors to find the ones that give the most satisfying data. See Figure 5 for examples of different secondary antibody performance.

**Note:** When evaluating the performance of the primary and secondary antibodies, try different blocking buffers, as the choice of blocker can affect the antibodies’ performance. For optimal results do not dilute the HRP-conjugated secondary antibodies with blocking buffer containing sodium azide as a preservative (e.g., Odyssey Blocking Buffer), as it will inhibit peroxidase activity and result in less light production.
E. Concentrations of Primary and Secondary Antibodies

The concentration of the HRP-labeled secondary antibody on a chemiluminescent Western blot directly affects the appearance of the bands. Too little HRP enzyme will result in low chemiluminescent signal and light or missing bands. On the other hand, a concentration that is too high will deplete all the substrate in that area and result in bands with no signal in the middle. The duration of image acquisition will also make a difference, so the optimal concentrations may be different for a CCD-based imaging system than for film methods. Try different concentrations of the primary and secondary antibodies to find the combination that gives the best data on the Odyssey Fc Imaging System (Figure 6).

![Figure 6. Two-fold serial dilutions of C32 lysate (10μg-1.25μg/lane) were resolved by SDS-PAGE and transferred to nitrocellulose. In Panel A, blots were detected with varying dilutions of primary antibody and a constant dilution of secondary antibody. In Panel B, a constant dilution of primary antibody and varying dilutions of secondary antibody were used. Optimal results were achieved with a 1:1,000 dilution of primary antibody and a 1:10,000 dilution of secondary antibody (center blot of each panel).]

F. Washing the Membranes

Adequately washing the membranes after incubating with the primary and secondary antibodies will greatly improve the appearance of the chemiluminescent Western blot. Wash the membrane with a saline-buffered solution or another suitable wash buffer. Including a non-ionic detergent (e.g., Tween® 20) at a final concentration of 0.05 to 0.1% may also help reduce background signal. Wash four to six times for at least five minutes each time with ample wash solution on a shaker or rotator.
G. Chemiluminescent Substrates

There is a wide variety of chemiluminescent substrates for HRP detection and some are better suited for digital imaging than others (Figure 7). In general, choose a substrate with a faster rate of reaction for use with the Odyssey Fc Imaging System. Some substrates that are designed for optimal performance on film may not be suitable for detection on a CCD-based imaging system. Try different substrates to find the one that gives the most desirable image.

The manufacturers for some chemiluminescent substrates may recommend that the substrates be stored at 4°C and warmed to room temperature before use. If so, ensure the substrate is at room temperature before use for optimum signal. Cold temperatures slow the activity of the HRP enzyme, resulting in less light production and less intense or missing bands (Figure 8).
IV. Troubleshooting Guide

A. High Background Due to Substrate
Pools of excess substrate on the membrane can lead to areas of high background, as can adding more substrate to a membrane that has dried. Apply the substrate carefully and wick off any pools of substrate before imaging. Do not allow the membrane to dry.

Placing a clear, flat plastic covering on the chemiluminescent Western blot will prevent the blot from drying out by keeping the substrate in contact with the HRP enzyme. It will also minimize pooling of the substrate. Image the plastic covering by itself first to determine if it scatters light, causing high background. You may need to try several types of plastic coverings before finding the best one.

B. Contaminated Odyssey Fc Imaging Tray
It is important to image with a clean tray to prevent unwanted background, as shown in Figure 10. You can clean a previously used tray with ultrapure water or methanol to remove any traces of substrate or dye. If you have cleaned a used tray, image the tray by itself first to see if there is any contamination left. If there is still signal detected, clean the tray again with ultrapure water or methanol and re-image. Dispose of the contaminated tray and use a new tray if necessary.
C. Wrapping the Blot in Plastic Wrap

Wrapping the blot in plastic wrap may cause unwanted background, especially if it is folded or handled roughly. When using plastic wrap it is important to avoid wrinkles, as they scatter light, resulting in high background. In addition, try to avoid leaving fingerprints from pressing on the blot (see top of the blot in Figure 11). An alternative to plastic wrap is to use a clear, flat plastic covering to keep the membrane from drying out and to minimize pooling of the substrate. Refer to High Background Due to Substrate on page 9 for more information.

You can image the plastic wrap alone first to determine if the plastic itself scatters light. If it does, try different brands of plastic wrap to find the best one.

D. Optimizing the Image Display

Use the Lookup Table (LUT) in Image Studio to adjust how the data are mapped to the display pixels of your computer screen.

Overlaying the Lookup Table histogram is a curve with three adjustable points. Move the Max Point to the left to map more of the higher intensity data to the brighter display pixels and make the bands appear darker. Move the Min Point to the right to map the lower intensity data to the background color, creating a visually cleaner background. The middle point (K value) smoothly adjusts the mapping from linear to logarithmic. Changing to a more logarithmic mapping reduces the contrast between the lower and higher intensity data, so the appearance of less intense bands becomes less prominent.
is improved while avoiding overly dark bands (LI-COR, *How to Adjust the Lookup Tables in Image Studio for an Optimal Image Display*).

For more information, refer to the Help system in Image Studio software.

![Figure 13. The curve overlaying the histogram in the Lookup Table was adjusted by moving the Max point to the left to make the bands appear darker in the second image.](image)

### V. References


LI-COR Biosciences, (2009) *Technical Note: Protein Electrotransfer Methods and the Odyssey Infrared Imaging System*

LI-COR Biosciences, (2011) *How to Adjust the Lookup Tables in Image Studio for an Optimal Image Display*
Imaging Nucleic Acid Gels on the Odyssey® Fc Imager

Developed for:
Odyssey Fc Imaging System

Published August 2011. The most recent version of this protocol is posted at: http://biosupport.licor.com
I. Introduction

The Odyssey Fc Imager, with 600 channel capabilities, can image agarose gels stained with popular DNA stains, such as ethidium bromide and SYBR Safe DNA stain, with sub-nanogram sensitivity. The Odyssey Fc Imager contains a 532 nm diffuse source with an excitation maximum of 520 nm and a detection maximum of 600 nm. These instrument parameters are within the range of the excitation and emission wavelengths of ethidium bromide (Ex/Em = 302 & 518/605 nm\textsuperscript{1,2}) and other visible fluorescent nucleic acid stains and provide a sensitive gel documentation option.

SYBR Safe DNA stain (Ex/Em = 502/530 nm) has also been tested on the Odyssey Fc Imager (using the 600 channel) with sensitivities exceeding ethidium bromide detection. The maximum fluorescence emission wavelength of SYBR Safe is very close to the maximum excitation wavelength. However, the Odyssey Fc 600 channel collects excitation light about 50 nm above the maximum excitation wavelength of SYBR Safe, decreasing the background and improving the signal-to-noise ratio for nucleic acid detection.

Specific instructions are given in this technical note for ethidium bromide and SYBR Safe use. There are a variety of commercial DNA stains that may be appropriate for fluorescent imaging with the Odyssey Fc 600 channel. SYBR Green I (Life Technologies), GelStar (FMC), Gel Red\textsuperscript{TM} (Biotium), Gel Green\textsuperscript{TM} (Biotium) and Nancy-520 (Sigma) stains have also been tested at LI-COR (see example images). Other nucleic acid binding stains may also be compatible with the Odyssey Fc Imager. Please check the excitation and emission spectra to determine if other stains can be detected on the Odyssey Fc Imager.

The Odyssey Fc Imager is also equipped with two infrared channels (700 and 800) and a chemiluminescent detection channel. Nucleic acid detection in the 700 channel is achieved with Syto\textsuperscript{®} 60 stain, a cell-permeant cyanine dye. A detailed protocol is available for the use of Syto 60 with the Odyssey and Aerius family of imagers (LI-COR, Syto 60 Staining of Nucleic Acids in Gels).

\textbf{Note:} Any questions regarding specific properties of the DNA binding stains should be directed to the representative vendors listed in this technical guide.
II. DNA Separation and Detection on Agarose Gels

A. Suggested Materials

This section is intended as a guideline; other materials may be substituted.

- High Grade or Molecular Biology Grade agarose
  (Low melting-point agarose may increase the degree of speckling on the digital image.) OR
- E-Gel® Pre-cast agarose gels from Life Technologies (Ethidium Bromide, SYBR® Safe, or Clear gel types)
- 1X TAE or TBE buffer
- Ethidium Bromide (EtBr, 10 mg/mL solution) OR
- SYBR Safe DNA stain (10,000X concentrate in DMSO)
- Gel tank and casting tray for running submersion gels
- Power supply

Note: Dispose of all gel and buffer solutions in accordance with the regulations of your facility.

B. In-Gel Pre-Staining Protocol

Gel Preparation
1. Prepare desired agarose (0.8%, 1.0%, 1.2%, etc.) in 1X TAE or 1X TBE buffer.
2. Heat to dissolve agarose.
3. Cool solution until warm to the touch (60°F) prior to adding DNA stain.
   • Ethidium Bromide— Stock solutions are typically 10 mg/mL. Add ethidium bromide to give a final concentration of 0.5 µg/mL.
   • SYBR Safe— Stock solutions are typically 10,000X. Add SYBR Safe to a final concentration of 1X.
4. Pour molten agarose solution into casting tray and set comb into place. Allow gel to solidify.
5. Remove comb and place gel in buffer tank.
   Note: Both Ethidium Bromide and SYBR Safe are positively charged stains and will migrate in the opposite direction of the DNA. If the stain is included only in the gel, but not the buffer, there will be an area of high background indicating the stain has not migrated out of the gel.

Buffer Preparation
6. Prepare enough buffer (1X TAE or 1X TBE) to fill the apparatus.
7. Add DNA stain to buffer.
   • Ethidium Bromide— Add 5µL of 10 mg/mL EtBr stock solution to 100mL of buffer (final concentration: 0.5 µg/mL).
SYBR® Safe— Add 10µL of 10,000X SYBR Safe stock solution to 100 mL of buffer (final concentration: 1X).

Electrophoresis and Destaining
8. Prepare samples with loading buffer and load in gel.
9. Electrophorese samples at 5-8 V/cm.
10. (Optional) Destain in water for 20 minutes. Repeat as necessary to remove background from the gel.
11. Image on the Odyssey Fc Imager using the 600 channel. Refer to III. Image Acquisition for more information.

C. Post-Electrophoresis Staining Protocol

Gel Preparation
1. Prepare desired agarose (0.8%, 1.0%, 1.2%, etc.) in 1X TAE or 1X TBE buffer.
2. Heat to dissolve agarose.
3. Cool solution until warm to the touch (60°F) prior to pouring in casting tray.
4. Pour molten agarose solution into casting tray and set comb into place. Allow gel to solidify.
5. Remove comb and place gel in buffer tank.

Buffer Preparation
6. Prepare enough buffer (1X TAE or 1X TBE) to fill the apparatus.

Electrophoresis and Destaining
7. Prepare samples with loading buffer and load in gel.
8. Electrophorese samples at 5-8 V/cm.

Staining Procedure
9. Prepare enough solution of 1X TAE or water to cover the agarose gel.
   • Ethidium Bromide— Add 5µL of 10 mg/mL EtBr stock solution to 100mL of buffer (final concentration: 0.5 µg/mL).
   • SYBR Safe— Add 10µL of 10,000X SYBR Safe stock solution to 100 mL of buffer (final concentration: 1X).
10. Soak gel for 20 minutes in the prepared solution.
11. Destain in water for 20 minutes. Repeat as necessary to remove background from the gel.
12. Image on the Odyssey Fc Imager using the 600 channel. Refer to III. Image Acquisition for more information.

D. E-Gel® Pre-Cast Agarose Gels
The E-Gel pre-cast agarose gels containing Ethidium Bromide or SYBR Safe are compatible with digital imaging on the Odyssey Fc Imager using the 600 channel. The clear versions of the E-Gel gels allow for post-staining with a DNA binding stain of your choice. Follow the manufacturer’s protocols for sample preparation and gel electrophoresis parameters.
III. Image Acquisition on the Odyssey Fc Imager

1. Place gel face-up on an Odyssey Fc Imaging Tray.
   
   **Note:** E-Gel® gel cassettes can be placed directly on the tray without removing the gel. The cassette has low background in the sample imaging area.

2. Open the imaging drawer by pressing the imaging drawer open/close button.

3. Place the Odyssey Fc Imaging Tray containing the gel in the imaging drawer. Close the drawer by pressing the imaging drawer open/close button again.

4. Open Image Studio software and connect to the Odyssey Fc Imager.

5. Click on the **Acquire** tab to show the Acquire ribbon.

6. In the Analyze Type group, select **DNA Gel** for automatic analysis or select **None**.

7. In the Channels group, select the 600 channel and deselect the other channels.

8. Select the acquisition time by dragging the slider in the 600 box. Typical acquisition times for agarose gels are from 0.5 to 2 minutes.

9. Once the parameters have been set, click on **Acquire Image** to start the acquisition. The Status group provides information on the imaging process.

   **Note:** To end an acquisition before it is completed, click on **Stop Acquiring**. All existing and pending channel images will be discarded.

10. Adjust the Lookup Table for the 600 channel to optimize the image display. Refer to How to Adjust the Lookup Tables in Image Studio for an Optimal Image Display (LI-COR Biosciences) for more information.

To excise a DNA band from the gel, carefully lift or slide the prepared gel onto an ultraviolet transilluminator (if using ethidium bromide), or a blue light transilluminator (if using SYBR® Safe). If using an E-Gel pre-cast agarose gel cassette, first remove the gel by opening the cassette with the E-Gel Opener.
IV. Results — Ethidium Bromide and SYBR Safe

A. Sensitivity of the Odyssey Fc Imager, 600 Channel
The images in Figure 1 were prepared following the post-electrophoresis staining protocol on page 4 with Ethidium Bromide and SYBR® Safe DNA stains. These images show the sensitivity of the Odyssey Fc Imager.

<table>
<thead>
<tr>
<th>Total DNA (ng)</th>
<th>200</th>
<th>50</th>
<th>20</th>
<th>10</th>
<th>5</th>
</tr>
</thead>
<tbody>
<tr>
<td>Ethidium Bromide</td>
<td>![Image]( ethicum bromide)</td>
<td>![Image]( SYBR Safe)</td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

**Sensitivity of the Odyssey Fc Imager**

Figure 1. Dilutions (200 - 5 ng) of a 2-log DNA ladder (0.1 - 1kb; New England Biolabs) were loaded on a 1% agarose gel. Gels were post-stained with 0.5 µg/mL ethidium bromide or 1X SYBR Safe DNA stain in 1X TAE buffer. Images were collected on the Odyssey Fc Imager (600 channel) using a 2 minute acquisition time.

B. DNA Samples — Plasmid Digests and PCR Products
DNA samples were loaded on 1.2% E-Gel® gels (Ethidium Bromide and SYBR Safe), electrophoresed for 30 minutes, and imaged on the Odyssey Fc Imager (2 minutes) in E-Gel cassettes.

<table>
<thead>
<tr>
<th>Lane</th>
<th>Description</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>380ng 1kb Ladder</td>
</tr>
<tr>
<td>2</td>
<td>100ng pUC19</td>
</tr>
<tr>
<td>3</td>
<td>500ng pUC19 + pUC19/XmnI/HindIII</td>
</tr>
<tr>
<td>4</td>
<td>150ng pUC19/XmnI/HindIII</td>
</tr>
<tr>
<td>5</td>
<td>380ng pUC19/XmnI/HindIII + 50ng PCR product</td>
</tr>
<tr>
<td>6</td>
<td>380ng pUC19/XmnI/HindIII + 75ng PCR product</td>
</tr>
<tr>
<td>7</td>
<td>80ng pUC19/XmnI/HindIII</td>
</tr>
<tr>
<td>8</td>
<td>100ng pUC19/XmnI/HindIII + 100ng PCR product</td>
</tr>
<tr>
<td>9</td>
<td>125ng PCR product</td>
</tr>
<tr>
<td>10</td>
<td>400ng 50bp Ladder</td>
</tr>
</tbody>
</table>

Figure 2. 1.2% E-Gel gels (Ethidium Bromide and SYBR Safe) run for 30 minutes and then imaged on the Odyssey Fc Imager (2 minutes) in E-Gel cassettes. Lane contents shown in the following guide.
C. Examples of Other DNA Stains

The same DNA samples from Figure 2 were loaded on 1.2% agarose gels pre-stained with the DNA stains as specified. Images were acquired on the Odyssey Fc Imager using the 600 channel.

![Images](image-url)

**Figure 3:** Gels were pre-stained using these DNA stains according to the manufacturers’ recommendations. Images were acquired on the Odyssey Fc Imager in the 600 channel (2 minutes). Lane contents are identical to those described in Figure 2.
V. References


LI-COR Biosciences, (2010) Syto 60 Staining of Nucleic Acids in Gels

LI-COR Biosciences, (2011) How to Adjust the Lookup Tables in Image Studio for an Optimal Image Display
Syto® 60 Staining of Nucleic Acids in Gels

Published June 2010. The most recent version of this pack insert is posted at http://biosupport.licor.com/support
The Syto® 60 stain is a red fluorescent nucleic acid stain supplied as a 5 mM solution in DMSO by Invitrogen, P/N S-11342. Any questions regarding the Syto® 60 stain should be directed to Invitrogen (www.invitrogen.com).

I. Introduction

Invitrogen’s patented Syto® dyes are cell-permeant cyanine dyes that bind to nucleic acids. Several Syto dyes are available with varying cell permeability, fluorescence enhancement upon binding to nucleic acids, excitation and emission spectra, and nucleic acid selectivity and binding affinity. The Syto 60 stain has absorption and fluorescence emission maxima of 652/678 nm. Nucleic acids stained with the Syto 60 stain can be detected and quantified on the Odyssey® Infrared and Odyssey Fc Imaging Systems using the 700 nm channel.

In the procedures outlined, the Syto 60 dye was used to stain serial dilutions of a 1 kb DNA ladder and a 50 bp DNA ladder (New England Biolabs, P/N N3232 and N3236, respectively). Three methods are presented for staining of DNA in this technical note. The Syto 60 stain can be included in the DNA sample for detection using an Odyssey Imaging system. The Syto 60 stain can also be combined with ethidium bromide (EtBr) and included in the DNA sample for visualization on an Odyssey Imaging System and on a UV transilluminator; or the Syto 60 stain can be diluted and used alone as a post-electrophoresis gel stain.

II. Methods

Method I. Electrophoretic Staining

Purpose: To obtain an archivable, digital image of a DNA agarose gel using an Odyssey Imaging System.

Method:

1. Dilute the Syto 60 stain 1:1000 in TE buffer, mix well. 
   Note: Syto 60 stain is stable for up to 1 week at 4°C when diluted.

2. Prepare DNA samples in loading dye and reserve an additional 1 µl in the final volume to accommodate the 1 µl of Syto 60 stain for loading.

3. To each sample, add 1 µl of the diluted Syto 60 stain and mix well with a pipettor.

4. Incubate at room temperature for 5 minutes.

5. Load the samples on the gel.

6. Run the gel at ~5-10 V/cm for ~1 hour or less.

7. Use the Odyssey Infrared or Odyssey Fc Imaging Systems to obtain a digital image of the Syto 60 stained DNA. 
   Odyssey Infrared Imaging System Settings:
   • Gel face down on scan bed 
   • 700 nm channel intensity: 5-8 
   • Focus offset: 0.5 mm 
   Odyssey Fc System Settings:
   • Gel face up on imaging tray 
   • Acquisition time: 2 min.

Figure 1. Two-fold dilutions of 1 kb ladder, from 1 µg to 0.125 µg, separated on a 1.2% agarose gel at 8V/cm in 1X TAE buffer for 1 hour. Panel A is the image of the gel obtained from the Odyssey Infrared Imaging System using an intensity of 5.0, gel face down. Panel B is the image of the gel acquired for 2 minutes using the Odyssey Fc Imaging System 700 nm channel, gel face up.
Method II. Dual Electrophoretic Staining

Purpose: To obtain a digital image using an Odyssey® Imaging System and then visualize DNA bands on a UV transilluminator for excision.

1. Dilute the Syto 60 stain 1:1000 in TE buffer, mix well.
   Note: The Syto 60 stain is stable for up to 1 week at 4°C when diluted.

2. Dilute EtBr (10 mg/ml solution) 1:500 in TE buffer, mix well (made fresh).

3. Prepare DNA samples in loading dye and reserve an additional volume of 2 µl to accommodate the volume of Syto 60 stain and EtBr for loading.

4. To each sample, add 1 µl of the diluted EtBr and mix with a pipettor.

5. To each sample, add 1 µl of the diluted Syto 60 stain and mix with pipettor.

6. Incubate at room temperature for 5 minutes.

7. Load the samples.

8. Run the gel at ~5-10 V/cm for ~1 hour or less.
   Note: Longer run times result in fading of the Syto 60 intensity.

9. Image on an Odyssey Imaging System in the 700 nm channel to obtain a digital image of Syto 60-stained DNA.
   Odyssey Infrared Imaging System Settings:
   • Gel face down on scan bed
   • 700 nm channel intensity: 5-8
   • Focus offset: 0.5 mm
   Odyssey Fc System Settings:
   • Gel face up on imaging tray
   • Acquisition time: 2 min.

   UV Transilluminator:
   • Place gel on UV transilluminator to identify bands for excision. If the band(s) to be excised are not bright enough, the gel can be soaked for a short time in a 2 mg/ml solution of EtBr in TAE or TBE buffer after imaging on an Odyssey System.

Figure 2. A 1.2% agarose gel was imaged using the Odyssey Infrared Imaging System (panel A), Odyssey Fc Imaging System (panel B) or a UV transilluminator and the image captured using Polaroid 667 film (panel C). Lane 1) 1 µg 1 kb ladder; Lane 2) 0.5 µg 1 kb ladder; Lane 3) 0.25 µg 1 kb ladder; Lane 4) 0.5 µg pUC 19; Lane 5) 0.5 µg pUC19/HindIII / XmnI; Lane 6) 1 µg 50 bp ladder; Lane 7) 0.5 µg 50 bp ladder; Lane 8) 0.25 µg 50 bp ladder. The gel was electrophoresed for 8 V/cm in 1X TAE buffer for 1 hr. The Odyssey intensity setting for the 700 nm channel was 8 and focus offset was 0.5 with the gel face down. The Odyssey Fc acquisition was 2 minutes, gel face up.
**Hints and Tips for Method I and II**

1. The range of dilution for the Syto 60 stain is 1:500 to 1:20,000. The dilution to use is dependent on the DNA size, concentration, and whether the Syto 60 stain will be used in combination with EtBr. **Warning:** Smaller bands may not be visualized (<100 bp).

2. The Syto 60 stain, diluted within the recommended range in TE buffer, is stable for 1 week at 4°C.

3. EtBr is not stable in TE and should be diluted fresh each time.

4. The grade of agarose is important. High grade or Molecular Biology grade agarose is less likely to cause "speckling" on Odyssey images.

5. When using the Odyssey® Infrared Imaging System to image DNA gels stained with Syto 60 stain, it may be necessary to scan the gel with the front side on the glass and/or adjust the focus offset, depending on the gel thickness. A 5 mm-7 mm thick gel is optimum.

6. Addition of EtBr to the gel and running buffer with the Syto 60 stain added in the sample is not recommended.

**Method III. Post-electrophoretic staining**

**Purpose:** To obtain an archivable, digital image of a DNA agarose gel using an Odyssey Imaging System.

**Method:**

1. Two parallel 1.3% agarose/TBE gels were loaded with serial two fold dilutions of 100 bp DNA ladder (New England Biolabs) from 1 µg to 0.3 µg per lane.

2. The gels were electrophoresed in 1X TBE running buffer at approximately 5 V/cm.

3. One gel was stained with Syto 60 dye diluted 1:2500 in water for 45 minutes at room temperature, rinsed briefly with double distilled water and then imaged in the 700nm channel using an Odyssey Imaging system.

4. The other gel was stained in 0.5 µg/ml ethidium bromide for 20 minutes at room temperature, rinsed briefly in water and imaged using a UV transilluminator and a standard CCD camera.

![Image](image-url)  

**Figure 3.** Panel A: Image of a Syto 60 stained gel using the Odyssey Infrared Imaging System. Panel B: Image of ethidium bromide-stained gel using a CCD camera.
**Recommended Dilutions and Time Requirements for Method III.**

**Gel Conditions:** A 10 x 10 cm agarose gel, 5-8 mm thick, made with high grade or molecular biology grade agarose in 1X TAE or TBE buffer

<table>
<thead>
<tr>
<th>Syto® 60 Nucleic Acid Stain Dilution</th>
<th>Minimum Staining Time</th>
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</thead>
<tbody>
<tr>
<td>1:2000</td>
<td>5-15 min</td>
</tr>
<tr>
<td>1:2500</td>
<td>15-30 min</td>
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<tr>
<td>1:5000</td>
<td>30-45 min</td>
</tr>
<tr>
<td>1:10000</td>
<td>45 min</td>
</tr>
<tr>
<td>1:15000</td>
<td>45 min</td>
</tr>
<tr>
<td>1:20000</td>
<td>45 min</td>
</tr>
</tbody>
</table>

The quickest staining time was 5 minutes using 1:2000 dilution of the Syto 60 stain in water. Gels were stained sufficiently in 15 minutes using a 1:2500 dilution. A 1:5000 dilution of Syto 60 stain requires at least 30-45 minutes of staining. The most dilute solution tested was 1:20,000 and the gel was stained sufficiently after 45 minutes. There was no significant improvement in sensitivity from 60 to 120 minutes using 1:10,000, 1:15,000 and 1:20,000 dilutions.

**Speckle Reduction**

The appearance of speckles on the gel may be present after post-electrophoretic staining. The Odyssey® Infrared Imaging System software’s “FILTER” then “Noise Removal” function and the Odyssey Fc Image Studio’s “NOISE REDUCTION” function can be used to improve the appearance of the images (see Figure 4). To reduce the appearance of speckles on the gel, cut off the wells before post-electrophoretic staining and rinse the gel in water.

**Note:** The type and concentration of agarose will affect the degree of speckling. For example, low melting-point agarose tends to be highly prone to speckling.

**Figure 4.** Image of agarose gel showing before and after using Odyssey Infrared Imaging System software’s “FILTER” then “Noise Removal” function.
Conclusions
A table of cost comparisons for the Syto 60 stain and the ethidium bromide staining reagents used for each method is provided below. The recommended dilution of the Syto 60 staining reagent makes it more competitive with ethidium bromide on a cost basis, and the inclusion of a small amount of the Syto 60 stain in the sample is environmentally friendly.

<table>
<thead>
<tr>
<th>Cost Comparisons</th>
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<tbody>
<tr>
<td>Dilution</td>
</tr>
<tr>
<td>---</td>
</tr>
<tr>
<td>Syto 60 stain</td>
</tr>
<tr>
<td>Syto 60 stain</td>
</tr>
<tr>
<td>Syto 60 stain</td>
</tr>
<tr>
<td>EtBr</td>
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<td>EtBr</td>
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Electrophoretic Mobility Shift Assay (EMSA) Using IRDye® Oligonucleotides

Developed for:

Aerius and Odyssey® Family of Imaging Systems

Please refer to your manual to confirm that this protocol is appropriate for the applications compatible with your Odyssey and/or Aerius Imager model.

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The most recent version of this protocol is posted at http://biosupport.licor.com
I. Introduction

Gel shift assays or electrophoretic mobility shift assays (EMSA) provide a simple method to study DNA-protein interactions. This assay is based on the principle that a DNA-protein complex will have different mobility during electrophoresis than non-bound DNA. These shifts can be visualized on a native acrylamide gel using labeled DNA to form the DNA-protein binding complex. To date, protocols require labeling DNA by (1) radioisotope, (2) digoxigenin, or (3) biotin. The Aerius and Odyssey® family of Imaging Systems (LI-COR® Biosciences) offer a quick and easily-adapted alternative method to radioisotopic and chemiluminescent detection methods for EMSA analysis and visualization.

A DNA oligonucleotide end-labeled with LI-COR IRDye is a good substrate for protein binding. LI-COR offers pre-annealed oligonucleotides specific to eight unique binding proteins. DNA detection using IRDye reagents is linear within a 50-fold dilution range, from 9.1 fmol to 0.18 fmol. Additional benefits include no hazardous radioisotope, no gel transfer to membrane or gel drying, no chemiluminescent substrate reagents, and no film exposure. Following electrophoresis, the gel can be imaged while remaining in the glass plates. If necessary, the gel can be placed back in the electrophoresis unit and run longer.

Existing mobility shift assay protocols can be easily transformed into infrared assays by replacing the existing DNA oligonucleotides with oligonucleotides end-labeled with IRDye reagents. The binding conditions and electrophoresis conditions will remain the same as with any other EMSA detection method.

II. General Methodology

EMSA Oligonucleotides Labeled with IRDye 700

<table>
<thead>
<tr>
<th>Part Number</th>
<th>Oligonucleotide</th>
</tr>
</thead>
<tbody>
<tr>
<td>829-07921</td>
<td>IRDye 700 p53 Consensus Oligonucleotide</td>
</tr>
<tr>
<td>829-07922</td>
<td>IRDye 700 STAT3 Consensus Oligonucleotide</td>
</tr>
<tr>
<td>829-07923</td>
<td>IRDye 700 CREB Consensus Oligonucleotide</td>
</tr>
<tr>
<td>829-07924</td>
<td>IRDye 700 NFkB Consensus Oligonucleotide</td>
</tr>
<tr>
<td>829-07925</td>
<td>IRDye 700 AP-1 Consensus Oligonucleotide</td>
</tr>
<tr>
<td>829-07926</td>
<td>IRDye 700 Sp-1 Consensus Oligonucleotide</td>
</tr>
<tr>
<td>829-07929</td>
<td>IRDye 700 HIF-1 Consensus Oligonucleotide</td>
</tr>
<tr>
<td>829-07933</td>
<td>IRDye 700 ARE (Androgen Receptor) Consensus Oligonucleotide</td>
</tr>
<tr>
<td>829-07910</td>
<td>EMSA Buffer Kit for the Odyssey</td>
</tr>
</tbody>
</table>

Labeling DNA Fragments with IRDye Infrared Dyes

To obtain DNA fragments end-labeled with IRDye infrared dyes, oligos labeled with IRDye infrared dyes are used. It is critical that the DNA fragment is end-labeled rather than having dye incorporated into the DNA, which interferes with the formation of the DNA-Protein complex.

Oligonucleotides are manufactured in single strand form; therefore, both forward and reverse DNA oligonucleotides must be purchased. Once oligonucleotides are obtained, they need to be annealed to form a double-stranded DNA fragment.

Oligonucleotides are annealed by placing the oligonucleotide set in a 100°C heat block for 5 minutes and then leaving the oligonucleotides in the heat block and turning it off to slowly cool to room temperature.

**Important:** Both oligonucleotide sequences should be end-labeled with the same IRDye infrared dye. There is a significant decline (~70%) in signal intensity when using only one end-labeled oligonucleotide.
III. Mobility Shift Sample Protocol (NFkB)

Each oligo labeled with IRDye 700 provided by LI-COR® Biosciences for EMSA reactions will have an optimized protocol to measure the protein-DNA interaction. See the specific EMSA oligo pack insert for more information. As an example, the p53 protein-DNA interaction will be described in this document.

**Gel Preparation:** Native pre-cast polyacrylamide gels such as 5% TBE (BioRad) or 4-12% TBE (Invitrogen) are recommended. Alternatively, the recipe below can be used to prepare a 4% native gel.

**Note:** The protein shift detected on each gel type (i.e., 5% vs 4-12%) will be unique.

Prepare 4% native polyacrylamide gel containing 50 mM Tris, pH 7.5; 0.38 M glycine; and 2 mM EDTA:

For 40 mL mix:

- 5 mL 40% polyacrylamide stock (Polyacrylamide-BIS ratio = 29:1)
- 2 mL 1 M Tris, pH 7.5
- 7.6 mL 1 M Glycine
- 160 μL 0.5 M EDTA
- 26 mL H2O
- 200 μL 10% APS
- 30 μL TEMED

Pour the gel between glass plates and wait about 1-2 hours to polymerize.

**Oligo Preparation:** EMSA oligonucleotides from LI-COR Biosciences are pre-annealed.

1. Dilute oligos in 1XTE for final concentration of 20 pmol/μL.
2. Place 5 μL of forward IRDye 700 oligo into a new tube and add 5 μL of reverse IRDye 700 oligo.
3. Anneal oligos by placing the oligo set in a 100°C heat block for 3 minutes. Leave the oligos in the heat block and turn it off to slowly cool to room temperature.
4. Dilute annealed oligos 1 μL in 199 μL water. This is your working DNA stock. Oligos can be stored at -20°C for up to a year if protected from light.

**Binding Reaction:** For NFkB IRDye 700 oligonucleotide, the following binding reaction is a good starting point.

<table>
<thead>
<tr>
<th>Reaction</th>
<th>µL</th>
</tr>
</thead>
<tbody>
<tr>
<td>10X Binding Buffer (100 mM Tris, 500 mM KCl, 10 mM DTT; pH 7.5)</td>
<td>2</td>
</tr>
<tr>
<td>Poly(d*I•dC) 1 μg/μL in 10 mM Tris, 1 mM EDTA; pH 7.5</td>
<td>1</td>
</tr>
<tr>
<td>25 mM DTT/2.5% Tween® 20</td>
<td>2</td>
</tr>
<tr>
<td>Water</td>
<td>13</td>
</tr>
<tr>
<td>IRDye 700 NFkB</td>
<td>1</td>
</tr>
<tr>
<td>Raji nuclear extract (Positive control) (5 μg/μL)</td>
<td>1</td>
</tr>
<tr>
<td><strong>TOTAL</strong></td>
<td><strong>20</strong></td>
</tr>
</tbody>
</table>

After the addition of the DNA to the protein-buffer mix, reactions are incubated to allow protein binding to DNA. A typical incubation condition is 20-30 minutes at room temperature. Since IRDye 700 infrared dye is sensitive to light, it is best to keep binding reactions in the dark during incubation periods (e.g., put tubes into a drawer or cover the tube rack with aluminum foil).

**Electrophoresis:**

1. Add 1 μL of 10X Orange loading dye (LI-COR, P/N 927-10100), mix, and load on a gel.
2. Run the gel at 10 V/cm for about 30 minutes in non-denaturing buffer (i.e., 1XTGE or TBE buffer).

**Note:** For best results, electrophoresis should be performed in the dark (simply put a cardboard box over the electrophoresis apparatus).
**Imaging:** Gels can be imaged either inside the glass plates or removed from the glass plate. When removing gel from the glass plates, take care not to deform or tear the gel. Scan the gel. Please refer to your manual for specific information on your model of imager.

**Figure 1.** IRDye 700 NFkB oligonucleotides were separated on a native polyacrylamide gel (4-12% TBE, Invitrogen EC62352BOX) and imaged on the Odyssey® Infrared Imaging System.
Lane 1) no nuclear extract;
Lanes 2 and 5) 10 μg Raji nuclear extract;
Lanes 3 and 6) 5 μg Raji nuclear extract;
Lanes 4 and 7) 2.5 μg Raji nuclear extract.

**Figure 2.** The uppermost shifted band in Lanes 2-7 of Figure 1 was analyzed to determine the level of NFkB binding to the IRDye 700 NFkB oligonucleotides.

One of the benefits of using the Odyssey® Infrared Imaging System for EMSA analysis is that it provides an easy method for quantification. However, there are issues to consider when using the Odyssey Imager to quantify EMSA results. The primary issue is that the free DNA fragment has much less signal than the DNA fragment when bound to a protein, making quantification of the unbound DNA inaccurate. The addition of DTT/Tween® 20 to the binding reaction stabilizes the dye and reduces this phenomenon. In addition, it is unrealistic to perform quantification analyses under the assumption that the free DNA band in the control, containing DNA only (no extract), should equal the sum of the signals of the free and bound DNA in the samples where the protein-DNA binding reaction occurs. Using end-labeled oligonucleotide duplexes as the DNA source and nuclear extract as a protein source renders this assumption impractical, due to the non-specific binding that occurs from using a nuclear extract. Oligonucleotides can also complicate quantification because the free oligonucleotides form a smear rather than a tight band. This makes it more difficult to assign an intensity value to bands.
Optimization

Binding Reaction

A universal binding condition that applies to every protein-DNA interaction cannot be recommended, since binding conditions are specific for each protein-DNA interaction. Thus, the user should establish binding reaction conditions for each protein-DNA pair. Binding buffer should be the same for this method as with any other mobility shift detection method used.

After the addition of DNA to the protein-buffer mix, reactions are incubated to allow protein to bind to DNA. Time required for binding is the same as when radioactively-labeled DNA fragments are used; a typical incubation condition is 20-30 minutes at room temperature. Since IRDye reagents are sensitive to light, it is best to keep binding reactions in darkness during incubation periods (e.g., put tubes into a drawer or simply cover the tube rack with aluminum foil). After the incubation period, native loading dye is added to the binding reaction.

**Note:** In some cases, it was observed that DNA control reactions (no protein) have lower signal than reactions containing protein. This may be due to lower stability of the dye in certain buffer conditions. The addition of 5 mM DTT and 0.5% Tween 20 to all reactions reduces this phenomenon.

**Important:** It is critical not to use any blue loading dye (e.g., bromophenol blue), as this will be visible on the Odyssey image. Use 10X Orange loading dye instead (LI-COR, Part #927-10100).

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**Figure 3. AP-1 EMSA using IRDye 700 end-labeled oligonucleotide duplex.**

It is common to use unlabeled DNA duplex to determine binding specificity. Excess unlabeled DNA is added to the binding reaction; therefore, it competes with the labeled DNA for binding sites. If competition eliminates labeled DNA binding, no shift is observed (see last three lanes in gel), indicating that the binding reaction is specific.

Competition reactions contained 100-fold molar excess of wild-type oligonucleotide duplex. Nuclear extracts of HeLa, HeLa 2-hour serum response, and HeLa 4-hour serum response, were used to visualize an increase in AP-1 binding as a result of the serum response treatment to the HeLa cells.

<table>
<thead>
<tr>
<th>Nuclear Extract</th>
<th>IRDye 700 AP-1 oligo</th>
<th>AP-1 wild-type competitor oligo</th>
</tr>
</thead>
<tbody>
<tr>
<td>No Extract</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>HeLa</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>HeLa 2 hr SR</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>HeLa 4 hr SR</td>
<td>+</td>
<td>+</td>
</tr>
</tbody>
</table>

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Competition using mutant DNA duplexes is another common method to determine binding specificity. A mutant DNA sequence is used to compete with the wild-type binding sequence. Specific binding is observed when mutant DNA (unlabeled) does not reduce the binding of labeled wild-type DNA. Two-color analysis of mutant vs. wild-type binding is done using the Odyssey Infrared Imaging System. The wild-type oligos are labeled with IRDye 700 phosphoramidite and mutant oligos with IRDye 800 phosphoramidite. In the figure above, the mutant non-specific binding is very intense (800 nm image); however, there is no decrease in wild-type binding (700 nm image).

Lane 1 – Free IRDye 700 AP-1 consensus oligonucleotide and IRDye 800 AP-1 mutant oligonucleotide with no nuclear extract;
Lane 2 – Nuclear extract with 0:1 ratio of IRDye 700 AP-1 consensus oligonucleotide to IRDye 800 AP-1 mutant oligonucleotide;
Lane 3 – Nuclear extract with 1:0 ratio of IRDye 700 AP-1 consensus oligonucleotide to IRDye 800 AP-1 mutant oligonucleotide;
Lane 4 – Nuclear extract with 1:1 ratio of IRDye 700 AP-1 consensus oligonucleotide to IRDye 800 AP-1 mutant oligonucleotide;
Lane 5 – Nuclear extract with 1:2 ratio of IRDye 700 AP-1 consensus oligonucleotide to IRDye 800 AP-1 mutant oligonucleotide;
Lane 6 – Nuclear extract with 1:3 ratio of IRDye 700 AP-1 consensus oligonucleotide to IRDye 800 AP-1 mutant oligonucleotide;
Lane 7 – Nuclear extract with 1:4 ratio of IRDye 700 AP-1 consensus oligonucleotide to IRDye 800 AP-1 mutant oligonucleotide;
Lane 8 – Nuclear extract with 1:5 ratio of IRDye 700 AP-1 consensus oligonucleotide to IRDye 800 AP-1 mutant oligonucleotide;

References
