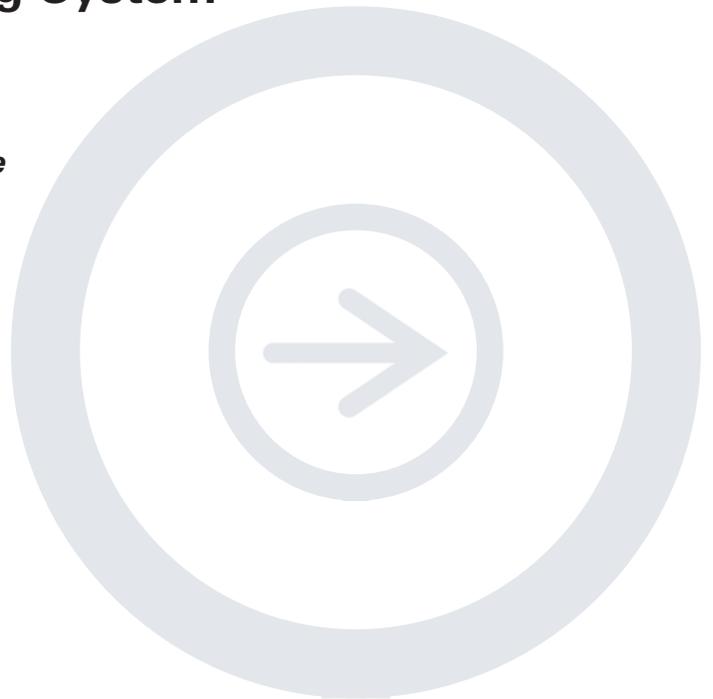# Good Westerns Gone Bad:

Tips to Make Your NIR Western Blot Great

Developed for:

**Aerius,
Odyssey® Classic,
Odyssey CLx, and
Odyssey Sa Infrared Imaging Systems,
and the Odyssey Fc Imaging System**

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

LI-COR®

Contents

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.[1] 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.

Near-infrared fluorescence detection on the Odyssey Classic, Odyssey CLx, Odyssey Fc, or Odyssey Sa Imaging Systems provides a quantitative two-color detection method for Western Blots. This document will discuss some factors that may alter the performance of a near-infrared (NIR) Western blot, resulting in "good Westerns, gone bad."

II. Important Factors That Affect Western Blot Results

A. Membrane

A low-background membrane is essential for NIR Western blot success. Background can result from membrane autofluorescence or non-specific binding of antibodies. Polyvinylidene fluoride (PVDF) and nitrocellulose membranes are typically used for Western blotting applications. There are many brands and vendors for both types of membranes.

Before using your blotting membrane for the full Odyssey Western blot protocol, cut a small sample of membrane for testing. Image this sample (both wet and dry) to evaluate membrane autofluorescence. If possible, include a sample of membrane that is known to work well with the Odyssey system, so you can compare background levels.

LI-COR has evaluated many different transfer membranes for Western blotting; examples of membrane performance are shown in Figure 1. PVDF membranes typically display higher membrane autofluorescence than nitrocellulose, and more variability in background levels. In general, nitrocellulose membranes offer the lowest membrane autofluorescence. However, PVDF has many

advantages (including higher binding capacity, higher target retention, and better tensile strength) that make it an appropriate choice for many experiments.

NOTE: Not all sources of PVDF and nitrocellulose have been evaluated by LI-COR, and lot-to-lot variation can occur. It is important to evaluate the membrane before use. Samples of membrane can be quickly evaluated by imaging them both wet and dry with any Odyssey® System.
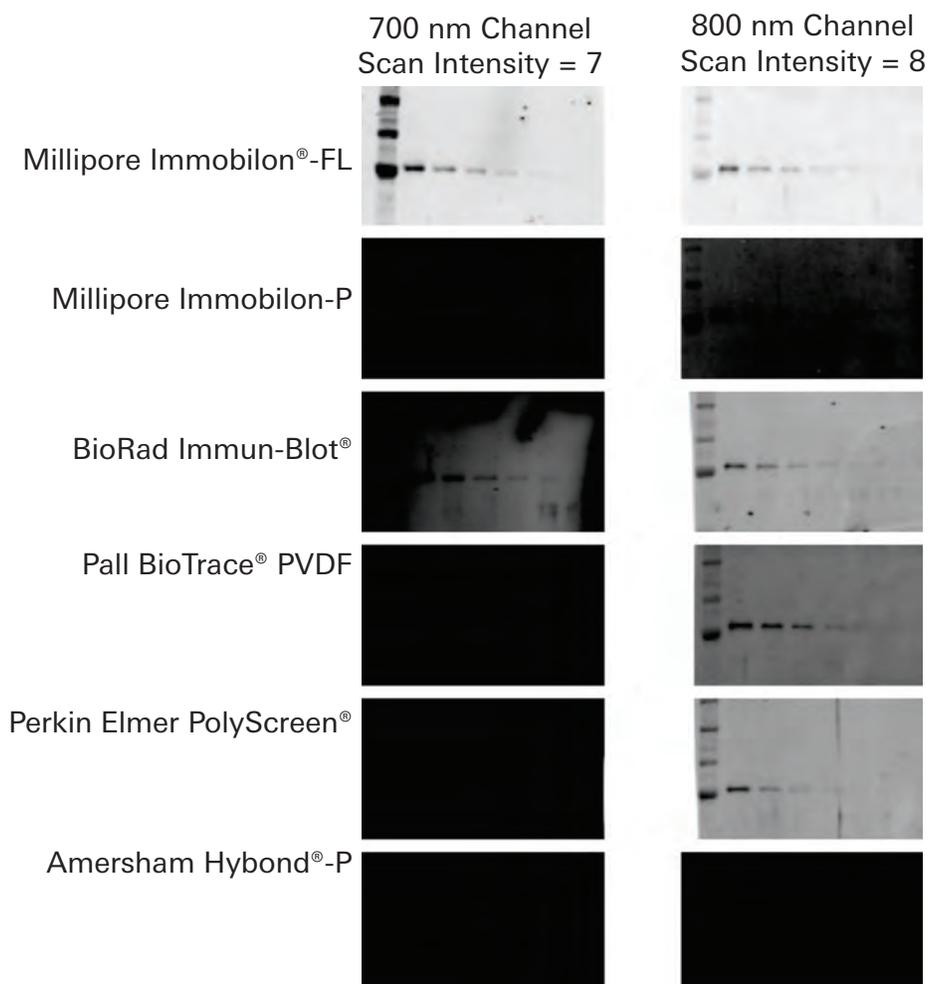

Figure 1. **Membrane autofluorescence from PVDF affects Western blot performance.** Transferrin was detected by Western blotting, using various vendors and brands of PVDF membrane. Blots were imaged with the Odyssey Classic Infrared Imaging System in both 700 and 800 nm channels.

B. Blocking Reagent

Many different sources and types of blocking reagents are used for Western blot applications. Antibody performance can sometimes be compromised by the blocking reagent chosen. Blocking buffer choice may affect antibody specificity and non-specific binding, and can dramatically increase the number of background bands (Figure 2).[2] Excessive blocking (for example, with high concentrations of nonfat dry milk) may cause loss of blotted proteins or mask the desired antibody-antigen interactions.[3]

Detection reagents may cross-react with certain blocking buffers. 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, trying another blocker may solve the problem. No single blocking reagent will be suitable for ALL antigen-antibody pairs. Refer to the Odyssey® Western Blot Blocker Optimization Protocol (*www.licor.com/optimize*) to help design an experiment to optimize your blocking conditions. Figure 2 shows the behavior of a PKCα antibody in 5% BSA, 5% Milk, and Odyssey Blocking Buffer on nitrocellulose membrane. For this primary antibody, choice of blocking buffer had a dramatic effect on non-specific banding, particularly when 5% BSA was used.
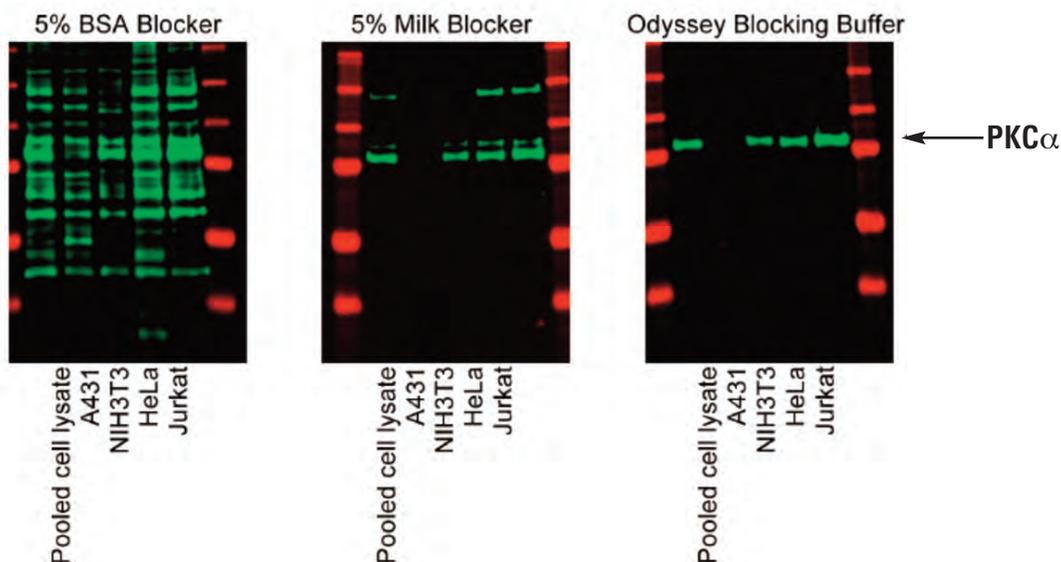

Figure 2. **Effects of blocking buffer on detection of PKC**α. Western blots were detected with anti-PKCα primary antibody and IRDye® 800CW Goat anti-Mouse IgG, using different blocking buffers. All blots were processed identically, with the exception of blocking reagent. Images were generated with an Odyssey Classic Infrared Imager (scan intensity=5, sensitivity=5).

C. TBS- vs. PBS-Based Blocking Buffers

Many blocking reagents are available in Phosphate Buffer or Tris Buffer (PBS/TBS) systems. In general, TBS blocking reagents are used for detection of phospho-proteins, because the phosphate present in PBS blocking reagents may competitively bind with antibodies to phospho-proteins. Some phospho-proteins can be detected with PBS-based blocking reagents, depending on the antibody specificity and affinity; however, it is important to optimize the blocking reagent for the specific antibody that is being used for optimal Western blot performance.

The same buffer system should be used throughout the Western blot process; for example, if you are using a TBS-based buffer system, choose Odyssey Blocking Buffer (TBS). If you are using a PBS-based buffer system, use Odyssey Blocking Buffer (PBS).

The experiments below show the importance of optimizing for the proper blocking reagent. Figure 3 shows Jurkat cell lysate stimulated with Calyculin (+) and a non-stimulated control (-). Phospho-Akt (green bands) was clearly detected with TBS-based Odyssey® Blocking Buffer, but could not be detected with the PBS-based Odyssey Blocking Buffer. This suggests that binding of the pAkt primary antibody may be affected by the phosphates in the PBS blocking reagent. Detection of total Akt (red bands) was also less robust with the PBS buffer system.
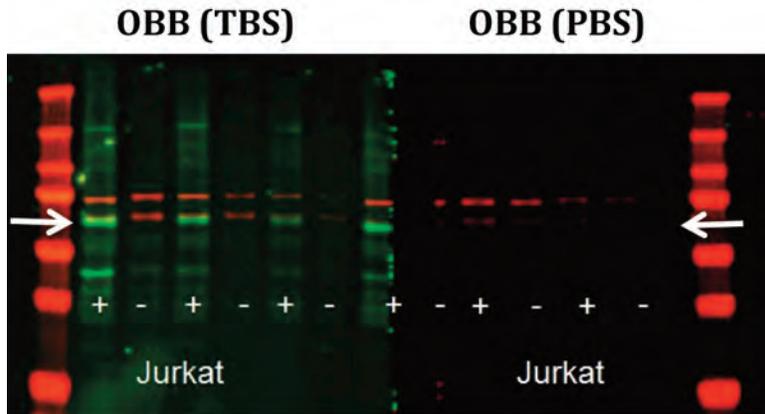

Figure 3. **Phospho-Akt detection is affected by TBS and PBS blocking buffers systems**. Jurkat cells were treated with Calyculin A (+) or left untreated (-), and lysates were loaded at 10 µg, 5 µg, and 2.5 µg/well. Odyssey Blocking Buffer (TBS) and Odyssey Blocking Buffer (PBS) were used for blocking and antibody dilution, as indicated; appropriate TBS and PBS buffers were used for washes. Blot was probed with anti-phospho-Akt mAb (rabbit; Santa Cruz P/N sc-135650) and anti-Akt mAb (mouse; CST P/N 2967). IRDye® 800CW Goat anti-Rabbit IgG (LI-COR P/N 926-32221) and IRDye 680RD Goat anti-Mouse IgG (LI-COR P/N 926-68070) were used for detection. Blot was imaged with an Odyssey CLx Imager (auto-scan settings, 700 and 800 nm channels). Akt bands are shown in red (700 nm), and phospho-Akt bands are shown in green (800 nm). White arrows indicate position of Akt band.

Figure 4 shows detection of phospho-EGFR in A431 cell lysate stimulated with EGF (+) and in a nonstimulated (-) control. Both the PBS and TBS Odyssey Blocking Buffer systems enabled detection of phospho-EGFR. Because no single blocking reagent works well with all antigen-antibody pairs, it is important to test various buffer systems to ensure optimal detection of the protein target.
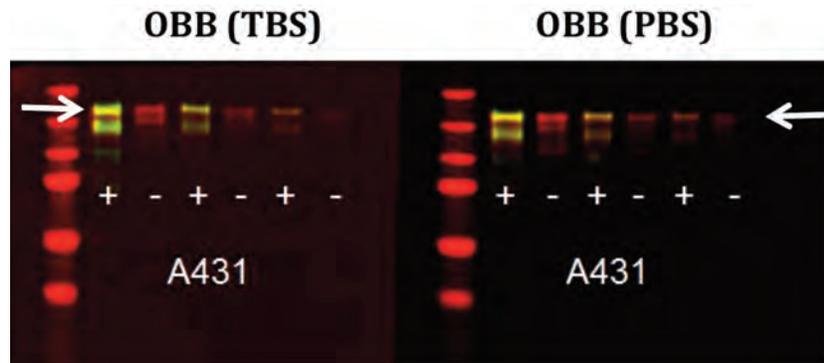
Figure 4. **Phospho-EGFR is efficiently detected with both TBS and PBS blocking buffer systems.** A431cells were treated with EGF (+) or left untreated (-), and lysates were loaded at 10 µg, 5 µg, and 2.5 µg/well. Odyssey® Blocking Buffer (TBS) and Odyssey Blocking Buffer (PBS) were used for blocking and antibody dilution, as indicated; appropriate TBS and PBS buffers were used for washes. Blot was probed with anti-phospho-EGFR mAb (rabbit; Epitomics P/N 1139-1) and anti-EGFR mAb (mouse; Life Technologies P/N AHR5062). IRDye® 800CW Goat anti-Rabbit IgG (LI-COR P/N 926-32211) and IRDye 680RD Goat anti-Mouse IgG (LI-COR P/N 926-68070) were used for detection. Blot was imaged with Odyssey CLx Imager (auto-scan settings, 700 and 800 nm channels). EGFR bands are shown in red (680 nm), and phospho-EGFR bands in green (800 nm). Phospho-EGFR bands appear yellow because 800 nm and 700 nm signals overlap. White arrows indicate position of EGFR band.

D. 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.

Tween® 20
- Blocking buffer – do not add Tween 20 during blocking.
- Diluted primary and secondary antibodies should contain Tween 20.
  Use a final concentration of 0.1 - 0.2% Tween 20 for PVDF or nitrocellulose membranes.
- Wash solutions should contain 0.1% Tween 20.

SDS
When using PVDF membrane, addition of SDS will dramatically reduce overall membrane background, especially in the 700 nm channel. It is critical to use only a very small amount, because SDS is an ionic detergent and can disrupt antibody-antigen interactions if too much is present at any time during the protocol.
SDS *should not* be used with nitrocellulose membranes.

- Blocking buffer - do not add SDS to the blocking reagent during blocking.
- Diluted primary antibodies *should not* contain SDS.
- ***Diluted secondary antibodies should contain a final concentration of 0.01 – 0.02% SDS and 0.1 – 0.2% Tween 20, when PVDF membrane and IRDye 680LT secondary antibodies are used.***
- SDS is essential when using IRDye 680LT antibodies with PVDF membrane, but optional when using IRDye 680RD antibodies with PVDF.
- Wash solutions *should not* contain SDS.

E. Primary Antibody

An antibody produced to detect a specific antigen is called the primary antibody, and 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 from different vendors and sources for the same antigen may perform very differently. It may be necessary to test more than one primary antibody for optimal detection of the target protein in your Western blot system. Figure 5 is an example of how different primary antibodies may react.
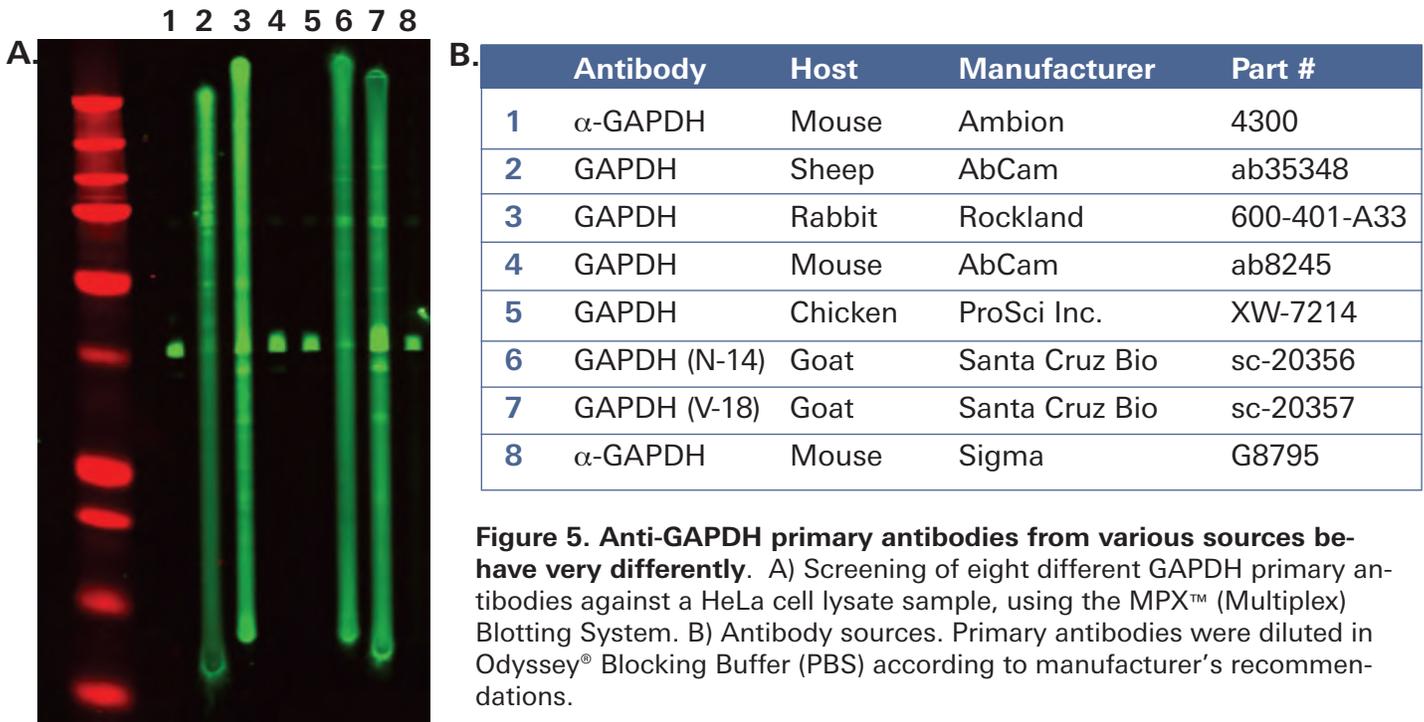
A.

B.

	Antibody	Host	Manufacturer	Part #
1	α-GAPDH	Mouse	Ambion	4300
2	GAPDH	Sheep	AbCam	ab35348
3	GAPDH	Rabbit	Rockland	600-401-A33
4	GAPDH	Mouse	AbCam	ab8245
5	GAPDH	Chicken	ProSci Inc.	XW-7214
6	GAPDH (N-14)	Goat	Santa Cruz Bio	sc-20356
7	GAPDH (V-18)	Goat	Santa Cruz Bio	sc-20357
8	α-GAPDH	Mouse	Sigma	G8795

Figure 5. Anti-GAPDH primary antibodies from various sources behave very differently. A) Screening of eight different GAPDH primary antibodies against a HeLa cell lysate sample, using the MPX™ (Multiplex) Blotting System. B) Antibody sources. Primary antibodies were diluted in Odyssey® Blocking Buffer (PBS) according to manufacturer's recommendations.

F. Secondary Antibody Quality

Fluorescent Western blot methods and the Odyssey® Imager can detect two protein 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 IgG and anti-mouse IgG secondary antibodies). One secondary antibody must be labeled with IRDye® 680LT or IRDye 680RD, and the other with IRDye 800CW. IRDye Subclass-Specific Antibodies offer a special exception to this rule. IRDye Goat anti-Mouse IgG_1, Goat anti-Mouse IgG_{2a}, and Goat anti-Mouse IgG_{2b} subclass-specific antibodies enable two-color detection of monoclonal primary antibodies derived from the same species (mouse). IRDye Subclass-Specific antibodies react only with the heavy (gamma) chain of the primary antibody. For details and a complete description, refer to Western Blot and In-Cell Western™ Assay Detection Using IRDye Subclass-Specific Antibodies. (*www.licor.com/subclass*).

Always use highly cross-adsorbed secondary antibodies for two-color detection. Failure to use cross-adsorbed antibodies may result in increased cross-reactivity (spurious bands that appear in the other detection channel; see Figure 6). LI-COR IRDye conjugated secondary antibodies are highly cross-adsorbed and optimized for two-color Western blot detection.
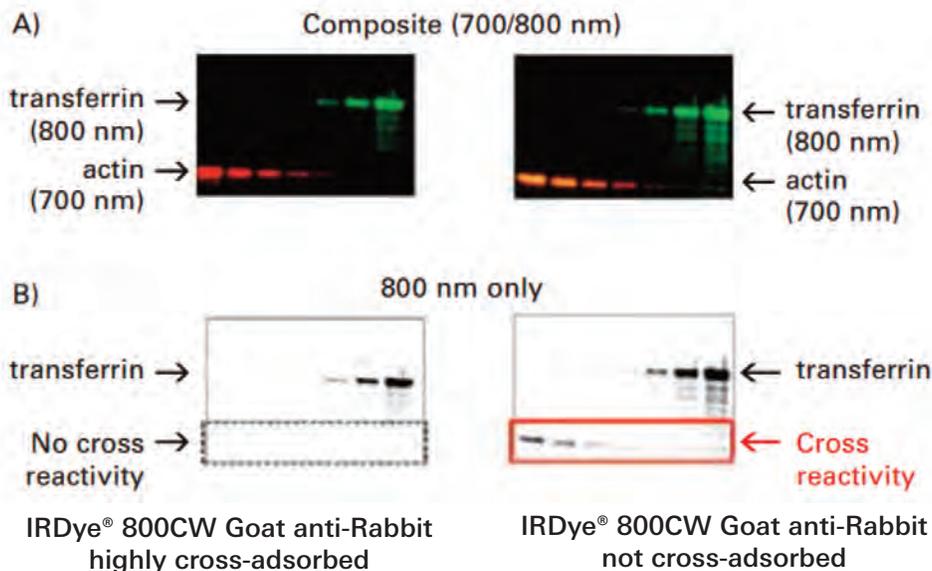
Figure 6. Highly cross-adsorbed secondary antibodies eliminate cross-reactive bands on multiplex Western blots. A) Actin was detected with mouse anti-actin primary and goat anti-mouse secondary antibody (700 nm; red). Transferrin was detected with rabbit anti-transferrin and goat anti-rabbit IRDye 800CW secondary antibody (LI-COR; 800 nm, green). Odyssey® Classic Imager was used, with detection sensitivity setting = 1.5 for both channels. Goat anti-Rabbit secondary antibody was highly cross-adsorbed (left; LI-COR product) or not cross-adsorbed (right; obtained from alternate supplier). B) In the 800 nm image, the LI-COR cross-adsorbed secondary antibody (left) shows no cross-reactivity with the mouse anti-actin primary. The non-cross-adsorbed antibody (right) cross-reacts with mouse anti-actin, generating spurious bands (which appear yellow in the composite image (A)).

Many types of secondary antibodies may be used for Western blot detection. The MPX™ (Multiplex) Blotting System can be used to choose the appropriate secondary antibody and optimal dilution. Figure 7 demonstrates the performance of LI-COR IRDye 800CW Goat anti-Mouse IgG compared to various other secondary antibody options for detection of a mouse IgG primary antibody.
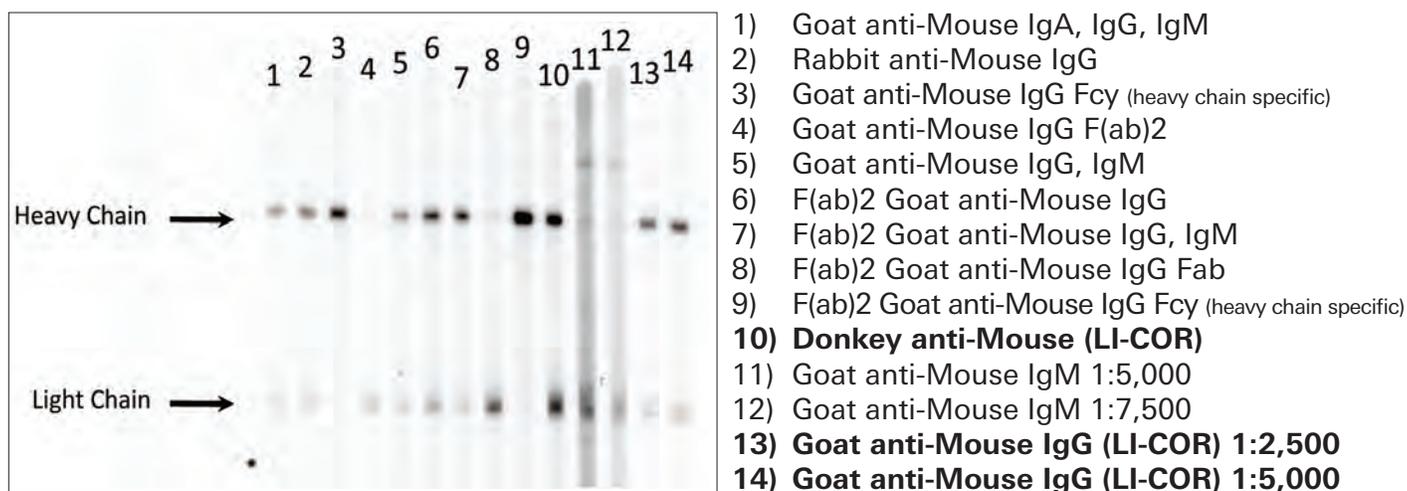

1) Goat anti-Mouse IgA, IgG, IgM
2) Rabbit anti-Mouse IgG
3) Goat anti-Mouse IgG Fcγ (heavy chain specific)
4) Goat anti-Mouse IgG F(ab)2
5) Goat anti-Mouse IgG, IgM
6) F(ab)2 Goat anti-Mouse IgG
7) F(ab)2 Goat anti-Mouse IgG, IgM
8) F(ab)2 Goat anti-Mouse IgG Fab
9) F(ab)2 Goat anti-Mouse IgG Fcγ (heavy chain specific)
10) Donkey anti-Mouse (LI-COR)
11) Goat anti-Mouse IgM 1:5,000
12) Goat anti-Mouse IgM 1:7,500
13) Goat anti-Mouse IgG (LI-COR) 1:2,500
14) Goat anti-Mouse IgG (LI-COR) 1:5,000

Figure 7. **Evaluation of secondary antibodies with the MPX Multiplex Blotting System.** Mouse IgG primary antibody was electrophoresed and blotted to nitrocellulose membrane. Various secondary antibodies were labeled with IRDye 800CW, and compared to IRDye 800CW Donkey anti-Mouse IgG (lane 10) and IRDye 800CW Goat anti-Mouse IgG (lanes 13 and 14). Secondary antibodies were used at a 1:5,000 dilution unless otherwise indicated. Data were generated using the MPX (Multiplex) Blotting System and Odyssey Classic Imager.

G. Secondary Antibody Dilution

The amount of secondary antibody used for NIR Western blot detection can vary widely. When using LI-COR IRDye® 800CW and IRDye 680RD 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 1:20,000 to 1:50,000. The dilution should be optimized for the primary antibody being used and the preferred appearance of the Western blot. Image Studio™ or Odyssey® imaging software can be used to optimize the appearance of the image, for a variety of secondary antibody dilutions (Figure 8). Higher dilutions (1:20,000 – 1:100,000) provide lower membrane background and fewer background bands than 1:1,000 or 1:5,000 dilutions (Figure 8).

If too much secondary antibody is used, bands may be very strong and may show saturation of signal (white pixels in Fig. 8A). Re-imaging the blot at a lower scan intensity setting can often relieve saturation (Fig. 8B). Image display settings can be adjusted to optimize the brightness and contrast, and make background bands less visible. In Figure 8B, image display adjustments were used to make all four blots appear similar to the eye. However, image display adjustments affect visual presentation only and do not alter the raw data in any way.
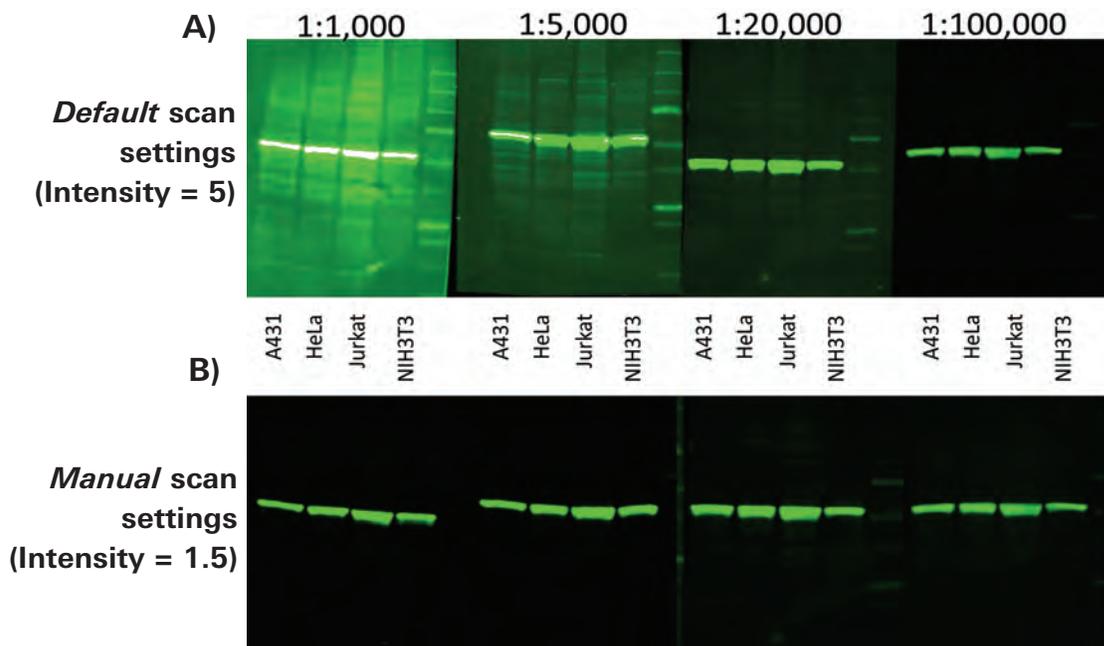

Figure 8. **Effect of secondary antibody dilution on image data.** IRDye 800CW secondary antibody (LI-COR) was used at the dilutions shown, to detect the target protein in various cell lysates. A) Blots were imaged with the Odyssey Classic system with the default scan settings (intensity = 5). The 1:1,000 antibody dilution shows saturation of strong bands (left; white pixels in the bands). Higher dilutions do not show saturation. Increased background banding is observed with 1:1,000 and 1:5,000 dilutions. All images were displayed with "Auto" image display settings. B) Manual scan settings were used to re-image the same blots with Odyssey Classic (Intensity = 1.5). The lower scan intensity setting eliminates saturated pixels. Image display settings were optimized for each individual blot.

H. Miscellaneous Contamination

Many factors can cause contamination and increase the background levels of a near-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 9.
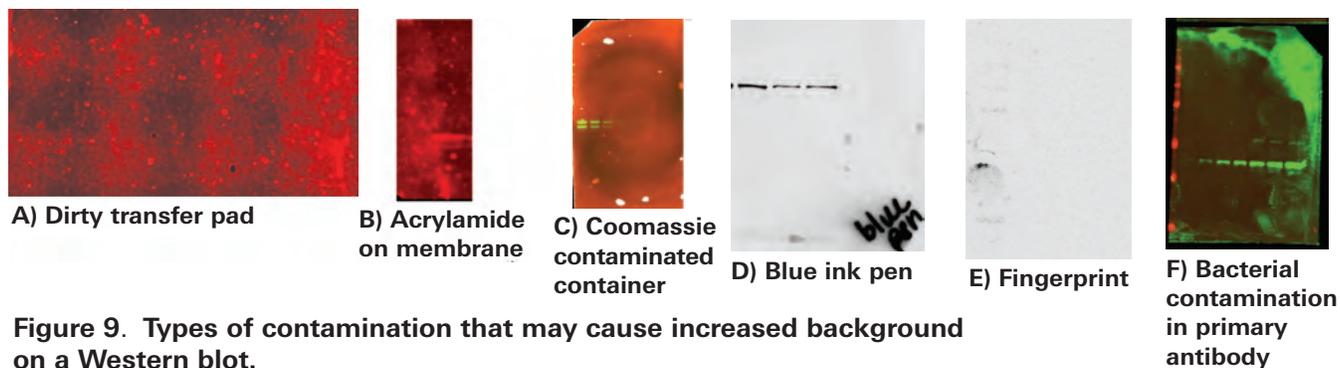

A) Dirty transfer pad   B) Acrylamide on membrane   C) Coomassie contaminated container   D) Blue ink pen   E) Fingerprint   F) Bacterial contamination in primary antibody

Figure 9. Types of contamination that may cause increased background on a Western blot.

Table 1.

Contamination Source	Appearance	Solution
Blue loading buffer used during gel electrophoresis	Smeared signal in the 700 nm channel	Use LI-COR 4X Protein Sample Loading Buffer (P/N 928-40004).
Dirty transfer pads	Blotches can be seen on the blots that align with the transfer cassette holes	Replace transfer pads.
Acrylamide residue on membrane after transfer	Speckles and blotches can be seen in 700/800 nm channels	Carefully rinse off membrane in 1X PBS before it dries.
Blue pen used on membrane	Smeared signal in the 700 nm channels	Use pencil to mark blots.

Dirty processing containers:
  1. Coomassie Stain/gel stain/anything blue
 	
  2. Bacterial Growth
 	
  3. Acrylamide Residue
	1. In the 700 nm channel, entire membrane dark, smeared signal, or speckles, depending on the amount of stain residue in container.
	
	2. Speckles and blotches can be seen in 700/800 nm channels.
	
	3. Speckles and blotches can be seen in 700/800 nm channels.
		1. Use different containers for gel staining and Western blot detection.
		
		2. Wash containers with detergent, rinse thoroughly with distilled water, and a final rinse with methanol.
		
		3. Wash containers as indicated above.

Fingerprints	Blotches can be seen in 700/800 nm channels where gloved/ungloved hands have touched the membrane.	Handle Western membrane with clean forceps only.
Dirty Forceps	Blotches can be seen in 700/800 nm channels where forceps have touched the membrane.	Do not use rusty forceps. Forceps can be washed with detergent, rinsed with water, and a final rinse with methanol.
Bacterial growth in Antibodies (primary or secondary)	Speckles and blotches can be seen in 700/800 nm channels.	Replace antibodies.

III. Imaging Factors That Can Affect Western Blot Results

A. Handling of the blot

- ALWAYS start with a clean scan bed or imaging tray. If you capture an image and any empty area (not covered by membrane) shows 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 residual dye.
- Air bubbles can cause reduced signal detection during imaging. Flatten the membrane with a roller (such as LI-COR P/N 926-71000) to remove bubbles and excess liquid between the blot and the scan surface. See Figure 10.

Figure 10. **Air bubbles affect image appearance.**
Examples of bubbles in the transfer sandwich or on the Odyssey® Imager scan bed.

- A Western blot can be imaged either wet or dry on any Odyssey Imaging System. Typically, the signal is higher when a dry blot is imaged; however, background levels will increase. See Figure 11.
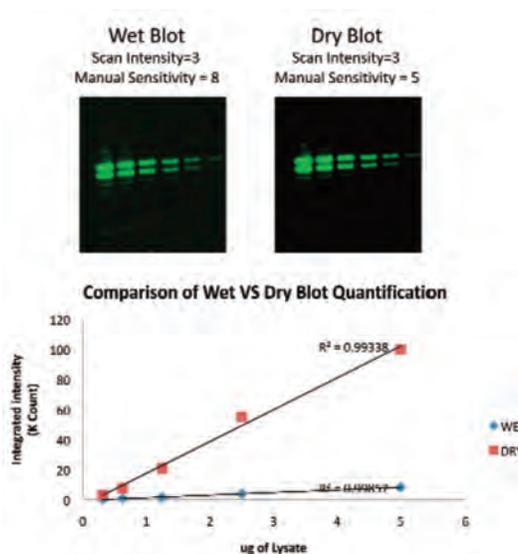 *NOTE: Once a blot has dried, or partially dried, stripping of the membrane for reuse cannot be performed.*

Figure 11. **Scanning of wet and dry Western blots**. The same Western blot was scanned wet and dry with an Odyssey Classic Imager. Optimal image display settings are shown. Quantification (graph below images) demonstrates higher signal intensity on the dry blot.

B. Imaging Parameters

Odyssey® Classic, Odyssey CLx, and Odyssey Sa

Focus Offset – Improper adjustment of the Focus Offset can result in reduced signal collection from the membrane. The Focus Offset should be set at 0 mm for scanning a Western blot. For details, see the *User Guide*.

Scan Intensity – Improper optimization of the **Scan Intensity** can result in saturation of signal and reduced linear dynamic range. Figure 12 demonstrates how changes in the Scan Intensity setting affect the resulting signals on the Odyssey Classic Imager.
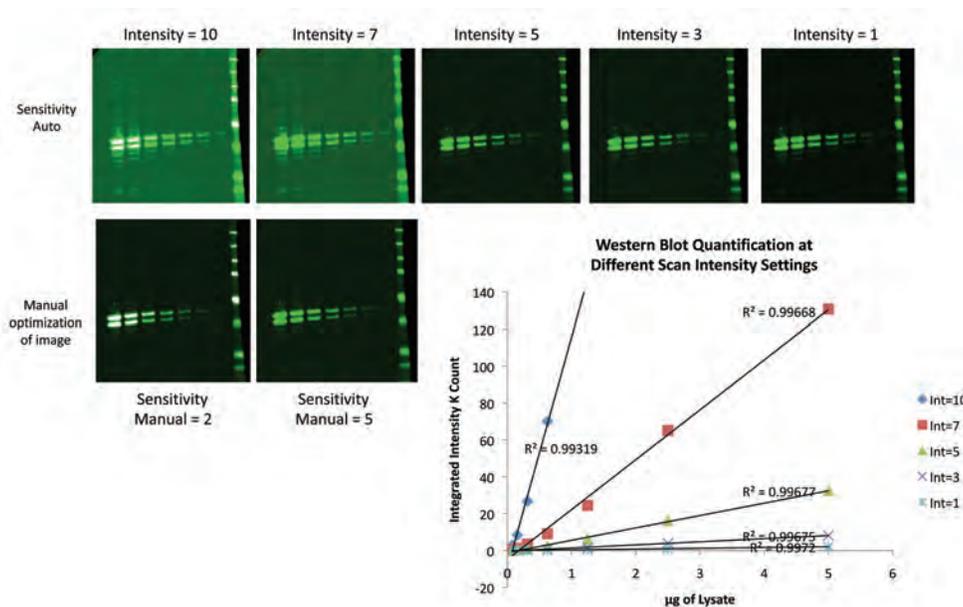
Figure 12. **Effect of Scan Intensity settings on Western blot signals.** The same Western blot was scanned at 5 different intensity settings with the Odyssey Classic Imager. A) Images shown with Auto Sensitivity settings for image display. Scan intensity setting is indicated above each imager. B) The same images (Intensity=10 and Intensity=7) are shown with optimized image display settings. C) Quantification of signals from the blots in A. Note that saturated signals at Intensity=10 (white pixels) cannot be quantified.

AutoScan function – The **AutoScan** imaging feature of the Odyssey CLx system is demonstrated in Figure 13. When Scan Intensity settings are manually adjusted, multiple scans may be required to generate an image without signal saturation. The Auto Intensity function automatically optimizes scan intensity to generate an image with no saturation (Figure 13A). For details, see the *Help System* of Image Studio™ Software.
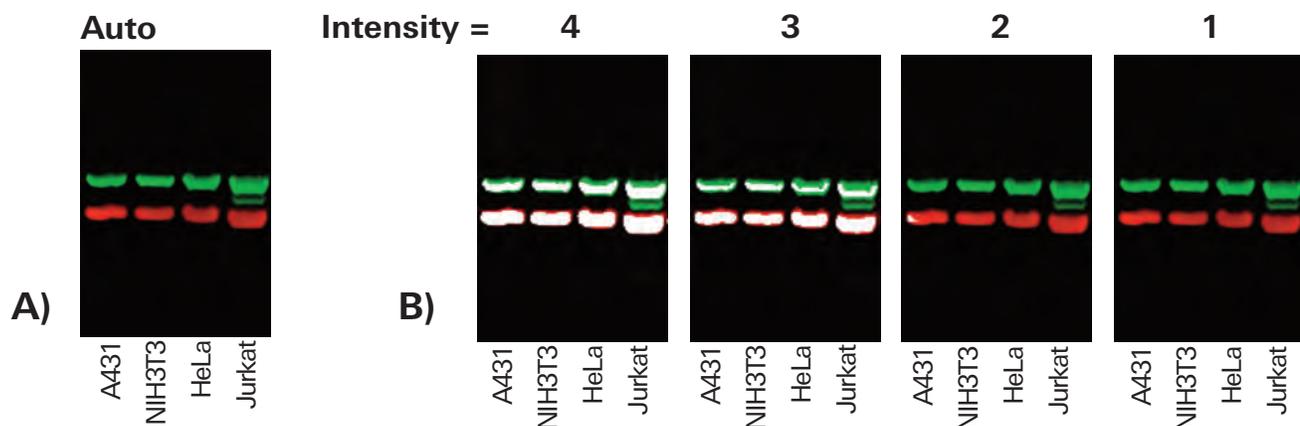
Figure 13. **The AutoScan function prevents signal saturation.** A) A two-color Western blot was imaged with the Odyssey® CLx system, using the AutoScan feature. No saturated signals were observed. B) The same blot was imaged repeatedly with manual Scan Intensity settings, beginning with Intensity=4. Pixel saturation is shown in white. Four separate scans were required to generate the desired image. In these lysates, tubulin (green bands) was detected with rabbit anti-Tubulin and IRDye® 800CW Goat anti-Rabbit IgG (LI-COR P/N 926-32211); actin (red bands) was detected with mouse anti-actin and IRDye 680LT Goat anti-Mouse IgG (LI-COR P/N 926-68051).

It is important to note that saturated pixels (which appear white in these pseudo-color images) cannot be accurately quantified. Signal saturation can also result in "crosstalk" (detection of signal in the alternate fluorescence channel). This is easily eliminated by scanning at a lower intensity setting.

Odyssey® Fc – The Odyssey Fc Imaging System is optimized to automatically acquire Western blot images without saturated pixels, and no user adjustments are needed.

IV.   Software Adjustments for Image Optimization

There are two common problems that can be corrected with a few adjustments of the software.

*   Blots that exhibit **no fluorescence**
*   Blots with **dim bands**

These software enhancements will not correct issues that result from binding chemistry problems (blocking buffer, antigen, or antibody).

**Odyssey Classic (ver. 1.x – 3.x application software) and
Odyssey Sa (ver. 1.x application software)**

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 **View** menu, then **Alter Image Display** menu. To enhance the image, simply click the **Linear Manual** radio button and adjust the slider. By manually adjusting the sensitivity settings, the most desirable image can be chosen. Image display adjustments affect appearance only, and do not alter the raw data. For details, see the *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 5. Adjust **Brightness** and **Contrast** sliders until the image is optimal. Each fluorescence channel can be adjusted independently. Image adjustments can also be made in grayscale, and very faint bands are visualized more easily when bands are displayed in black against a white background. For details, see the *User Guide*.

Odyssey® Classic, Odyssey CLx, and Odyssey Fc (Image Studio™ Software, ver. 1.x and higher)

No Fluorescence – Click on the Auto **Adjust button** in the **Image Look-Up-Tables (LUTs)** Tab. For details, see the *Help System*.

Dim Bands – Click and drag the **min, max,** and **K value** dots on the histogram or adjust the Brightness and Contrast slider bars (**Image LUTs** tab) to adjust the intensity of the image. Each channel can be adjusted independently. Image adjustments can be shown in grayscale and pseudo-color. Very faint bands are visualized more easily when black bands are displayed on a white background. For details, see the *Help System*.
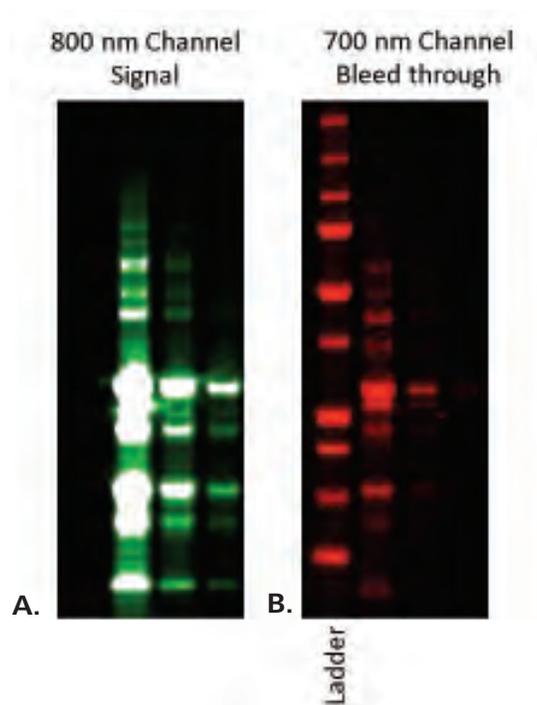

Figure 14. Saturated signal in the 800 nm channel (A) of the Odyssey Classic Infrared Imaging System can be visualized in the 700 nm channel (B). The only signal that should be seen in the 700 nm channel is the ladder on the far left of the image. The bands in the adjoining lanes are due to saturation in the 800 nm channel. Optimizing scan intensity can eliminate this.

V. Data Analysis with Image Studio™ Software: Background Subtraction

Image Studio (ver. 1.x and higher)

The *Background settings* tool is located in the **Background** group on the **Analyze** ribbon. To implement **Background** subtraction in Image Studio software, you can specify a localized background using the Median or Average. This method will use the area surrounding each band shape (top/bottom, left/right or all) to calculate the background. Alternatively, a user-defined background may be used, in which you draw one or more shapes over an area of typical background. Select the shape(s) and click **Assign Shape** in the Background group in the Analyze ribbon.

With the **Western Key**, the Background group on the Western and MPX™ Western Analysis ribbons includes the option of Lane background subtraction. This setting subtracts the background of the Lane from each Band.

VI. Data Analysis with Odyssey® Applications Software: Background Subtraction

Odyssey Classic or Odyssey Sa (ver. 3.x application software)

For accurate Western blot quantification, the **Background** setting must be applied appropriately. This tool measures the intensity of the pixels selected as the background region. It is important to choose the most suitable Background calculation for your blot. There are several methods for background subtraction, and each method is 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 blot experiments.
 ii. **Average Background** takes the average value of pixels on all four sides of the feature. A subset of sides (**All, Top/Bottom**, or **Right/Left**) can be selected to further optimize quantification. This customized background subtraction approach is used to address issues such as smears in lanes, or bands that are very close together. 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.
 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 saturated pixels), select the feature, choose the **Background** icon from the toolbar, and change the background method to **User Defined**. Click **Save**, and then **OK**. Notice that the feature is now designated as 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.

VII. Summary

There are many ways to maximize the performance of a Western blot. Full optimization of your membrane and detection reagents is the best place to start. LI-COR provides high-quality reagents designed for optimal Western blot detection with the Odyssey® Family of Imaging Systems. For a detailed Western blot protocol for your Odyssey Imager, see the *Western Blot Analysis* (*www.licor.com/wbanalysis*) protocol.

VIII. References

1. Towbin, et al., (1979) *Proc. Natl. Acad. Sci.* USA 76; 4350-54.
2. Ambroz, et al., (2008) *Proteomics* 8; 2379-83.
3. DenHollander and Befus, (1989) *J Immunol Methods* 122(1); 129-35.