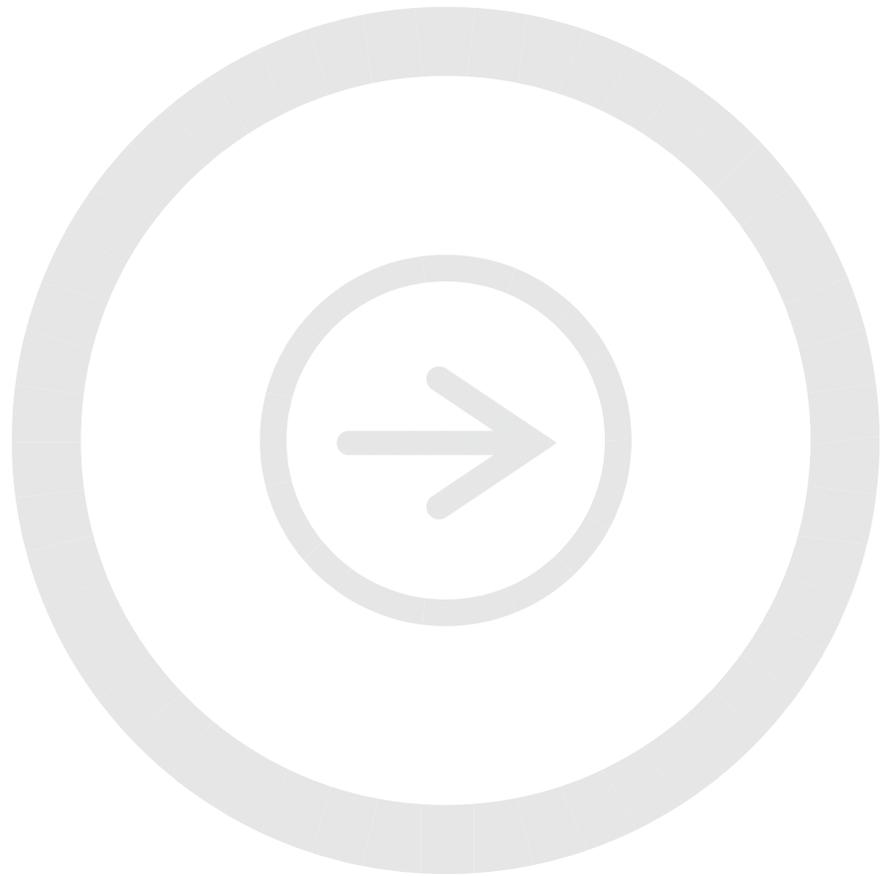


Optimizing Chemiluminescent Western Blots

Developed for:

Odyssey[®] Fc Imaging System



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

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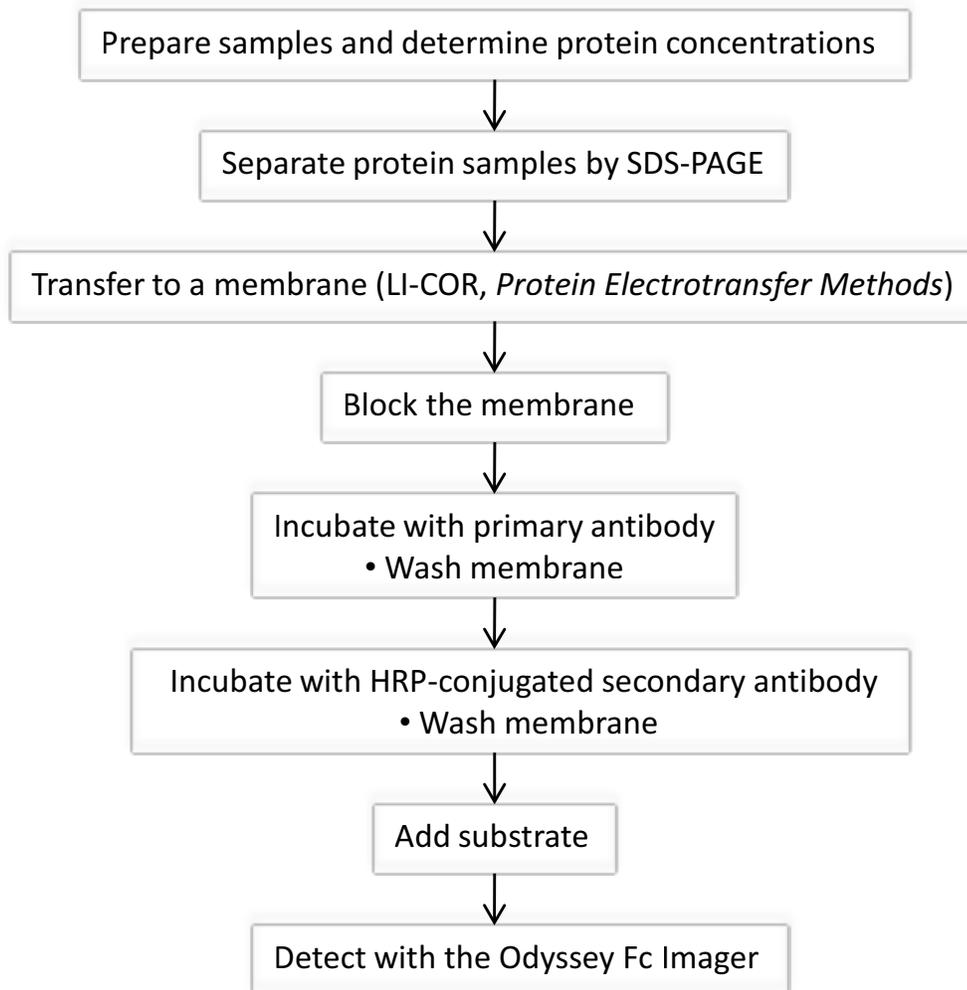
I. Introduction to Chemiluminescent Western Blotting on the Odyssey Fc Imager

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



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.

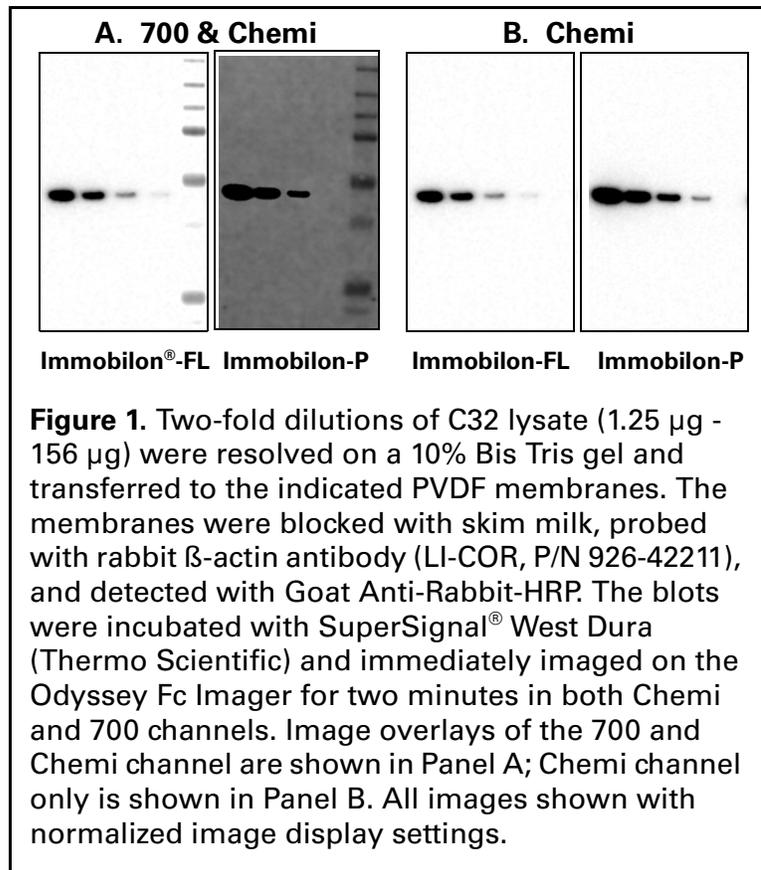
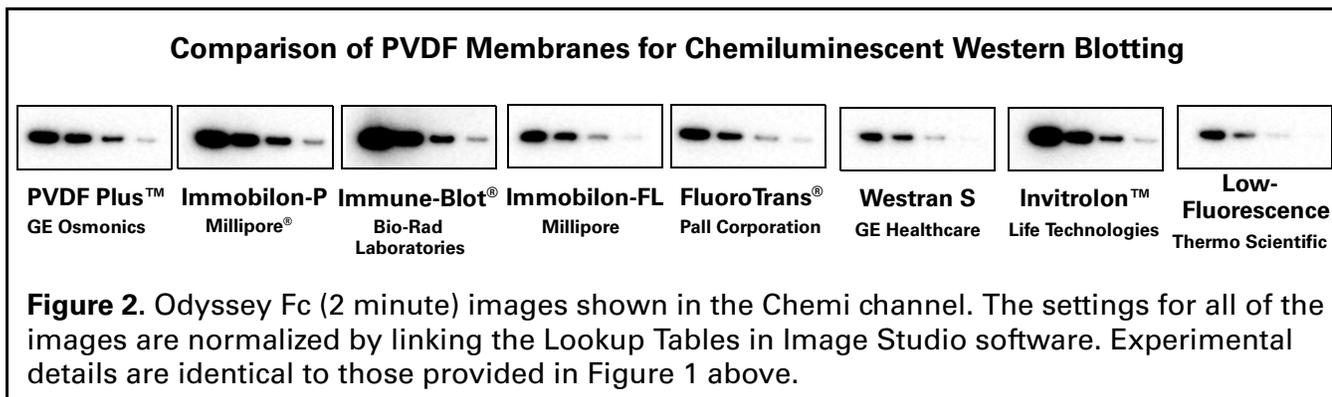
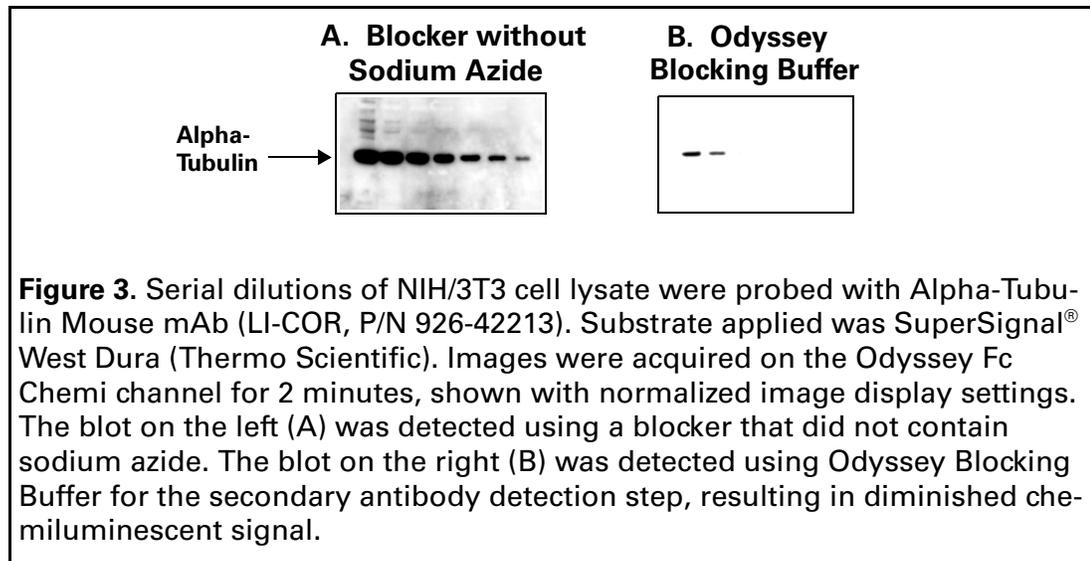


Figure 2 shows a thorough comparison of Western blot performance on a wide selection of PVDF membranes. Detection sensitivities can be greatly affected by the choice of membrane, and multiple membranes should be evaluated. Protein characteristics such as molecular weight, amino acid composition, and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



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.



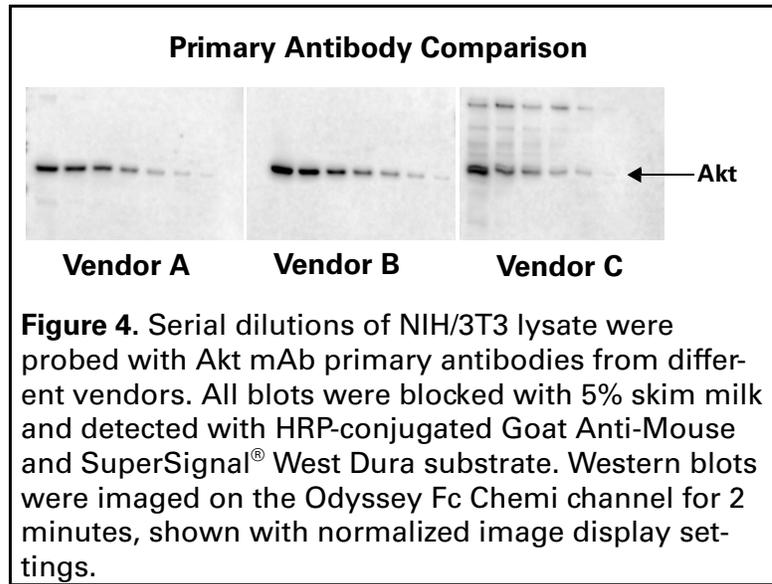
Blocking buffer is also often used as a diluent for the primary and secondary antibodies.

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

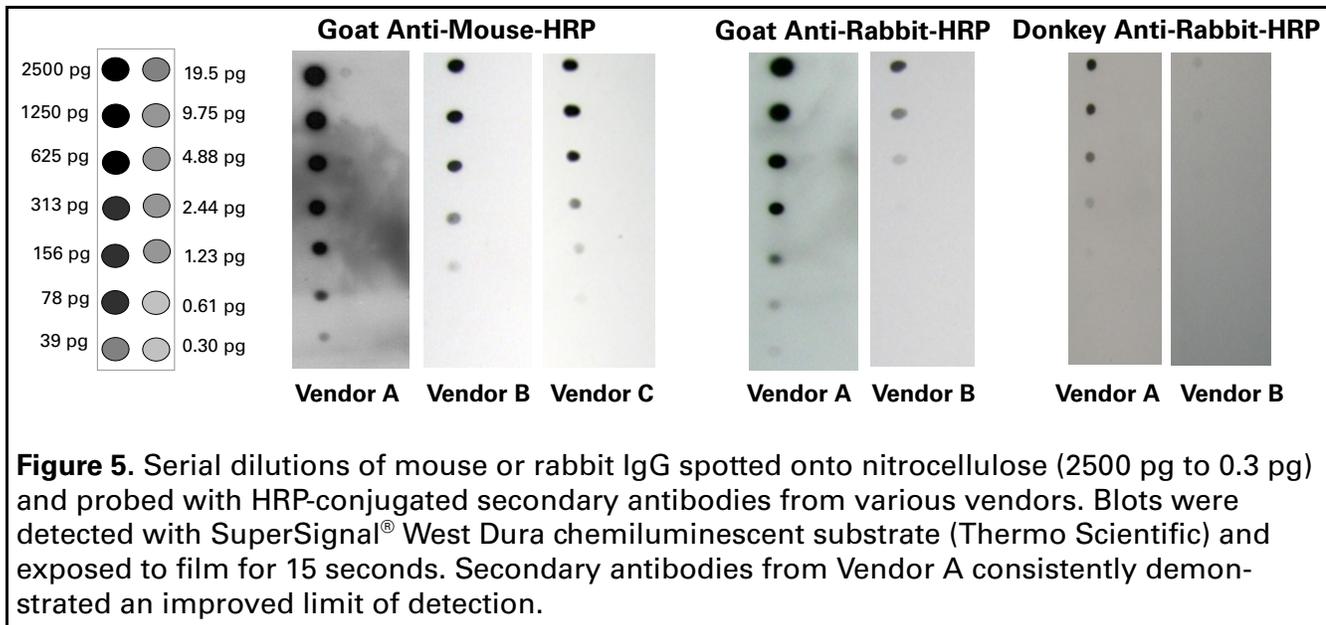
An antibody produced to detect a specific antigen is called a primary antibody. It binds directly to the molecule of interest. Primary antibodies can be produced in a wide variety of species such as mouse, rabbit, goat, chicken, rat, guinea pig, human, and many others. Primary antibodies for the same 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

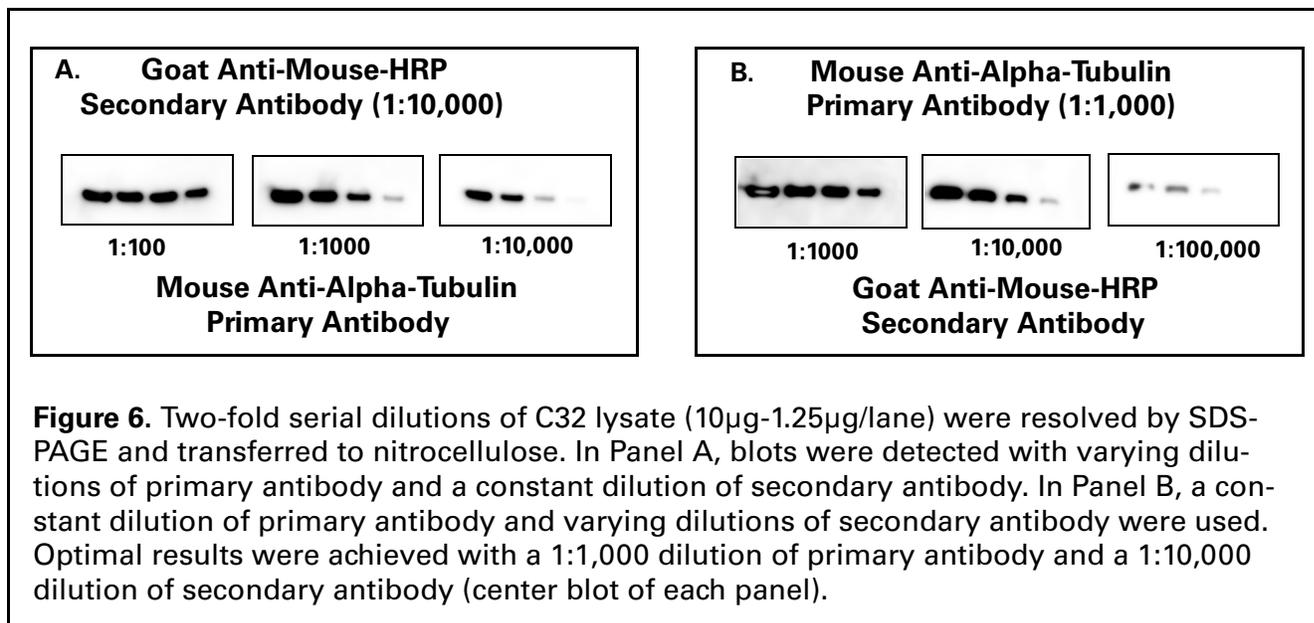
The reactivity of secondary antibodies ranges widely between vendors, even within the same species and especially between host species. The ratio of HRP enzyme to antibody varies, and may affect the detection of the target. Try secondary antibodies from several vendors to find the ones that give the 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).

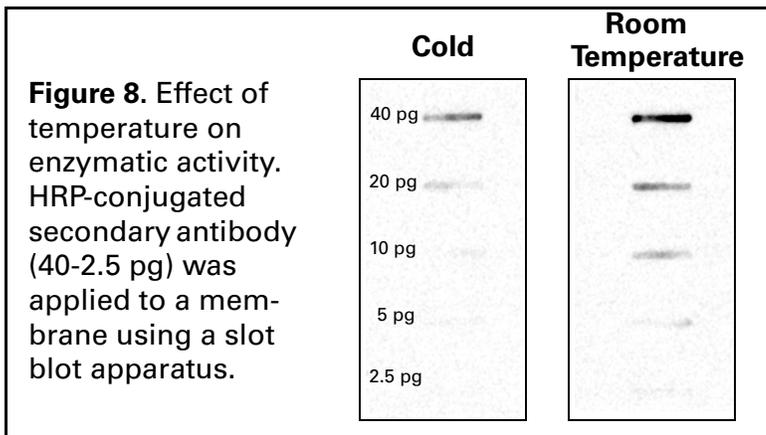
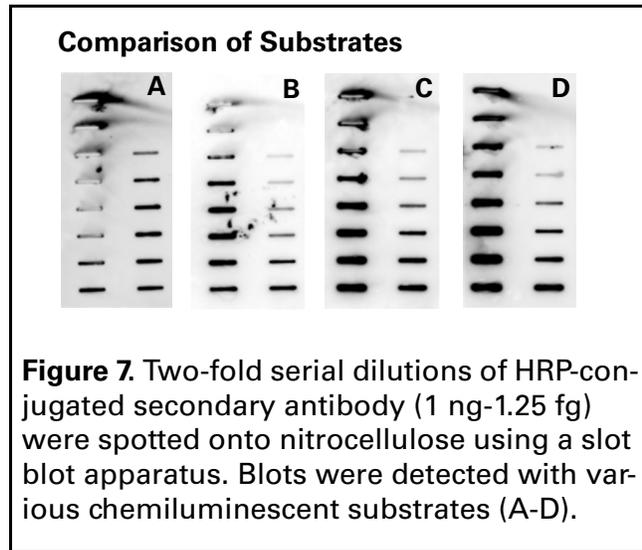


F. Washing the Membranes

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.

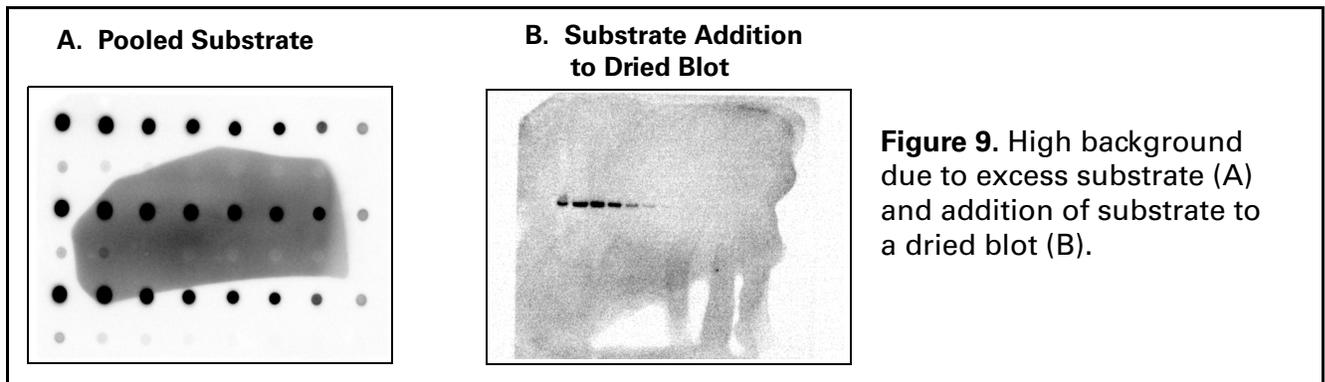


The manufacturers for some chemiluminescent substrates may recommend that the substrates be stored at 4°C and warmed to room temperature before use. If so, ensure the substrate is at room temperature before use for optimum signal. Cold temperatures slow the activity of the HRP enzyme, resulting in less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

IV. Troubleshooting Guide

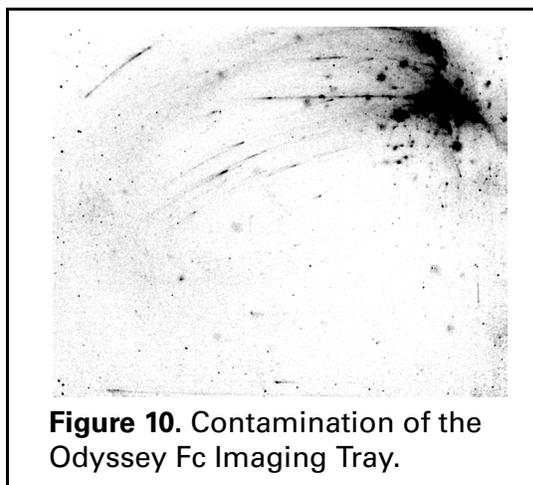
A. High Background Due to Substrate

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.

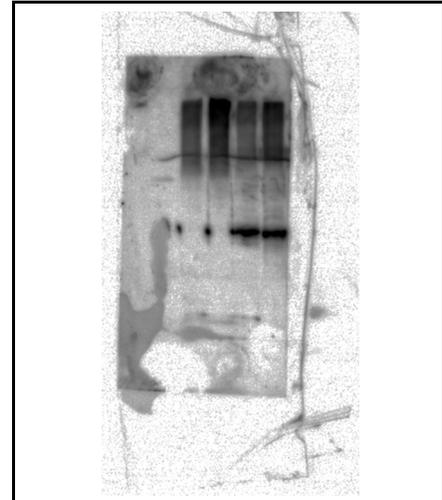


Figure 11. Unwanted background caused by plastic wrap.

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.

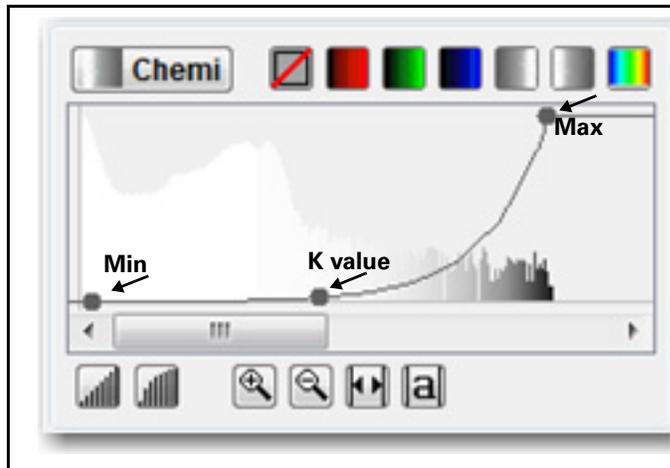


Figure 12. Use the three adjustable points to optimize the displayed image.

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

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.

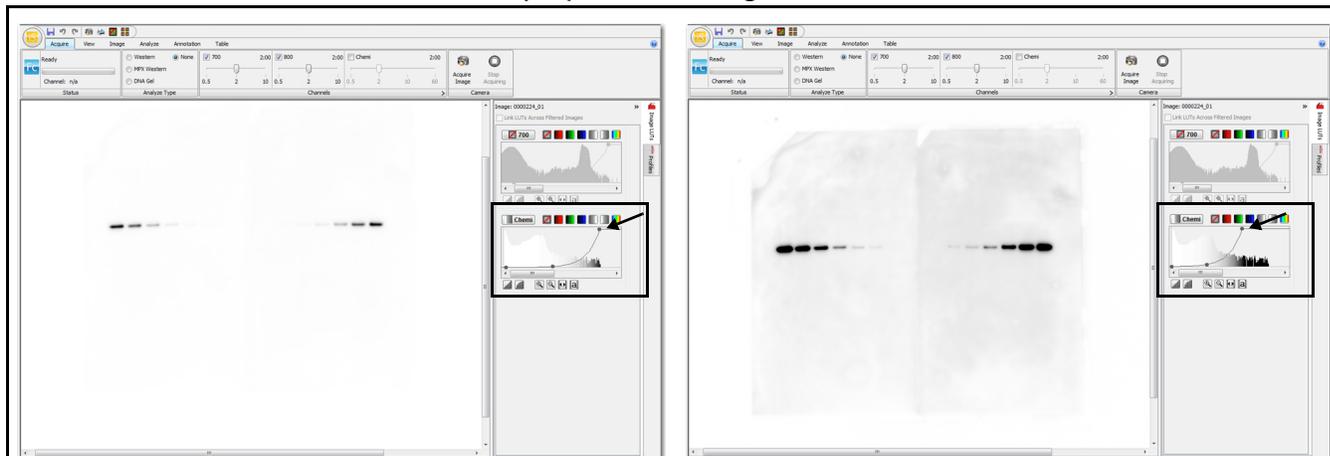


Figure 13. The curve overlaying the histogram in the Lookup Table was adjusted by moving the Max point to the left to make the bands appear darker in the second image.

V. References

Towbin, *et al.*, (1979) *Proc. Natl. Acad. Sci USA* 76; 4350-4

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*



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