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.

LI-COR®
Biosciences

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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. 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.

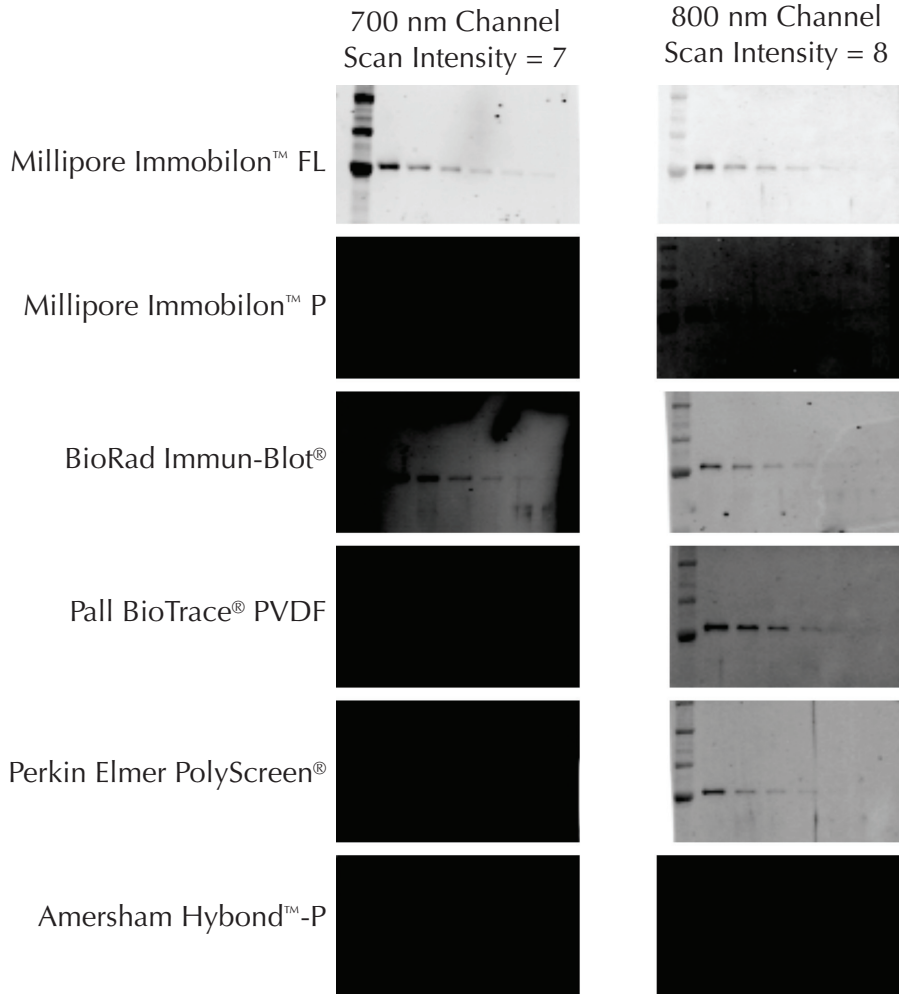


Figure 1. Western blot detection of transferrin using various vendors and brands of PVDF membrane on the Odyssey Infrared Imaging System in both 700 and 800 nm channels.

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.

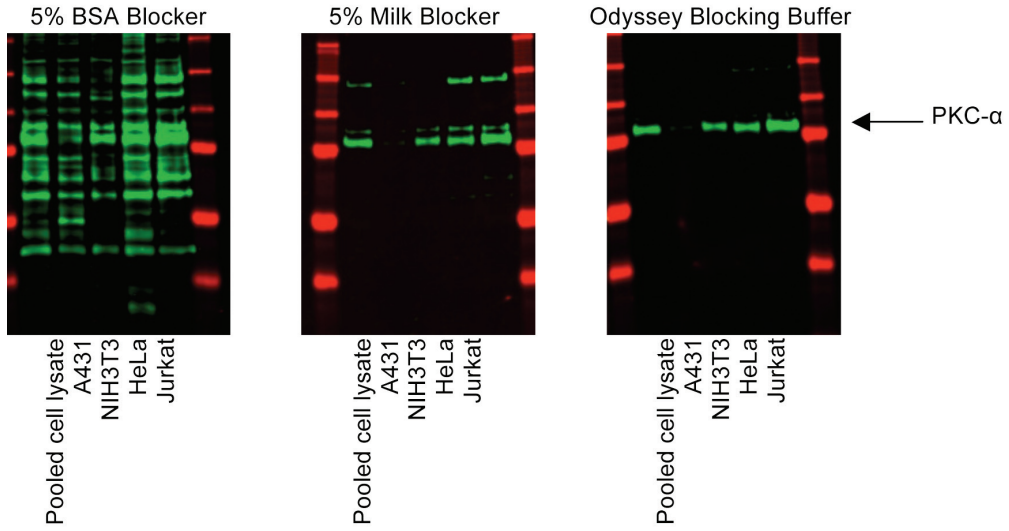


Figure 2. Western blots detected with anti-PKCα and IRDye® 800CW Goat anti-mouse. All blots were treated equally, with the exception of blocking reagent. All images were generated on the Odyssey® Infrared Imager with scan intensity setting of 5, sensitivity of 5.

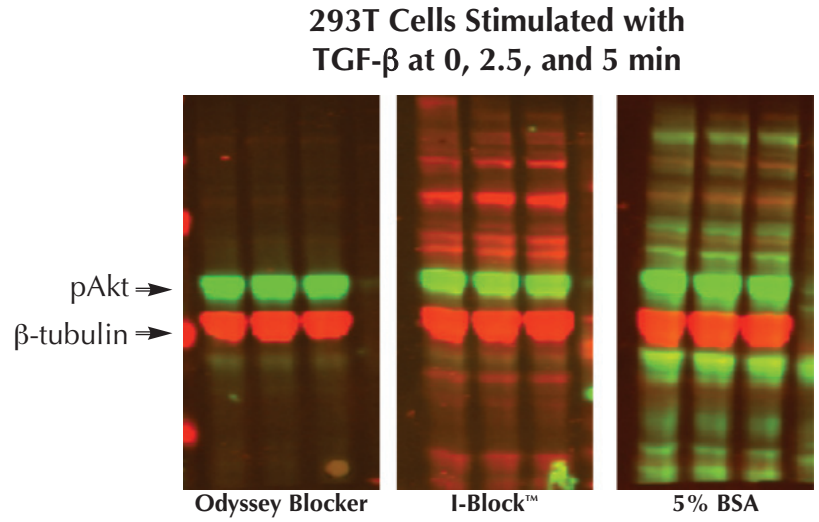


Figure 3. Western blots of 293T Cells stimulated with TGF-β (0, 2.5, and 5 minutes) detected with anti-pAkt and β-tubulin. All blots were treated equally, with the exception of blocking reagent. All images were generated on the Odyssey Infrared Imager with scan intensity setting of 3.5/5 (700/800 nm), sensitivity of 5.

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 deter gent. Never expose the membrane to detergent until blocking is complete, as this may cause high membrane background.

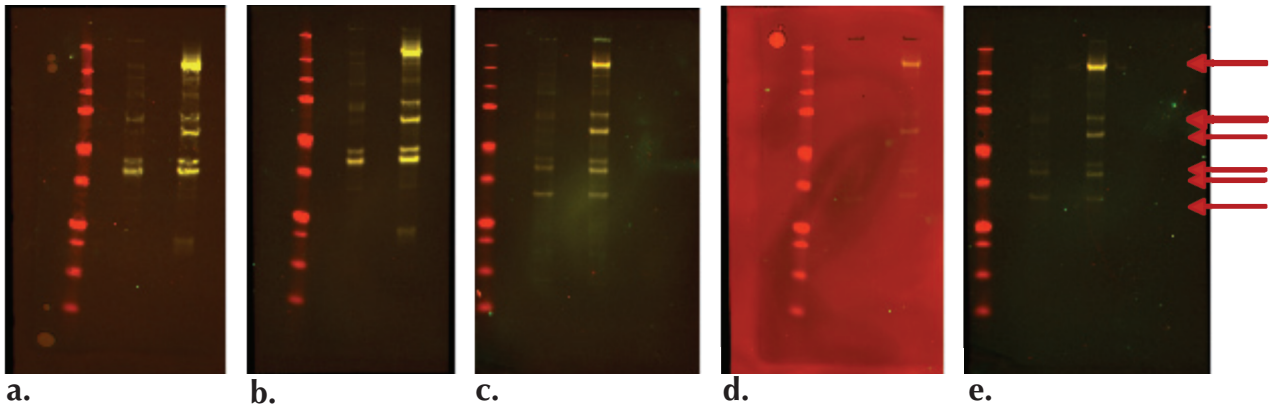
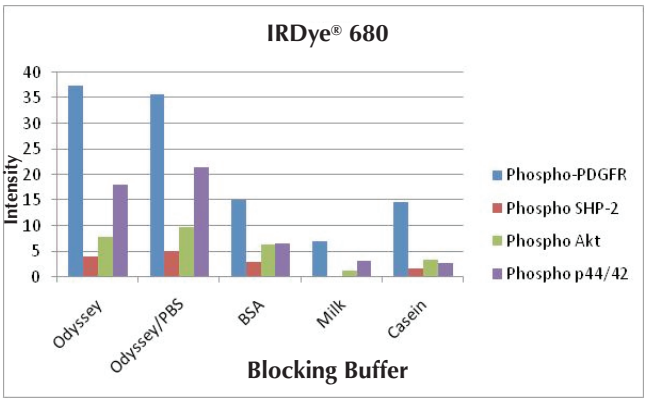
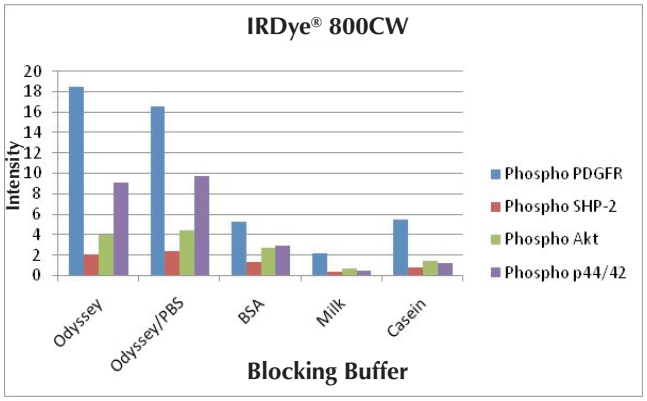


Figure 4. Above: Western blots utilizing PathScan Multiplex primary antibody and both IRDye® 680 and IRDye 800CW goat anti-rabbit for detection. Five different solutions were used for blocking and antibody dilution (antibody dilutions included 0.2% Tween® 20):
a. Odyssey® Blocking Buffer;
b. Odyssey + PBS (1:1);
c. 5% BSA;
d. 5% Skim Milk;
e. 0.5% Casein.
In each image, the arrows indicate the band positions for each of the detected proteins. Starting from the top: Phospho-PDGFR, phospho-SHP2, phospho-Akt, phospho-p44/p42, and S6.
f. Quantification of 700 nm signal in each blocking solution.
g. Quantification of 800 nm signal in each blocking solution.



f.



g.

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

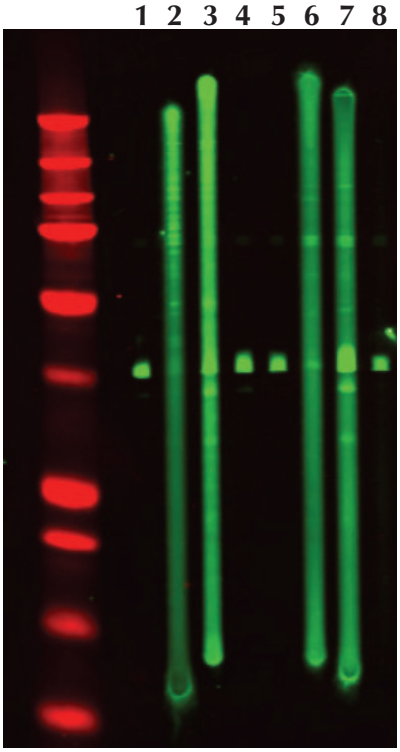
An antibody produced to detect a specific antigen is called the primary antibody. It binds directly to the molecule of interest. Primary antibodies can be produced in a wide variety of species such as mouse, rabbit, goat, chicken, rat, guinea pig, human, and many others. Primary antibodies for the same 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 IgG_1, Goat anti-Mouse IgG_{2a}, and Goat anti-Mouse IgG_{2b}, 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; IgG_1, IgG_{2a}, IgG_{2b}, IgG_{2c}, and IgG_3. 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 IgG_1 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.



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. 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.

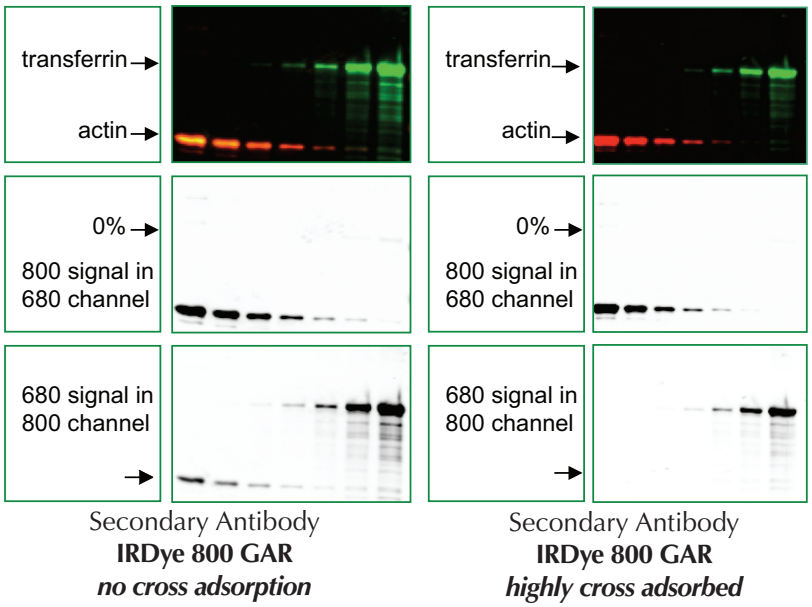


Figure 6. Example of a secondary antibody not cross adsorbed, cross-reacting with the second antibody pair in a two-color Western blot.

There are many choices in secondary antibodies for Western blot detection. LI-COR offers IRDye whole IgG (H + L) secondary antibodies and IRDye Subclass 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.

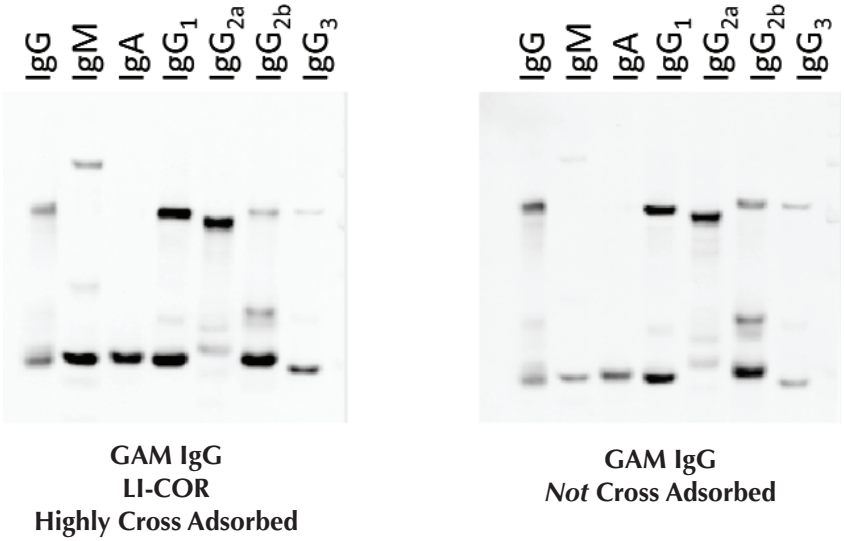


Figure 7. Mouse IgG Subclass detection comparing LI-COR IRDye goat anti-mouse antibody to a similar antibody that was not cross-adsorbed.

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).

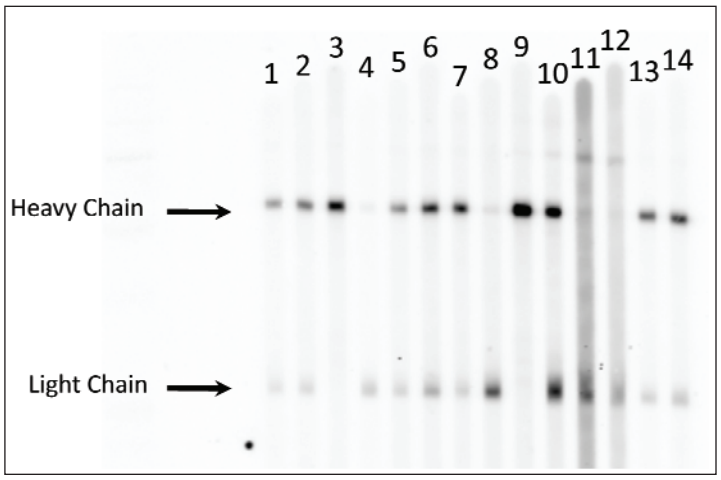
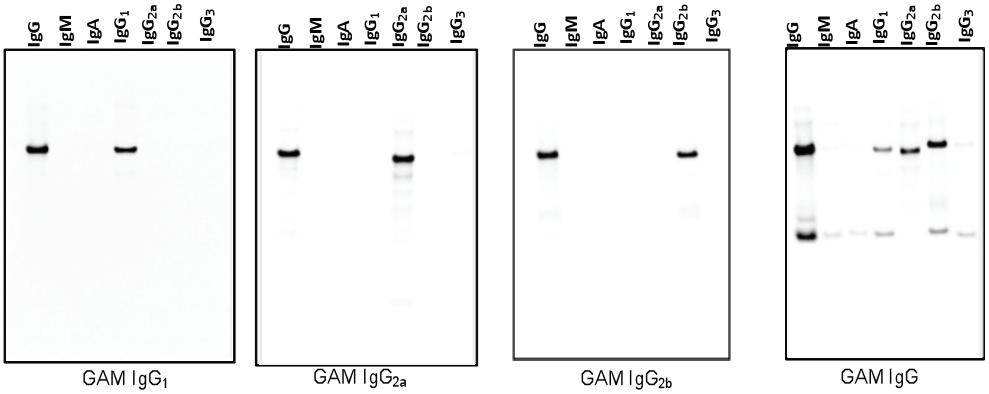


Figure 8. IRDye® 800CW labeled anti-mouse antibodies against purified mouse IgG.

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



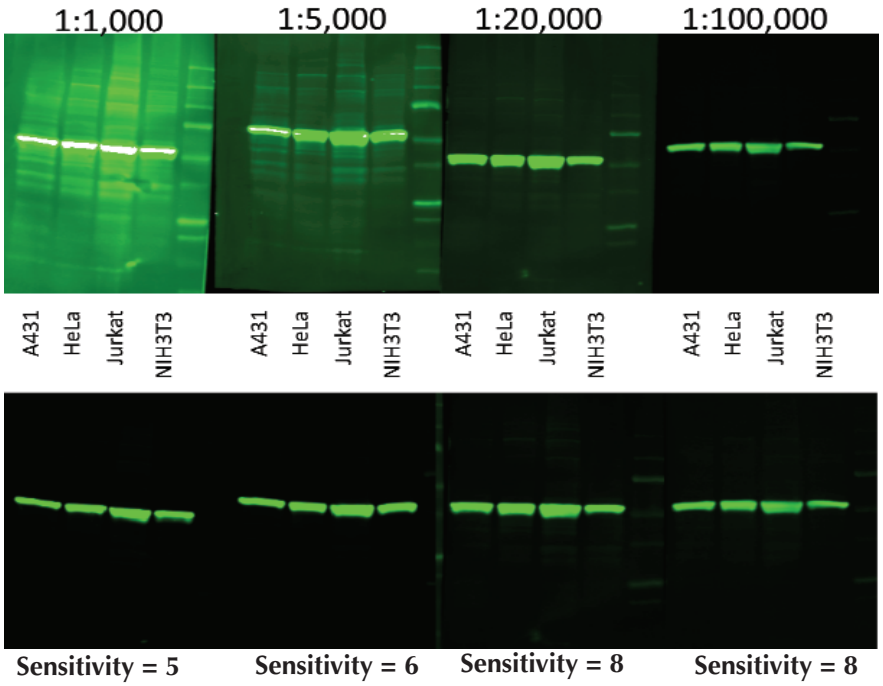


Figure 10. Secondary Antibody Concentration of IRDye 800CW goat anti-mouse with maximized Odyssey imaging capabilities.

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.

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 (Part #928-40004).
Dirty transfer pads	Blotches can be seen on the blot 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 channel	Carefully rinse off membrane in 1X PBS before it dries.
Blue pen used on membrane	Smeared signal in the 700 nm channel	Use pencil to mark blots.
Dirty processing containers: 1. Coomassie Stain/gel stain/ anything blue	1. In the 700 nm channel, entire membrane dark, smeared signal, or speckles, depending on the amount of stain residue in container.	1. Use different containers for gel staining and Western blot detection.
2. Bacterial Growth	2. Speckles and blotches can be seen in 700/800 nm channel.	2. Wash containers with detergent, rinse thoroughly with distilled water and a final rinse with methanol.
3. Acrylamide Residue	3. Speckles and blotches can be seen in 700/800 nm channel.	3. Wash containers as indicated above.
Fingerprints	Blotches can be seen in 700/800 nm channel 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 channel 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 channel.	Replace antibodies.

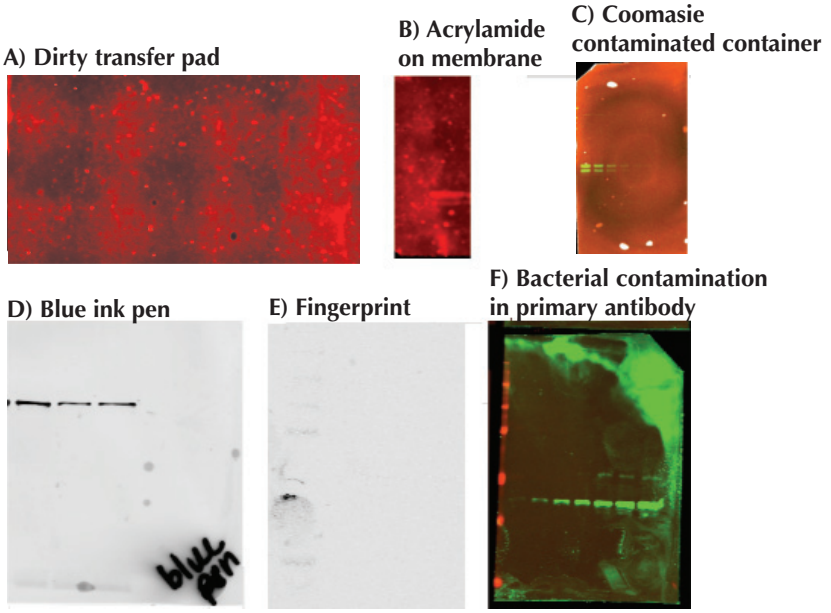


Figure 11. Examples of contamination events that may cause background on a Western blot.

Figure 12. Examples of air bubbles in the transfer vs. on the Odyssey® Infrared Imager scan bed.

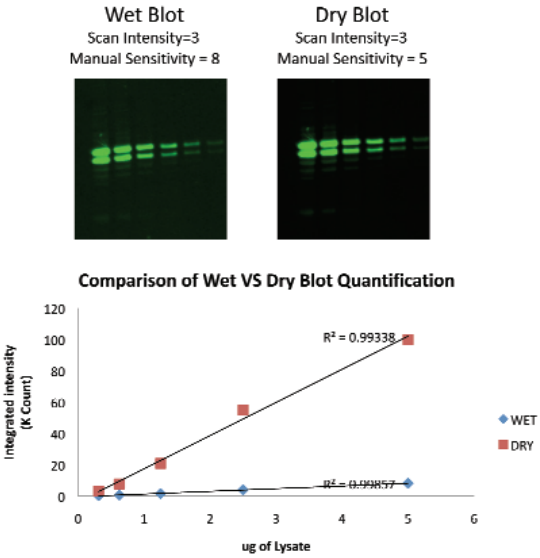


Figure 13. The same Western blot scanned on an Odyssey system wet and dry. The images are represented using the optimal display settings. Quantification is shown in the chart below the images.

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.

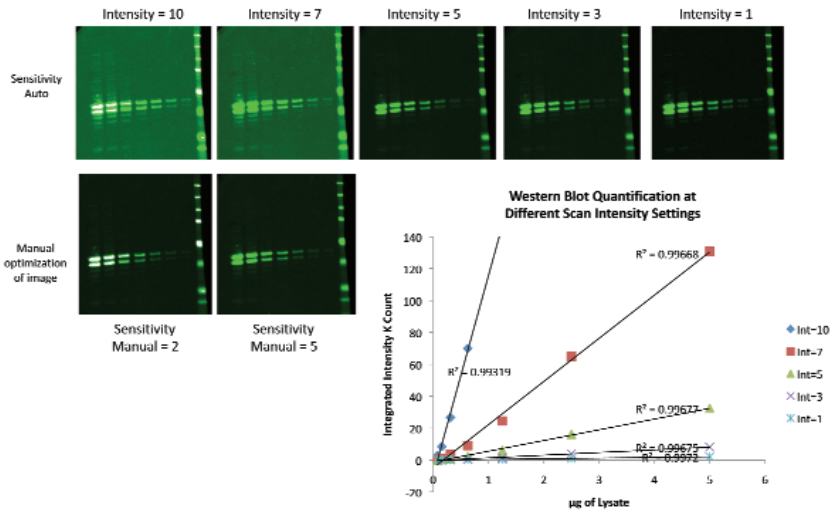


Figure 14. The same Western blot scanned on the Odyssey Infrared Imaging System at 5 different intensity settings. The top row of images are displayed using the auto sensitivity setting in the Odyssey Software. The bottom images were optimized using the manual sensitivity option for display. Quantification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

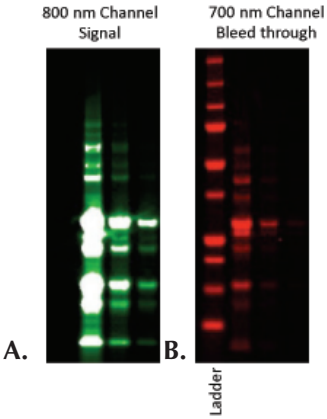


Figure 15. Saturated signal in the 800 nm channel (A) of the Odyssey Infrared Imaging System can be visualized in the 700 nm channel (B). The only detection that should be seen in the 700 nm channel is the ladder on the far left of the image. Optimizing scan intensity can eliminate this.

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

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

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