Application Guide

Good Westerns Gone Bad

Tips to Make Your Near-Infrared Western Great

Published December 2008. Revised March 2020. The most recent version of this document is posted at licor.com/bio/support.

Table of 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. Key Factors That Affect Western Blot Results

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.

Test Membrane Background

Before using a membrane for the Western blot protocol, cut a small sample of membrane to test the background of the membrane by itself.

- Image this sample (both wet and dry) to evaluate the level of membrane autofluorescence.
- If possible, include a sample of membrane that is known to work well with the Odyssey Imaging System, so you can compare background levels.

Membrane Comparison

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.

  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.

- **Nitrocellulose** membranes generally offer the lowest membrane autofluorescence.

> **Note:** LI-COR has not evaluated all sources of PVDF and nitrocellulose membranes, and lot-to-lot variation can occur. It is important for you to evaluate membranes before you use them.

PVDF Membrane Comparison

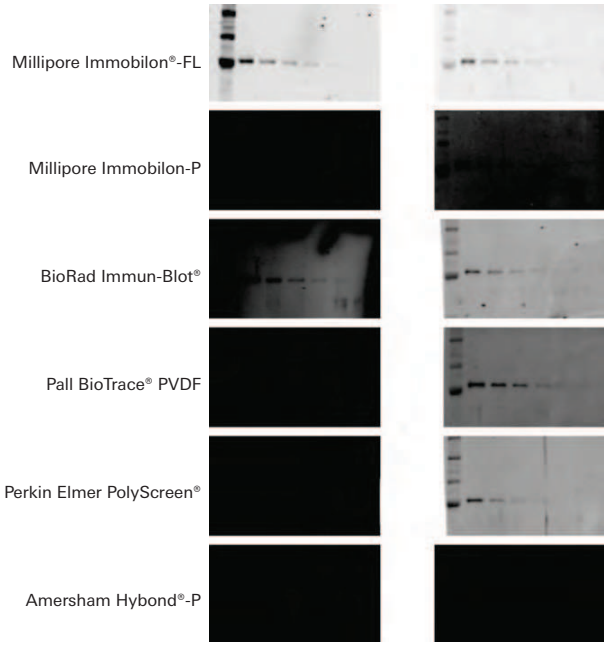


Figure 1. Membrane autofluorescence from PVDF affects Western blot performance. Transferrin was detected by Western blotting, using various vendors and brands of PVDF membrane. Blots were imaged with the Odyssey Classic Infrared Imaging System in both 700 and 800 nm channels.

Blocking Reagent

No single blocking reagent will be suitable for all antigen-antibody pairs.

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 1). 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).

If an antibody fails with one blocking condition, trying another blocker may solve the problem.

Milk-Based Blocking Buffer

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. It is best not to use milk-based blocking buffers when detecting phospho-targets or other post-translational modifications.

TBS vs PBS 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.

Be sure to keep your buffer system consistent throughout the protocol for blocking, antibody dilutions, and washes. For example, if you use a TBS-based buffer system, choose Intercept® (TBS) Blocking Buffer. If you use a PBS-based buffer system, choose Intercept® (PBS) Blocking Buffer.

Detergents

Adding detergents to diluted antibodies can significantly reduce background on the blot.

- **The optimal detergent concentration will vary**, depending on the antibodies, membrane type, and blocker used. Keep in mind that some primary antibodies do not bind as tightly as others and may be washed away by too much detergent.
- It is generally best not to expose the membrane to detergent until blocking is complete, as this may cause high membrane background.

Tween® 20

- Blocking buffer – It is best not to 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 and nitrocellulose membranes.
- Wash solutions should contain 0.1% Tween 20.

SDS

When using a PVDF membrane, adding SDS will dramatically reduce overall membrane background in the 700 nm channel. 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.

> **Important:** SDS should not be used with nitrocellulose membranes.

- Do **not** add SDS to the blocking reagent during blocking.
- Diluted primary antibodies should **not** contain SDS.
- Wash solutions should **not** contain SDS.
- When using a PVDF membrane, your secondary antibody diluent should contain a final concentration of 0.01 – 0.02% SDS and 0.1 – 0.2% Tween® 20.

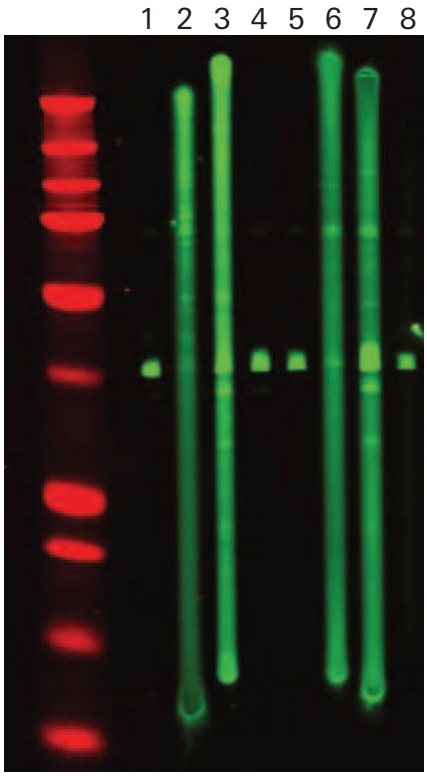
Primary Antibody

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.

Primary Antibody Variation Example

Figure 2 is an example of how different primary antibodies may react.

A)



B)

	Antibody	Host	Manufacturer	Part Number
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 2. Anti-GAPDH primary antibodies from various sources behave very differently. A) Screening of eight different GAPDH primary anibodies against a HeLa cell lysate sample, using the MPX™ Multiplexer Blotting System. B) Antibody sources. Primary antibodies were diluted according to manufacturer's recommendations.

Secondary Antibody

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* (licor.com/subclass).

Highly Cross-Adsorbed Secondary Antibodies

Not using cross-adsorbed antibodies may result in increased cross-reactivity (spurious bands that appear in the other detection channel, see Figure 3). LI-COR IRDye conjugated secondary antibodies are highly cross-adsorbed and optimized for two-color Western blot detection.

Example of Highly Cross-Adsorbed Secondary Antibodies

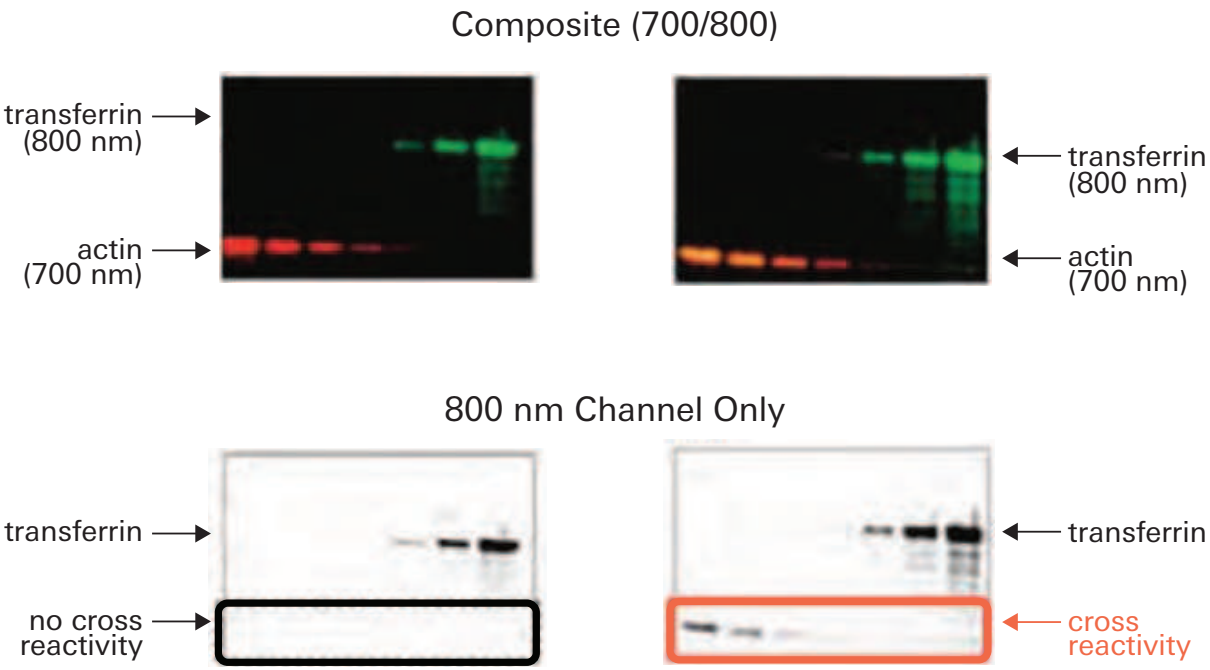
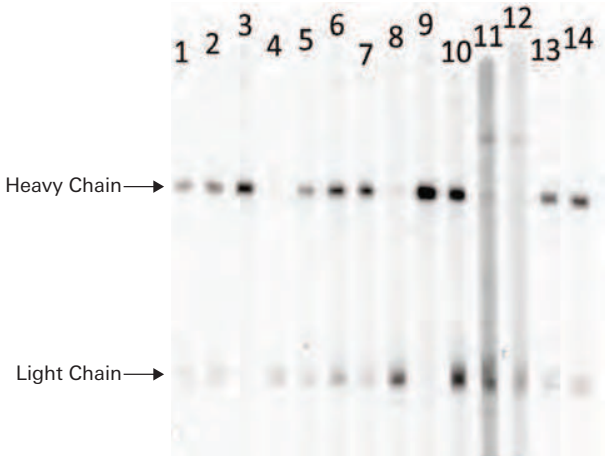


Figure 3. Highly cross-adsorbed secondary antibodies eliminate cross-reactive bands on multiplex Western blots. A) Actin was detected with mouse anti-actin primary and goat anti-mouse secondary antibody (700 nm; red). Transferrin was detected with rabbit anti-transferrin and goat anti-rabbit IRDye 800CW 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).

MPX Multiplexer Blotting System for Testing Secondary Antibodies

Many types of secondary antibodies may be used for Western blot detection.

The MPX™ Multiplexer Blotting System can be used to choose the appropriate secondary antibody and optimal dilution. Figure 4 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 4. Evaluation of secondary antibodies with the MPX Multiplexer 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 Multiplexer Blotting System and an Odyssey Classic Imager.

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. The recommended starting dilution is 1:20,000.
- When using LI-COR IRDye 680LT secondary antibodies, the recommended dilution range is 1:10,000 – 1:40,000.

The dilution should be optimized for the primary antibody being used and the preferred appearance of the Western blot. Image Studio™ Software can be used to optimize the appearance of the image, for a variety of secondary antibody dilutions (Figure 5). 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 5).

> **Note:** If too much secondary antibody is used, bands may be very strong and may show saturation of signal (white pixels in Figure 5). Image display settings can be adjusted to optimize the brightness and contrast, and make background bands less visible. However, image display adjustments affect visual presentation only and do not alter the raw data in any way.

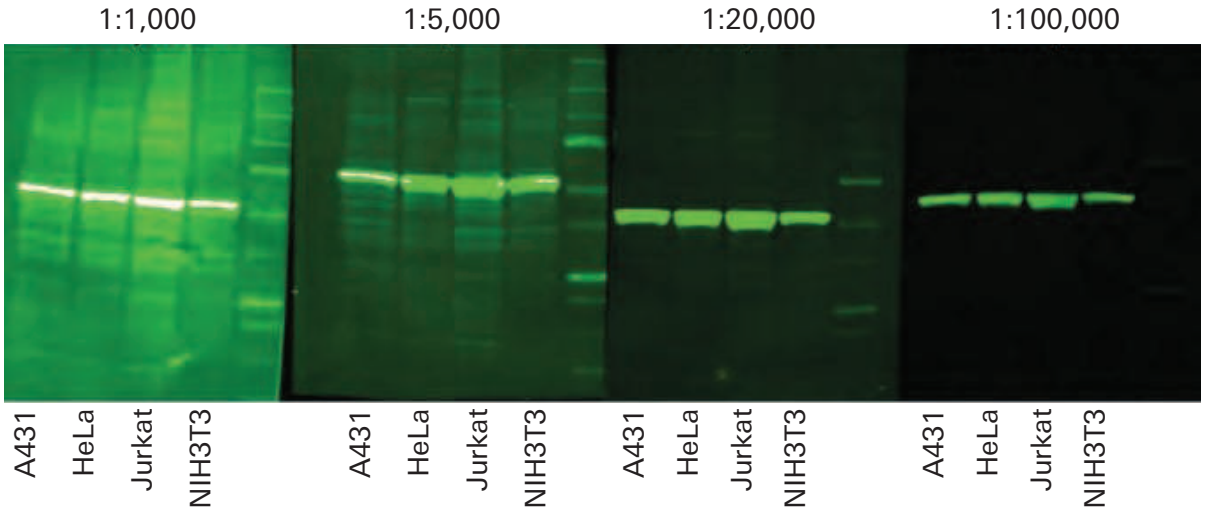


Figure 5. Effect of secondary antibody dilution on image data. IRDye 800CW secondary antibody was used at the dilutions shown, to detect the target protein in various cell lysates. Blots were imaged with the Odyssey Classic system with the default scan settings (intensity = 5). The 1:1,000 antibody dilution 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.

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.

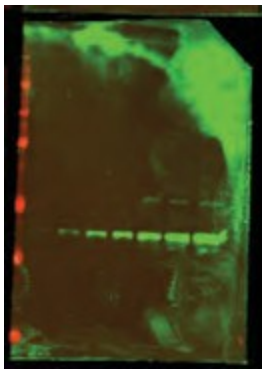
Blue Pen Used on Membrane

Appearance	Example Image	Solution
Smeared signal can be seen in the 700 nm channel.		Use a pencil to write on the membrane.

Acrylamide Residue on Membrane After Transfer

Appearance	Example Image	Solution
Speckles and blotches can be seen in 700/800 nm channels.		Carefully rinse off membrane in 1X PBS before it dries. Conditions may need to be optimized to prevent the gel from overheating during transfer.

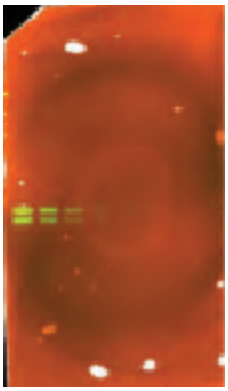
Bacterial Growth in Antibodies

Appearance	Example Image	Solution
Speckles and blotches can be seen in 700/800 nm channels.		Replace antibodies.

Blue Loading Buffer Used During Gel Electrophoresis

Appearance	Solution
Smeared signal in the 700 nm channel	Use LI-COR 4X Protein Sample Loading Buffer (P/N 928-40004).

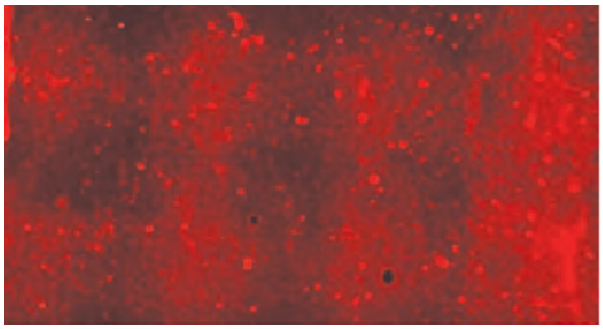
Dirty Processing Containers

Contamination Source	Appearance	Solution
Coomassie Stain/gel stain/ anything blue 	In the 700 nm channel, entire membrane dark, smeared signal, or speckles, depending on the amount of stain residue in container.	Use different containers for gel staining and Western blot detection.
Bacterial growth	Speckles and blotches can be seen in the 700/800 nm channels	Wash containers with detergent, rinse thoroughly with distilled water, and a final rinse with methanol
Acrylamide residue	Speckles and blotches can be seen in the 700/800 nm channels	Wash containers with detergent, rinse thoroughly with distilled water, and a final rinse with methanol

Dirty Forceps

Appearance	Solution
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.

Dirty Transfer Pads

Appearance	Example Image	Solution
Blotches can be seen on the blots that align with the transfer cassette holes		Cleaning the transfer pads in pure methanol may help, but the transfer pads may need to be replaced.

Fingerprints

Appearance	Example Image	Solution
Blotches can be seen in 700/800 nm channels where gloved/ungloved hands have touched the membrane.		Only handle membranes by the edges with clean forceps. Be careful not to touch the membrane with your hands or gloves.

III. Imaging Factors That Can Affect Western Blot Results

Start with A Clean Imaging Bed or Imaging Tray

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.

Carefully follow the cleaning instructions in the Operator's Manual for your imager to clean the scan bed or imaging tray.

Remove Air Bubbles

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 6.

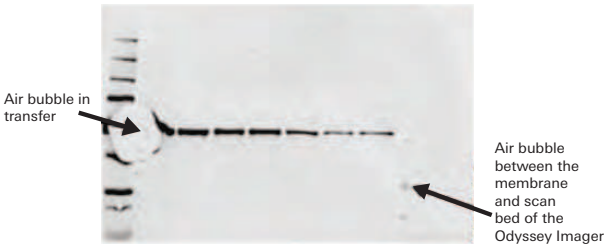


Figure 6. Air bubbles affect image appearance. Examples of bubbles in the transfer sandwich or on the Odyssey Imager scan bed.

Image Blot Wet or Dry

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 7.

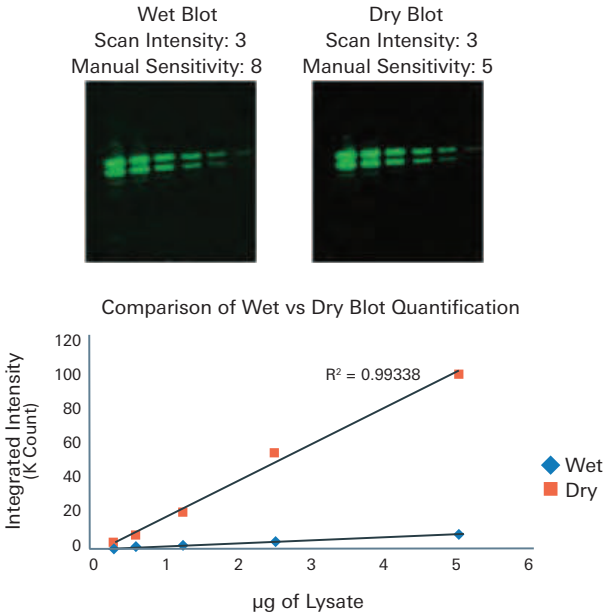


Figure 7. Scanning of wet and dry Western blots. The same Western blot was scanned wet and dry with an Odyssey Classic Imager. Optimal image display settings are shown. Quantification (graph below images) demonstrates higher signal intensity on the dry blot.

Imaging Parameters

The following information about imaging parameters applies to the 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 more information, see the manual for your imager.

Scan Intensity

Improper optimization of the Scan Intensity can result in saturation of signal and reduced linear dynamic range. Figure 8 demonstrates how changes in the Scan Intensity setting affect the resulting signals on the Odyssey Classic Imager.

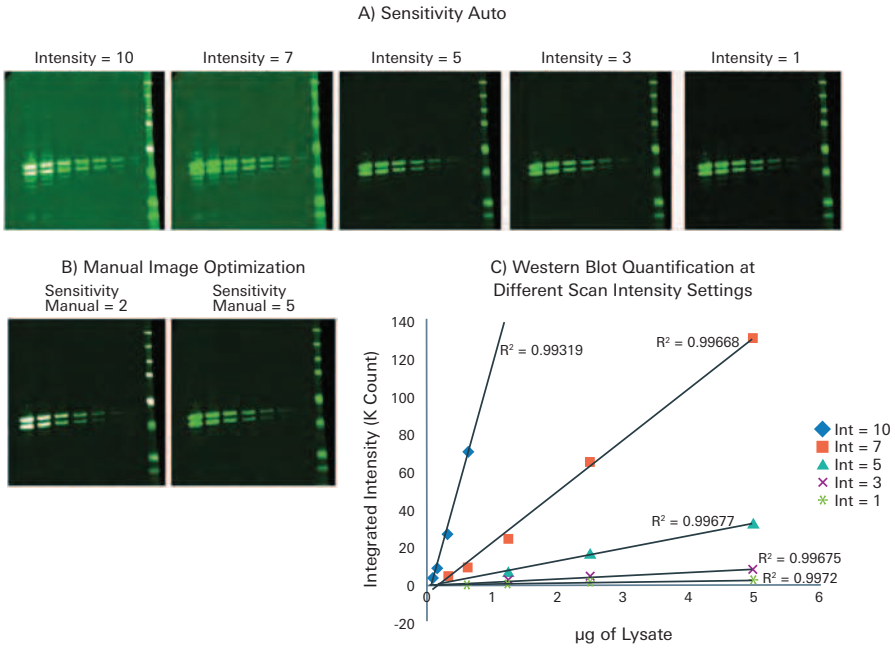


Figure 8. Effect of Scan Intensity settings on Western blot signals. The same Western blot was scanned at 5 different intensity settings with the Odyssey Classic Imager. A) Images shown with Auto Sensitivity settings for image display. Scan intensity setting is indicated above each imager. B) The same 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.

Auto Mode on the Odyssey® Imager

The Odyssey CLx Imager has two modes for dynamic range: Auto and Manual. A comparison between Auto Mode and Manual Mode is shown in Figure 9.

- In **Auto mode** the Odyssey CLx Imager acquires images with virtually no saturated pixels on the first attempt with no user adjustments. More than six logs (22 bits) of dynamic range are available for each image.
- In **Manual mode** the intensity of each channel can be manually adjusted across a 12 bit dynamic range.

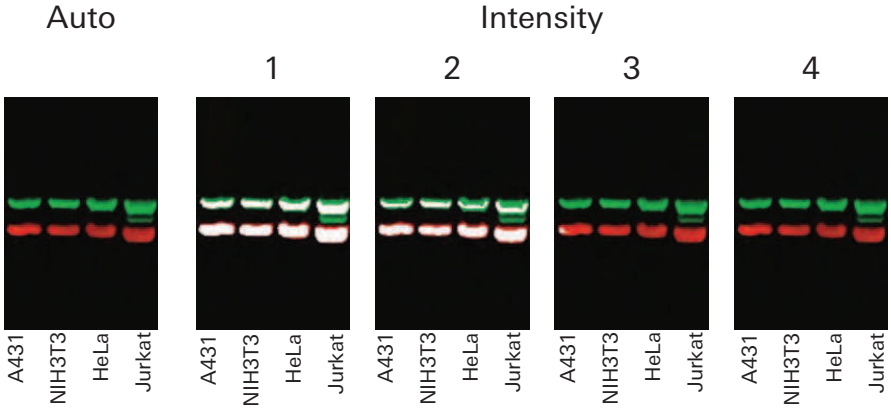


Figure 9. Auto Mode prevents signal saturation. A two-color Western blot was imaged with the Odyssey® CLx system, using Auto Mode. No saturated signals were observed. The same blot was imaged repeatedly with manual Scan Intensity settings, beginning with Intensity=4. Pixel saturation is shown in white. 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.

IV. 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.

LI-COR Biosciences

4647 Superior Street
Lincoln, NE 68504
Phone: +1-402-467-0700
Toll free: 800-645-4267
biosales@licor.com
licor.com/bio

Regional Offices

LI-COR Biosciences GmbH

Siemensstraße 25A
61352 Bad Homburg
Germany
Phone: +49 (0) 6172 17 17 771
bio-eu@licor.com

LI-COR Biosciences UK Ltd.

St. John's Innovation Centre
Cowley Road • Cambridge
CB4 0WS • United Kingdom
Phone: +44 (0) 1223 422104
bio-eu@licor.com

Doc # 988-19025
03/20