ODYSSEY® Fc

Application Protocols

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Good Westerns Gone Bad:

Maximizing sensitivity on chemiluminescent Western blots

Developed for:

Odyssey® Fc Imager and

C-DiGit® Blot Scanner

LI-COR®

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Maximizing sensitivity on chemiluminescent Western blots

The most important procedural difference between using film and digital imaging is related to the timing of image acquisition. Film is more tolerant in relation to processing time as you can always expose for a longer period of time if needed, whereas digital imaging requires that you capture the most photons within a finite imaging window. This window begins immediately after the addition of room temperature substrate (but do not compromise the five-minute incubation time!). ***Longer acquisitions in digital imaging do not correlate to results of longer exposures on film, as background noise can begin to contribute signal that impacts your overall limits of detection (LOD).***

If you are seeing weak signal in your data, the following information will help you assess what may be contributing factors.

Possible cause # 1: Substrate does not have a fast enough rate of reaction (e.g., SuperSignal® West Pico)

Solution: Use WesternSure™ PREMIUM or SuperSignal West Femto substrates

Why this matters: Different substrates have different rates of reaction. Some are developed to give off a lot of light quickly; others give off small amounts of light over longer periods of time. An alternate substrate may be required for digital imaging when imaging blots with low protein abundance. **See Figure 1.**

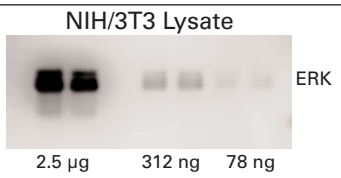
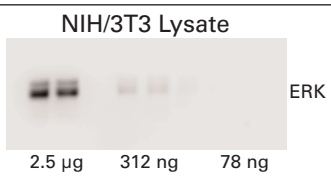
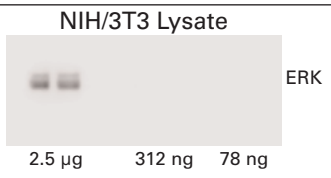
Figure 1	Optimal Blot	Satisfactory Blot	Unsatisfactory Blot
Images			
Conditions **Substrate**	**SuperSignal West Femto**	**SuperSignal West Dura**[1]	**SuperSignal West Pico**[2]
Substrate Volume	3.0 mL substrate	3.0 mL substrate	3.0 mL substrate
Imaging Method	Substrate placed directly on C-DiGit® Blot Scanner glass surface. Membrane placed on substrate, 1-ply sheet protector on top, incubate 5 min.	Substrate placed directly on C-DiGit Blot Scanner glass surface. Membrane placed on substrate, 1-ply sheet protector on top, incubate 5 min.	Substrate placed directly on C-DiGit Blot Scanner glass surface. Membrane placed on substrate, 1-ply sheet protector on top, incubate 5 min.
Scan Setting	High	High	High
Performance	LOD – 78 ng	LOD – 312 ng	LOD – 2.5 µg

Figure 1. Performance differences of three different substrate classifications using C-DiGit Blot Scanner. All images are normalized to the Lookup Table (LUT) settings of the optimal blot for accurate visual comparison.

[1] Comparable to WesternSure PREMIUM
[2] Comparable to WesternSure ULTRA

Possible cause # 2: Not enough substrate was added to the blot

Precaution/Solution:

For C-DiGit® Blot Scanner:

- Add at least 3 mL (7 x 4 cm blot, 0.1 mL/cm^2) of substrate to glass surface of the scanner, place blot protein side down into the substrate, place 1-ply sheet protector on top, incubate 5 min, then scan on High;
  OR,
- Add at least 3 mL (7 x 4 cm blot, 0.1 mL/cm^2) of substrate to blot surface, incubate 5 min, remove excess substrate, place blot protein side down onto the glass surface, cover with 1-ply sheet protector on top, then scan on High.

For Odyssey® Fc Imager:

- Add at least 3 mL (7 x 4 cm blot, 0.1 mL/cm^2) of substrate to blot surface, incubate 5 min, remove excess substrate, place blot protein side up onto the imaging tray, then image.

Why this matters: If you do not add enough substrate to your blot, the light-generating luminol will be depleted, leading to fewer photons (light) being released. **See Figures 2 and 3**.

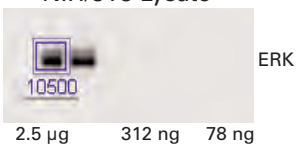
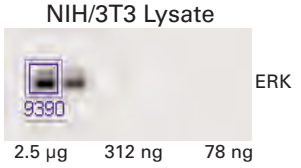
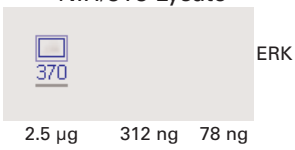
Figure 2	Optimal Blot	Satisfactory Blot	Unsatisfactory Blot
Images	NIH/3T3 Lysate 	NIH/3T3 Lysate 	NIH/3T3 Lysate 
Conditions			
Substrate	SuperSignal® West Pico^2	SuperSignal West Pico^2	SuperSignal West Pico^2
Substrate Vol.	**3.0 mL substrate**	**1.5 mL substrate**	**0.75 mL substrate**
Imaging Method	Substrate placed directly on C-DiGit Blot Scanner glass surface. Membrane placed on substrate, 1-ply sheet protector on top, incubate 5 min.	Substrate placed directly on C-DiGit Blot Scanner glass surface. Membrane placed on substrate, 1-ply sheet protector on top, incubate 5 min.	Substrate placed directly on C-DiGit Blot Scanner glass surface. Membrane placed on substrate, 1-ply sheet protector on top, incubate 5 min.
Scan Setting	High	High	High
Performance	Bright signal	Moderate signal	Low signal

Figure 2. Performance differences when incubating the blot in different volumes of SuperSignal West Pico. Three blots have the same LOD (2.5 µg/well); however, signal intensity varies. Blots were all imaged on the C-DiGit Blot Scanner. Images are normalized to the LUT of the Optimal blot.

[2] Comparable to WesternSure™ ULTRA

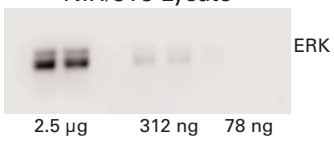
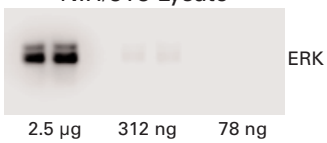
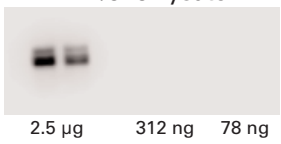
Figure 3	Optimal Blot	Satisfactory Blot	Unsatisfactory Blot
Images	NIH/3T3 Lysate 	NIH/3T3 Lysate 	NIH/3T3 Lysate 
Conditions **Substrate**	SuperSignal® West Dura[1]	SuperSignal West Dura[1]	SuperSignal West Dura[1]
Substrate Vol.	**3.0 mL substrate**	**1.5 mL substrate**	**0.75 mL substrate**
Imaging Method	Substrate placed directly on C-DiGit® Blot Scanner glass surface. Membrane placed on substrate, 1-ply sheet protector on top, incubate 5 min.	Substrate placed directly on C-DiGit Blot Scanner glass surface. Membrane placed on substrate, 1-ply sheet protector on top, incubate 5 min.	Substrate placed directly on C-DiGit Blot Scanner glass surface. Membrane placed on substrate, 1-ply sheet protector on top, incubate 5 min.
Scan Setting	High	High	High
Performance	LOD – 78 ng	LOD – 312 ng	LOD – 2.5 µg

Figure 3. Performance differences when incubating the blot in different volumes of SuperSignal West Dura. Blots were all imaged on the C-DiGit Blot Scanner. Images are normalized to the LUT of the Optimal blot.

[1] Comparable to WesternSure™ PREMIUM

Possible cause # 3: Membrane was placed on the detection system incorrectly

Solution: Ensure that your blot is placed with the proteins facing toward the detection system.

For the C-DiGit® Blot Scanner:

- Place blot protein side facing *down*

For Odyssey® Fc Imager:

- Place blot protein side facing *up*

Why this matters: If the blot is placed incorrectly, you may or may not be able to visualize bands. If bands are visualized, they will be substantially reduced in signal. **See Figure 4.**

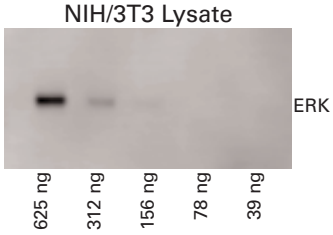
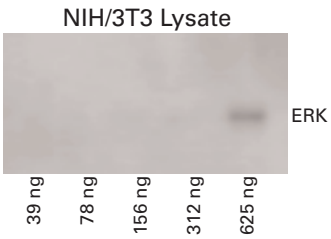
Figure 4	Correctly Imaged Blot	Incorrectly Imaged Blot
Images		
Conditions **Substrate**	SuperSignal® West Dura[1]	SuperSignal West Dura[1]
Imaging Method	**Blot imaged *protein side facing down***	**Blot imaged *protein side facing up***
Performance	LOD – 156 ng	LOD – 625 ng

Figure 4. Performance differences imaging the blot correctly (protein side down), compared to imaging the blot protein side up on the C-DiGit Blot Scanner. Images are normalized to the LUT of the correctly imaged blot.

[1] Comparable to WesternSure™ PREMIUM

Possible cause #4: Blot was not detected or processed on the same day it was imaged

Precaution/Solution: Blot should be processed and detected on the same day. The secondary antibody should be incubated the day of imaging and fresh substrate added before imaging.

Why this matters: Secondary antibody and/or substrate is not stable enough for acceptable photon emission when digitally imaged after the day it is applied. **See Figures 5 and 6.**

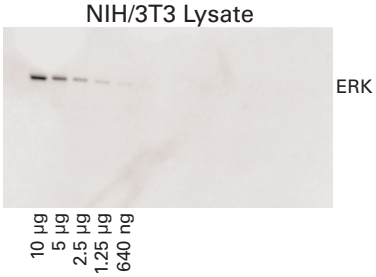
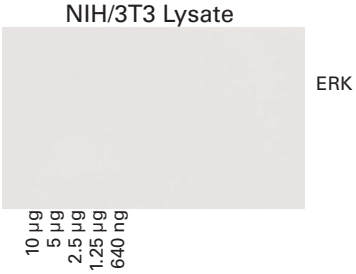
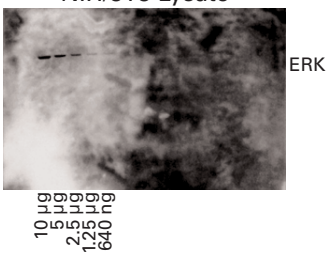
Figure 5	**Optimal Blot**	**Unsatisfactory Blot**	**Unsatisfactory Blot**
Images	NIH/3T3 Lysate  	NIH/3T3 Lysate  	NIH/3T3 Lysate 
Conditions			
Substrate	SuperSignal® West Dura[1]	SuperSignal West Dura[1]	SuperSignal West Dura[1]
Processing Time	**Same day**	**Next day**	**Next day**
Detection Process	HRP secondary incubated, washed, and substrate added immediately before imaging.	HRP secondary incubated, washed, and substrate added day before imaging.	HRP secondary incubated, washed, and substrate added day before imaging, **then re-incubated with HRP secondary and substrate added immediately before imaging.**
Storage Conditions		**Blot stored overnight dry, at room temperature**	**Blot stored overnight dry, at room temperature**
Performance	LOD – 640 ng	LOD – None detected	LOD – 1.25 µg

Figure 5. Performance differences when the same blot is imaged immediately after processing vs. stored overnight *dry* and then imaged. Blots were all imaged on the C-DiGit® Blot Scanner. Images are normalized to the LUT of the optimal blot.

[1] Comparable to WesternSure™ PREMIUM

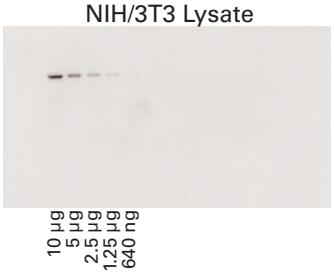
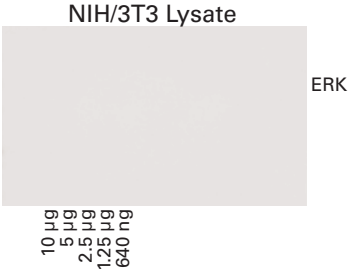
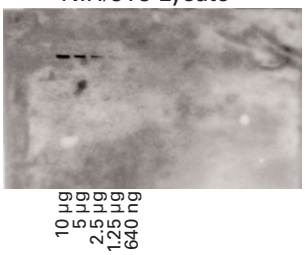
Figure 6	Optimal Blot	Unsatisfactory Blot	Unsatisfactory Blot
Images	NIH/3T3 Lysate  	NIH/3T3 Lysate  	NIH/3T3 Lysate 
<u>Conditions</u> **Substrate**	SuperSignal® West Dura[1]	SuperSignal West Dura[1]	SuperSignal West Dura[1]
Process Time	**Same day**	**Next day**	**Next day**
Detection Process	HRP secondary incubated, washed, and substrate added immediately before imaging.	HRP secondary incubated, washed, and substrate added day before imaging.	HRP secondary incubated, washed, and substrate added day before imaging, **then re-incubated with HRP secondary and substrate added immediately before imaging.**
Storage Conditions		**Blot stored overnight wet in PBS, at room temperature**	**Blot stored overnight wet in PBS, at room temperature**
Performance	LOD – 640 ng	LOD – None detected	LOD – 1.25 µg

Figure 6. Performance differences when the same blot is imaged immediately after processing vs. stored overnight *<u>wet</u>* and then imaged. Blots were all imaged on the C-DiGit® Blot Scanner. Images are normalized to the LUT of the optimal blot.

[1] Comparable to WesternSure™ PREMIUM

Possible cause # 5: Blot was not kept uniformly wet during the entire image acquisition

Precaution/Solution:
- Use more substrate prior to imaging
- Do not completely blot off all of the substrate before imaging

For C-DiGit® Blot Scanner:
- Wrap the blot in plastic wrap or cover with a plastic sheet protector
- Incubate blot with substrate directly on scanner bed

Why this matters: If enough substrate is not added, the membrane will not stay wet, and there will be no enzymatic activity. **See Figure 7.**

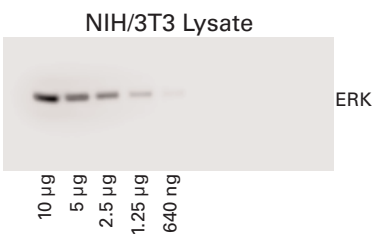
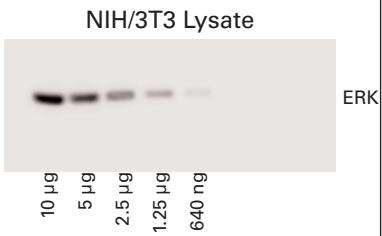
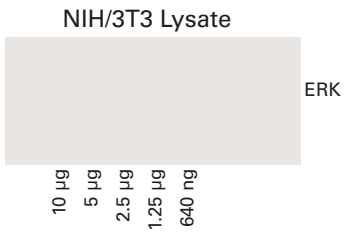
Figure 7	Optimal Blot	Optimal Blot	Unsatisfactory Blot
Images			
Conditions **Imaging Method**	**Wet blot** Imaged in 3.0 mL of SuperSignal® West Dura[1] substrate placed on the scan bed of the C-DiGit Blot Scanner with 1-ply sheet protector on top	**Damp blot** Excess SuperSignal West Dura[1] substrate removed, then imaged on the scan bed of the C-DiGit Blot Scanner with 1-ply sheet protector on top	**Dry blot** Blot dried before imaging
Performance	LOD – 640 ng	LOD – 640 ng	LOD – None detected

Figure 7. Performance differences when the same blot is imaged wet, damp and dry. Blots were all imaged on the C-DiGit Blot Scanner. Images are normalized to the LUT of the optimal wet blot.

Possible cause # 6: Blot was exposed to film BEFORE imaging on a digital imager

Precaution/Solution: Image on digital imager first, then expose blot to film.

Why this matters: Digital imaging requires capturing the most photons being generated, which is typically immediately after a 5-minute substrate incubation. Time may be more of an issue with some substrates. **See Figures 8, 9, and 10.**

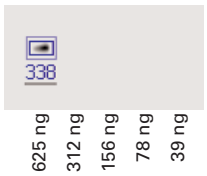
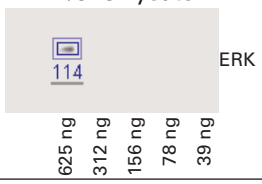
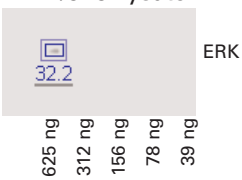
Figure 8	Optimal Blot	Unsatisfactory Blot	Unsatisfactory Blot
Images	NIH/3T3 Lysate 	NIH/3T3 Lysate 	NIH/3T3 Lysate 
Conditions **Imaging time** **Scan Setting**	**Immediately after incubation with SuperSignal® West Pico** High	**26 min after incubation** High	**51 min after incubation** High
Performance	LOD – 625 ng, Signal – 338	LOD – 625 ng, Signal – 114	LOD – 625 ng, Signal – 32.2

Figure 8. Performance differences of a Western blot detected with SuperSignal® West Pico[2] when the same blot is imaged over time. Blot was incubated 5 min in substrate before imaging on the C-DiGit® Blot Scanner. Images are normalized to the LUT of the optimal blot.

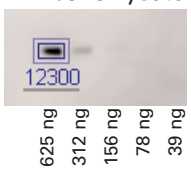
Figure 9	Optimal Blot	Satisfactory Blot	Satisfactory Blot
Images	NIH/3T3 Lysate 	NIH/3T3 Lysate 	NIH/3T3 Lysate 
Conditions **Imaging time** **Scan Setting**	**Immediately after incubation with SuperSignal West Dura** High	**24 min after incubation** High	**48 min after incubation** High
Performance	LOD – 156 ng, Signal – 12,300	LOD – 156 ng, Signal – 10,400	LOD – 156 ng, Signal – 9,090

Figure 9. Performance differences of a Western Blot detected with SuperSignal West Dura[1] when the same blot is imaged over time. Blot was incubated 5 min in substrate before imaging on the C-DiGit Blot Scanner. Images are normalized to the LUT of the optimal blot.

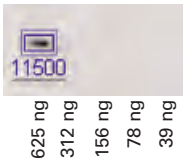
Figure 10	Optimal Blot	Satisfactory Blot	Satisfactory Blot
Images	NIH/3T3 Lysate 	NIH/3T3 Lysate 	NIH/3T3 Lysate 
Conditions **Imaging time** **Scan Setting**	**Immediately after incubation with SuperSignal West Femto** High	**24 min after incubation** High	**48 min after incubation** High
Performance	LOD – 156 ng, Signal – 11,500	LOD – 156 ng, Signal – 8,120	LOD – 156 ng, Signal – 6,860

Figure 10. Performance differences of a Western Blot detected with SuperSignal West Femto when the same blot is imaged over time. Blot was incubated 5 min in substrate before imaging on the C-DiGit Blot Scanner. Images are linked to the LUT of the optimal blot.

[1] Comparable to WesternSure™ PREMIUM
[2] Comparable to WesternSure ULTRA

Possible cause # 7: Blot was imaged using incorrect sensitivity setting

Precaution/Solution:

- On the C-DiGit® Blot Scanner, use High Sensitivity setting (12-min scan) for more sensitive detection
- On the Odyssey® Fc Imager, use a longer integration time (up to 10 min)

Why this matters: Digital imaging with the C-DiGit Blot Scanner or Odyssey Fc will not generally reach a saturation point. Begin with a longer acquisition time to ensure best sensitivity, then optimize to shorter scan times. **See Figure 11**.

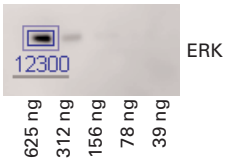
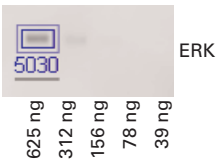
Figure 11	Optimal Blot	Satisfactory Blot
Images	NIH/3T3 Lysate 	NIH/3T3 Lysate 
Conditions	SuperSignal® West Dura[1] **Sensitivity High**	SuperSignal West Dura[1] **Sensitivity Standard**
Performance	Signal – 12,300	Signal – 5,030

Figure 11. Performance differences of a Western blot detected with SuperSignal West Dura on C-DiGit Blot Scanner when the same blot is imaged on High Sensitivity (12 min) versus Standard Sensitivity (6 min). Images are linked to the LUT of the optimal blot.

[1] Comparable to WesternSure™ PREMIUM

Possible cause # 8: Substrate was too cold

Precaution/Solution:

- Equilibrate substrate to room temperature before imaging on a digital imager.

Why this matters: Enzyme activity is greatly reduced when it is cold. Substrate needs to be equilibrated to room temperature for digital imaging. This is true with film as well, but there may be a period of time after adding substrate and exposing to film during which the substrate has had a chance to equilibrate to room temperature. **See Figure 12.**

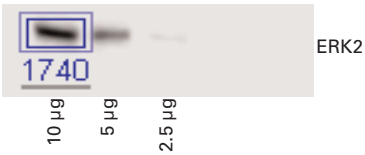
Figure 12	Optimal Blot	Unsatisfactory Blot
Images	NIH/3T3 Lysate 	NIH/3T3 Lysate 
Conditions	SuperSignal® Pico[2] Sensitivity Standard **Substrate room temperature**	SuperSignal Pico[2] Sensitivity Standard **Substrate cold**
Performance	Signal – 1,740	Signal – 200

Figure 12. Performance differences of a Western Blot detected with SuperSignal West Pico when the substrate has been equilibrated to room temperature versus being imaged with cold (4 °C) substrate. Image was acquired using Standard (6-min) sensitivity.

Possible cause # 9: Substrate was not incubated for 5 minutes

Precaution/Solution: Incubate substrate for five minutes prior to imaging on a digital imager.

Why this matters: Five minutes is the typical manufacturer's recommendation for optimal photon emission, for both film and digital imaging. **See Figure 13.**

[2] Comparable to WesternSure™ ULTRA

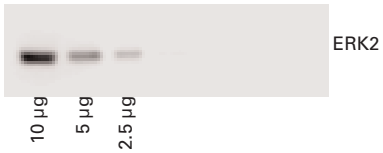
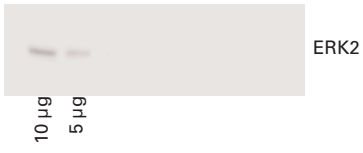
Figure 13	Optimal Blot	Unsatisfactory Blot
Images	NIH/3T3 Lysate 	NIH/3T3 Lysate 
Conditions	SuperSignal® Pico[2] **Incubation 5 min** Substrate room temperature	SuperSignal Pico[2] **No incubation** Substrate room temperature
Performance	LOD – 2.5 µg	LOD 5 µg

Figure 13. Performance differences of a Western Blot detected with SuperSignal West Pico when doing a 5-min substrate incubation as opposed to not doing a substrate incubation.

Possible cause # 10: Substrate was diluted

Precaution/Solution: Do NOT dilute your substrate.

Why this matters: Rate of reaction is determined by the ratio of enzyme to substrate. Diluting the substrate will dramatically impact the overall generation of light. **See Figure 14.**

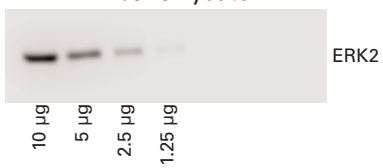
Figure 14	Optimal Blot	Unsatisfactory Blot
Images	NIH/3T3 Lysate 	NIH/3T3 Lysate 
Conditions	SuperSignal Dura[1] **Substrate not diluted**	SuperSignal Dura[1] **Substrate diluted 1:1 (in water)**
Performance	LOD – 1.25 µg	LOD 2.5 µg

Figure 14. Performance differences of a Western Blot detected with SuperSignal West Dura that has not been diluted compared to an identical blot in which the substrate has been diluted 1:1 in water.

[1] Comparable to WesternSure™ PREMIUM
[2] Comparable to WesternSure ULTRA

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0513

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 Odyssey Imager model.

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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 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 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; 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 any Odyssey System.

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 Buffer on a nitrocellulose membrane. Figure 3 is a similar example using Odyssey Blocking Buffer, I-Block™, and 5% BSA for detection of anti-pAkt and β-tubulin in 293T Cells stimulated with TGF-β.

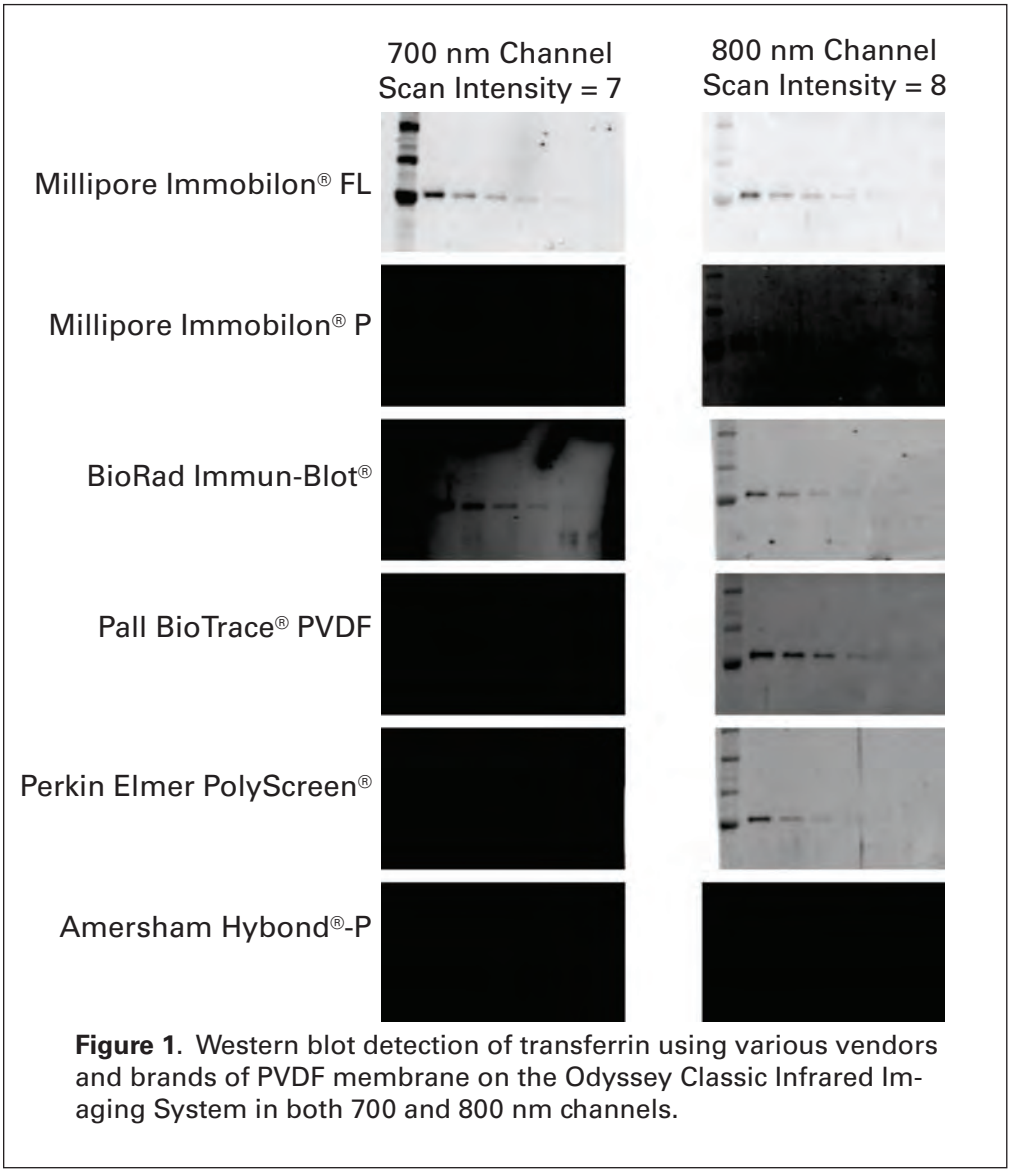


Figure 1. Western blot detection of transferrin using various vendors and brands of PVDF membrane on the Odyssey Classic Infrared Imaging System in both 700 and 800 nm channels.

We tested the PathScan® PDGFR Activity Assay: Phospho-PDGFR, Phospho-SHP2, Phospho-Akt, and Phospho-p44/42 MAPK (Erk1/2) Multiplex Western Detection Kit #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.

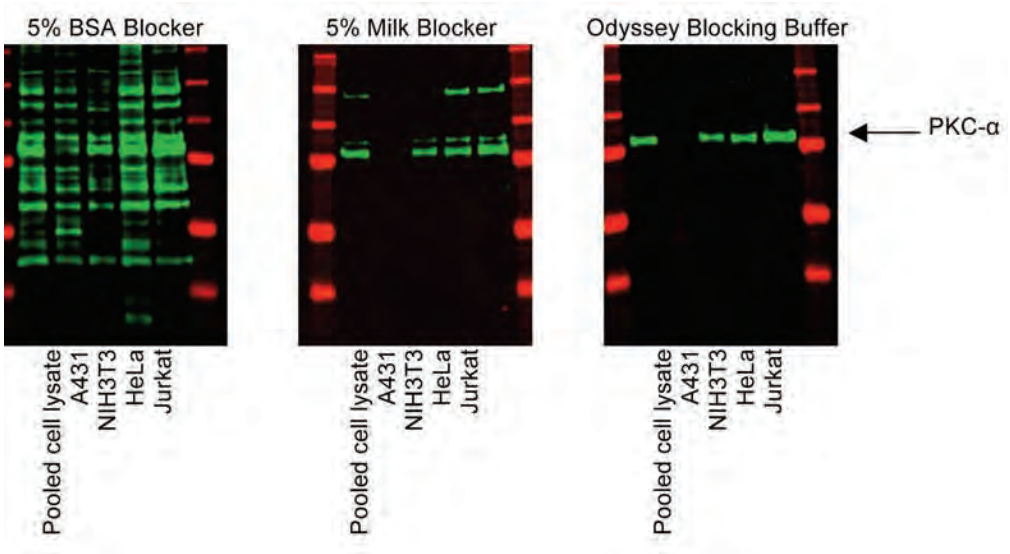


Figure 2. Western blots detected with anti-PKCα and IRDye® 800CW Goat anti-mouse. All blots were treated equally, with the exception of blocking reagent. All images were generated on the Odyssey Classic Infrared Imager with scan intensity setting of 5, sensitivity of 5.

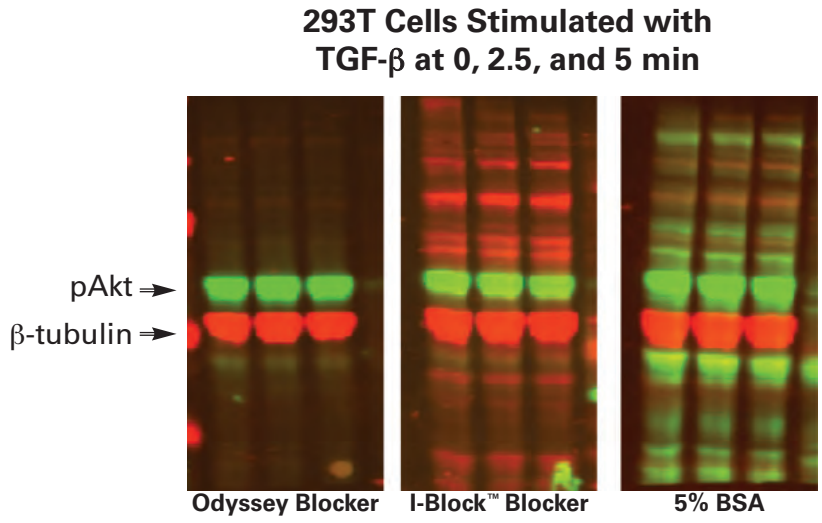


Figure 3. Western blots of 293T Cells stimulated with TGF-β (0, 2.5, and 5 minutes) detected with anti-pAkt and β-tubulin. All blots were treated equally, with the exception of blocking reagent. All images were generated on the Odyssey Classic Infrared Imager with scan intensity setting of 3.5/5 (700/800 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 detergent. Never expose the membrane to detergent until blocking is complete, as this may cause high membrane background.

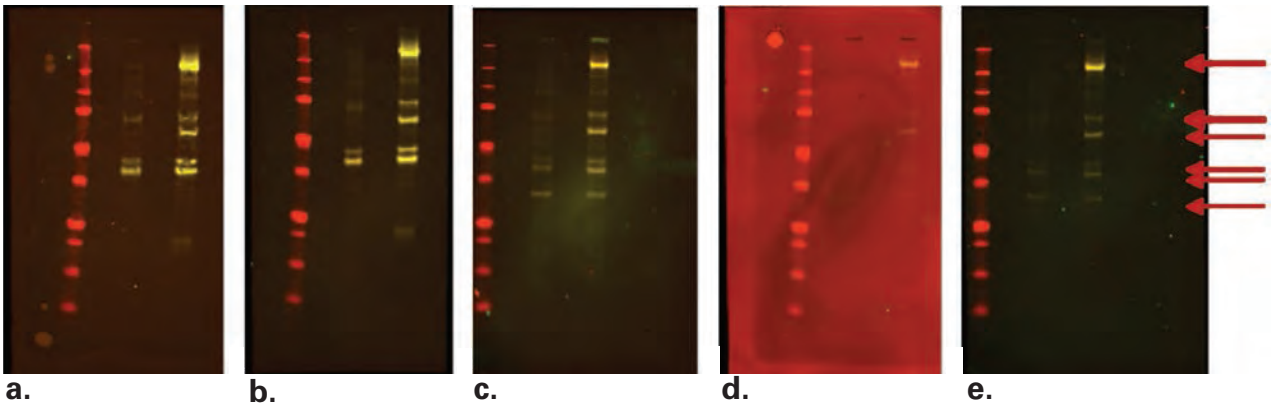


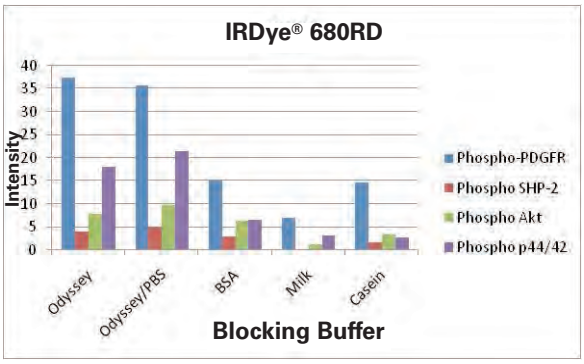
Figure 4. Above: Western blots utilizing PathScan® Multiplex primary antibody and both IRDye® 680 and IRDye 800CW goat anti-rabbit for detection. Five different solutions were used for blocking and antibody dilution (antibody dilutions included 0.2% Tween® 20):

a. Odyssey® Blocking Buffer;
b. Odyssey + PBS (1:1);
c. 5% BSA;
d. 5% Skim Milk;
e. 0.5% Casein.

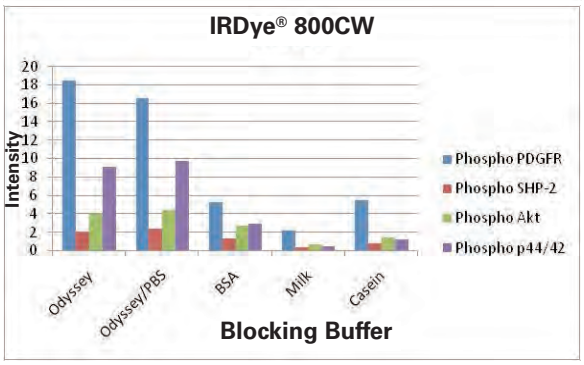
In each image, arrows indicate band positions for each of the detected proteins. Starting from top: Phospho-PDGFR, phospho-SHP2, phospho-Akt, phospho-p44/p42, and S6.

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. When working with **IRDye® 680LT** conjugates on **PVDF** membranes, SDS (final concentration of 0.01 - 0.02%) and Tween 20 (final concentration of 0.1. - 0.2%) **must** be added during the detection incubation step.
   c. Wash solutions should not contain SDS.

D. Primary Antibody

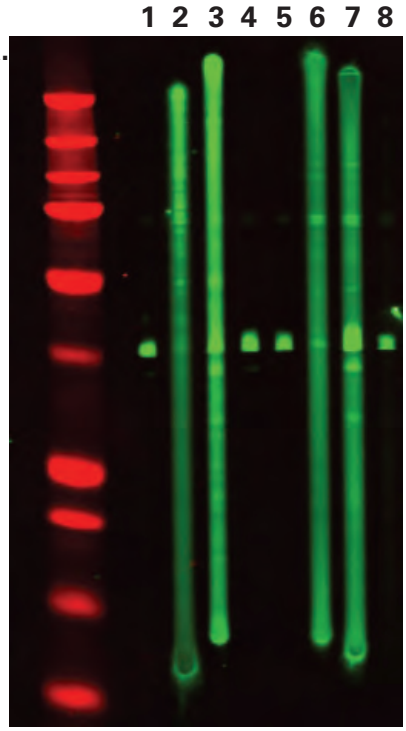
An antibody produced to detect a specific antigen is called the primary antibody, and it binds directly to the molecule of interest. Primary antibodies can be produced in a wide variety of species, such as mouse, rabbit, goat, chicken, rat, guinea pig, human, and many others. Primary antibodies for the 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 680RD, 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 only 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 and a complete description, refer to *Western Blot and In-Cell Western™ Assay Detection Using IRDye Subclass Specific Antibodies*.

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.

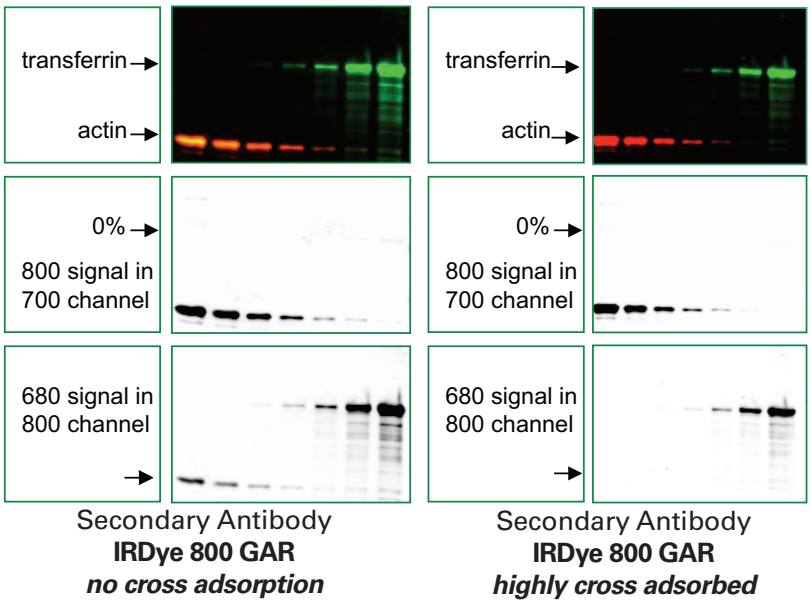
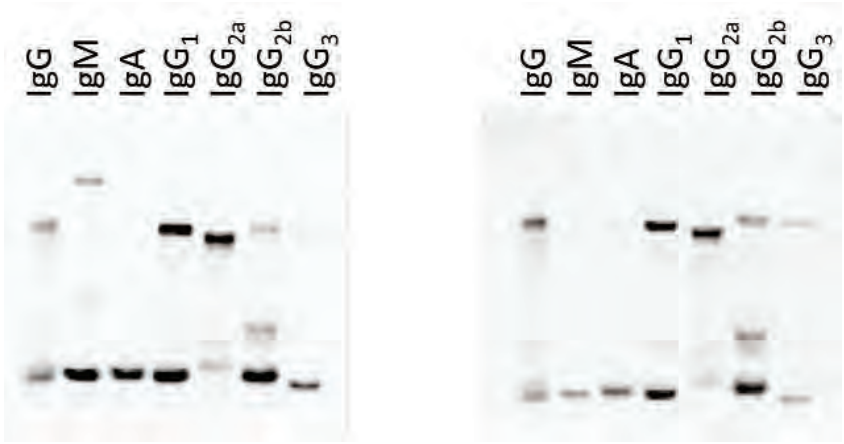


Figure 6. Example of a secondary antibody not cross adsorbed, cross-reacting with the second antibody pair in a two-color Western blot.

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.



GAM IgG
LI-COR®
Highly Cross Adsorbed

GAM IgG
***Not* Cross Adsorbed**

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

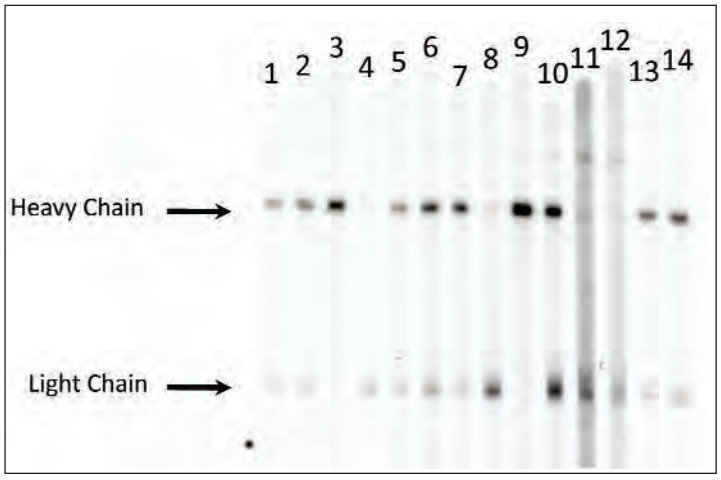
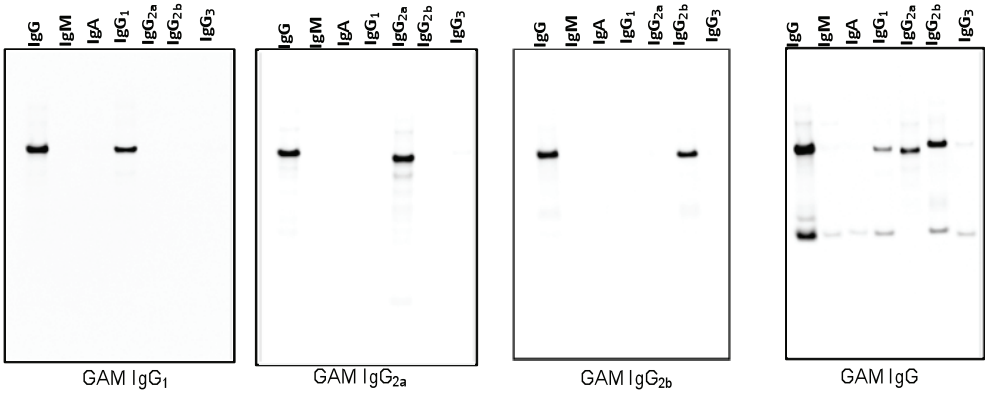


Figure 8. IRDye 800CW labeled anti-mouse antibodies against purified mouse IgG.

Secondary Antibodies used at a 1:5,000 dilution unless otherwise indicated

1) Goat anti-Mouse IgA, IgG, IgM
2) Rabbit anti-Mouse IgG
3) Goat anti-Mouse IgG Fcy (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 Fcy (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 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.



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 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. The Odyssey® imaging software can be used to maximize the appearance of the image using a wide range of secondary antibody dilutions (Figure 10).

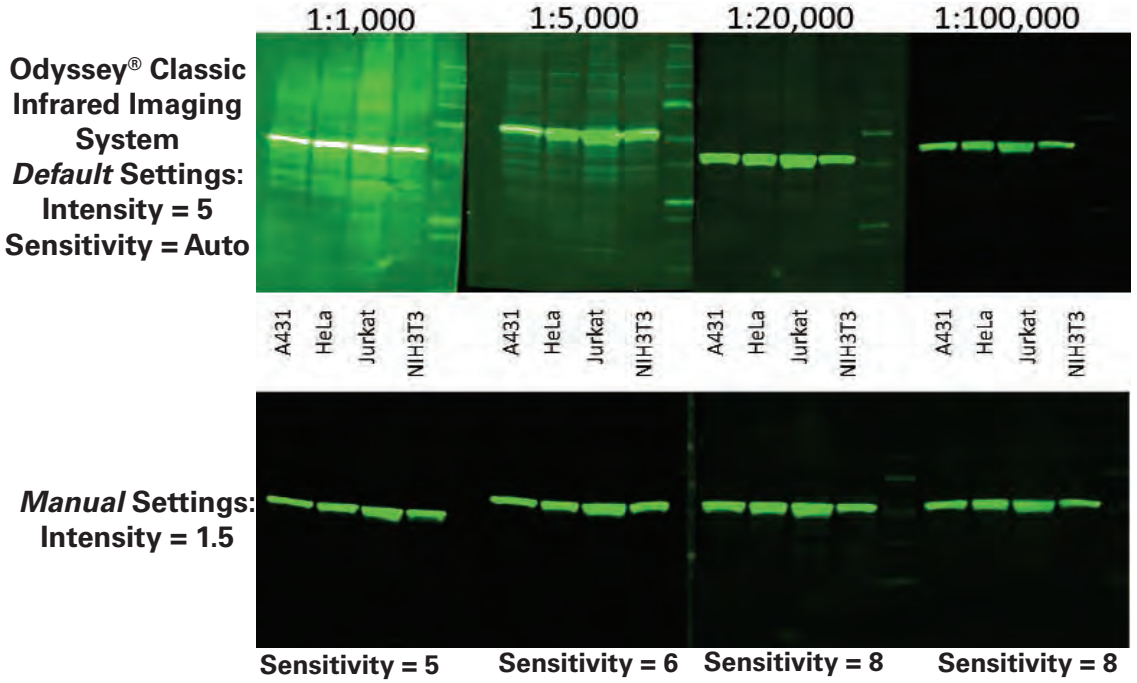


Figure 10. Secondary Antibody Concentration of IRDye 800CW goat anti-mouse with maximized Odyssey Classic imaging capabilities.

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.

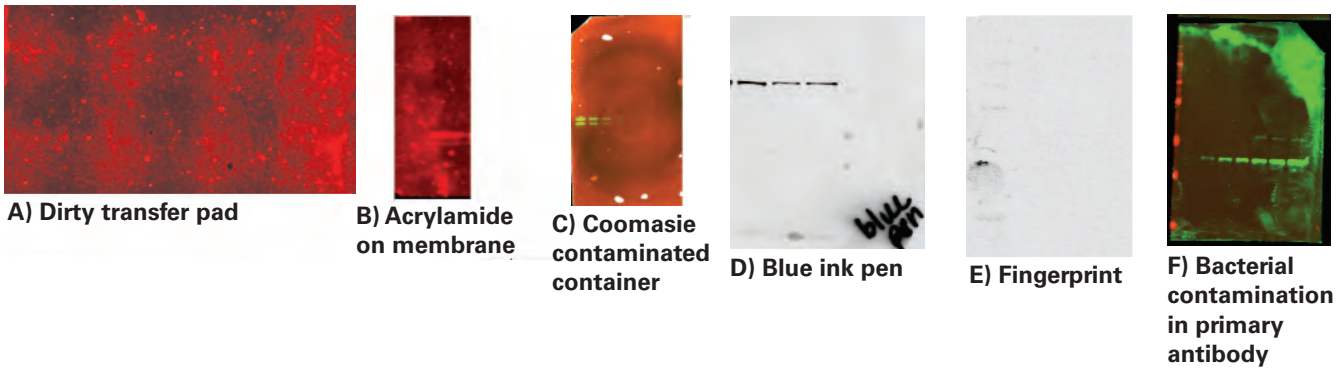


Figure 11. Examples of contamination events that may cause background on a Western blot.

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 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.

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 any Odyssey® Imaging System that can greatly influence 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 any Odyssey Imaging System. Typically, the signal is higher when a dry blot is imaged; however, background will increase.
NOTE: Once a blot is dry, or partially dried, stripping of the membrane for reuse is ineffective. See Figure 13.

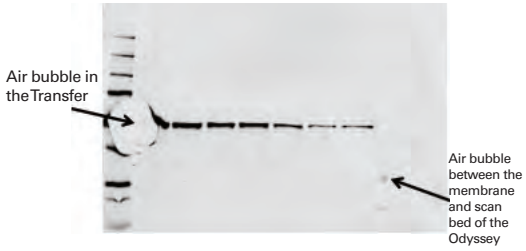


Figure 12. Examples of air bubbles in the transfer and on the Odyssey® Classic Infrared Imager scan bed.

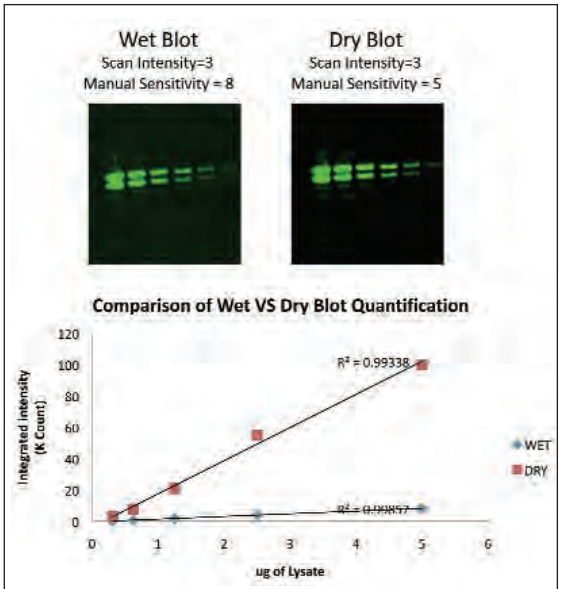


Figure 13. The same Western blot scanned wet and dry on an Odyssey Classic. The images are represented using the optimal display settings. Quantification is shown in the chart below the images.

Odyssey Classic, Odyssey CLx, and Odyssey Sa

Focus Offset – Improper adjustment of the Focus Offset can result in reduced signal collection from the imager. 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 14 shows the quantification variation that can occur by changing intensity settings in which the image is acquired on the Odyssey Classic. Figure 15 illustrates **AutoScan** imaging functionality on the Odyssey CLx. Multiple scans, at four intensity settings, are required to reduce saturation, compared to a non-saturated image from a single **Auto Intensity** setting. For details, see the Help System.

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. For example, saturated signal in the 800 nm channel can be seen as 700 nm signal in the 700 channel scan (see Figure 16). This can be easily eliminated by scanning at a lower intensity.

Odyssey Fc – The Odyssey Fc Imaging System is optimized for acquiring Western blot images without saturated pixels or further adjustments by the operator.

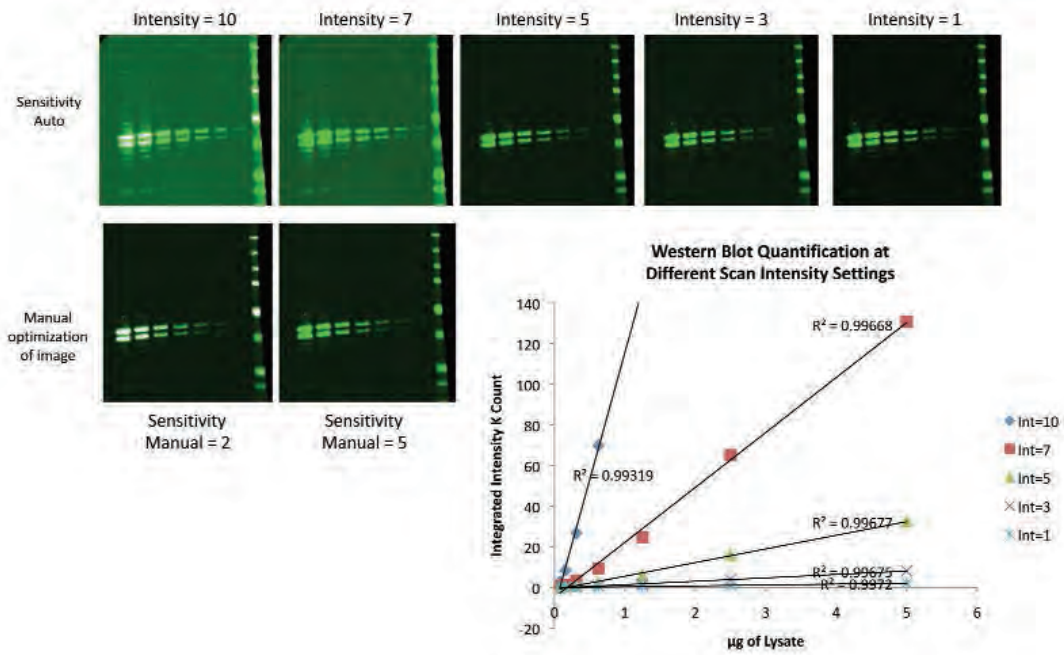


Figure 14. The same Western blot scanned at 5 different intensity settings on the Odyssey® Classic Infrared Imaging System. The top row of images are displayed using the Auto Sensitivity setting in the Odyssey Software. The bottom images were optimized using the Manual Sensitivity option for display.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

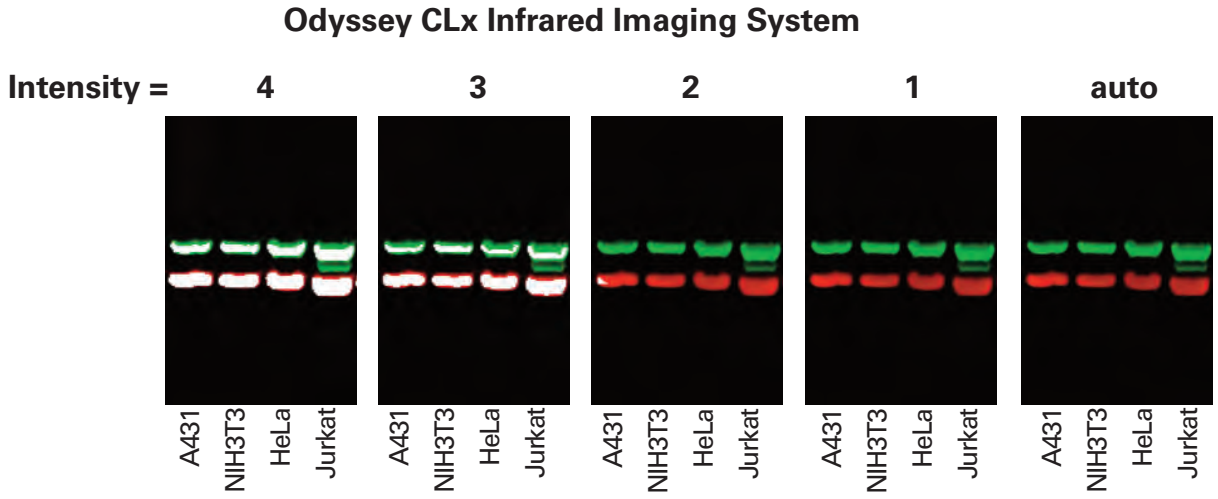


Figure 15. A single Western blot scanned on Odyssey CLx at decreasing Scan Intensity settings, and finally using AutoScan intensity. Pixel saturation appears in white. The antigen targets for each lysate sample are displayed in green (rabbit anti-Tubulin detected with IRDye® 800CW goat anti-rabbit) and red (mouse anti-Actin detected with IRDye 680LT goat anti-mouse).

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 only work on blots that are not experiencing binding chemistry problems.

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. 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 channel can be adjusted independently. Image adjustments can also be made in grayscale; very faint bands are visualized well when bands are displayed black on a white background. For details, see the *User Guide*.

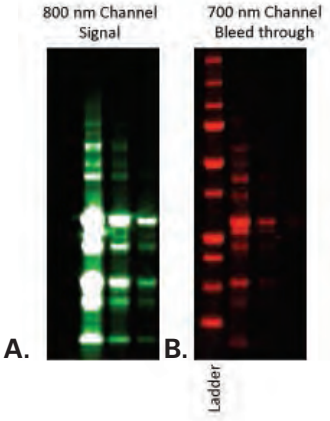
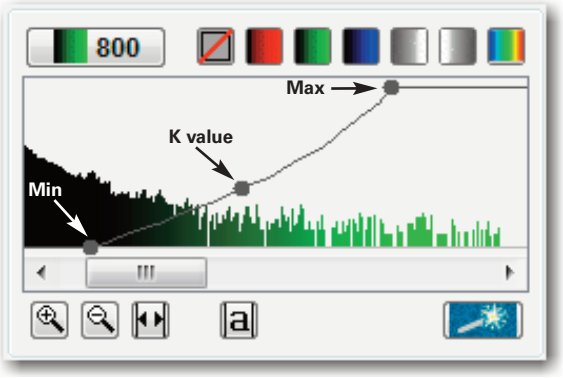


Figure 16. Saturated signal in the 800 nm channel (A) of the Odyssey Classic Infrared Imaging System can be visualized in the 700 nm channel (B). The only detection that should be seen in the 700 nm channel is the ladder on the far left of the image. Optimizing scan intensity can eliminate this.

Odyssey Classic, Odyssey CLx, and Odyssey Fc (Image Studio Software, ver. 1.x – 2.x)

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

Dim Bands – Click and drag the **min**, **max**, and **K value** dots on the histogram (**Image LUT**s 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 well when black bands are displayed on a white background. For details, see the Help System.



V. Data Analysis Using the Odyssey® Classic

Odyssey Classic (ver. 3.x application software)

Background

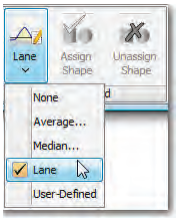
For accurate Western blot quantification, the **Background** setting must be applied effectively. The Background method sets the background calculation method for use in quantification, by measuring the intensity of the pixels selected as the background region. There are several methods for background 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 pixels on all four sides of the feature. The sides (**All**, **Top/Bottom**, or **Right/Left**) of the feature can be selected to optimize quantification. 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.
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 **OK** to the message. Notice that the 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.

Image Studio (ver. 1.x – 2.x)

Background settings can be found in the **Background** group on the **Analyze** ribbon. To implement **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 **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 includes the option of **Lane** background subtraction. This setting subtracts the background of the Lane from each Band. The same background settings used in Odyssey Classic 3.0 software can also be used in the Western and MPX Analysis ribbons.



VI. Data Analysis Using the Odyssey® CLx

Image Studio (ver. 1.x – 2.x)

Background considerations, using Image Studio, are identical to those described in Section V. for the Odyssey Classic Infrared Imager.

VII. Data Analysis Using the Odyssey Sa

Application Software (ver. 1.x)

Background considerations, using the application software, are identical to those described in Section V. for the Odyssey Infrared Imager.

VIII. Data Analysis Using the Odyssey Fc

Image Studio (ver. 1.x – 2.x)

Background considerations, using Image Studio, are identical to those described in Section V. for the Odyssey Classic Infrared Imager.

IX. 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 detailed protocol on how to do a Western blot with an Odyssey Family Imager, see the *Odyssey Western Blot Analysis* protocol.

X. Reference

Towbin, *et al.*, (1979) *Proc. Natl. Acad. Sci.* USA 76; 4350-54

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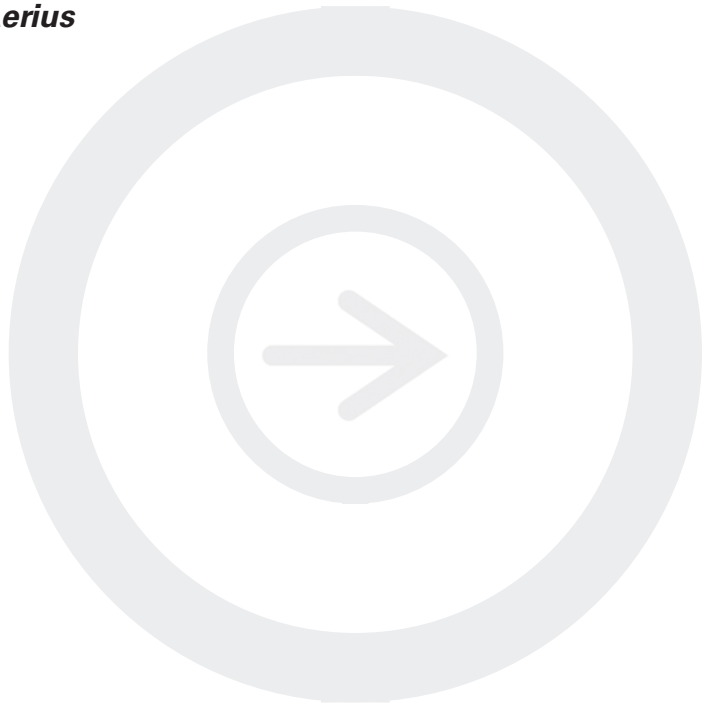
Doc #988-12623
0112

Western Blot Analysis

Developed for:

Aerius, and Odyssey® Family of Imagers

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



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Published 2003, revised January 2012. The most recent version of this document is posted at: http://biosupport.licor.com

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I. Required Reagents

- Blotted nitrocellulose (LI-COR, P/N 926-31090) or low-fluorescent PVDF membrane (LI-COR, P/N 926-31098)
- Odyssey Blocking Buffer (LI-COR, P/N 927-40000)
- Odyssey Pre-stained Molecular Weight Marker (LI-COR, P/N 928-40000)
- IRDye (680/800) Protein Markier (LI-COR, P/N 928-40006)
- Primary antibodies
- IRDye® secondary antibodies (LI-COR)
- Tween® 20
- PBS wash buffer (LI-COR, P/N 928-40018 or 928-40020)
- Ultrapure water
- Methanol for wetting of PVDF
- SDS (if desired)
- Other blocking buffers (if desired)
- NewBlot™ Stripping Buffer, if desired, for nitrocellulose (LI-COR, P/N 928-40030) or PVDF (LI-COR, P/N 928-40032) membranes

II. Western Detection Methods

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

After transfer, perform the following steps:

1. Wet the membrane in PBS for several minutes. If using a PVDF membrane that has been allowed to dry, pre-wet briefly in 100% methanol and rinse with ultrapure water before incubating in PBS.
   NOTES:
   - *Ink from most pens and markers will fluoresce on all Aerius or Odyssey Imagers. The ink may wash off and re-deposit elsewhere on the membrane, creating blotches and streaks. Pencil should be used to mark membranes. (The Odyssey pen doesn't fluoresce and can be used with nitrocellulose membranes, since the membrane will not be soaked in methanol, causing the ink to run.)*

2. Block the membrane in Odyssey® Blocking Buffer for 1 hour. Be sure to use sufficient blocking buffer to cover the membrane (a minimum of 0.4 mL/cm^2 is suggested).
   NOTES:
   - *Membranes can be blocked overnight at 4°C if desired.*
   - *Odyssey Blocking Buffer often yields higher and more consistent sensitivity and performance than other blockers. Nonfat dry milk or casein dissolved in PBS can also be used for blocking and antibody dilution, but be aware that milk may cause higher background on PVDF membranes. If using casein, a 0.1% solution in 0.2X PBS buffer is recommended (Hammersten-grade casein is not required).*
   - *Milk-based reagents can interfere with detection when using anti-goat antibodies. They also deteriorate rapidly at 4°C, so diluted antibodies should not be kept and re-used.*
   - *Blocking solutions containing BSA can be used, but in some cases they may cause high membrane background.* ***BSA-containing blockers are not generally recommended*** *and should be used only when the primary antibody requires BSA as blocker.*

3. Dilute primary antibody in Odyssey Blocking Buffer. Optimum dilution depends on the antibody and should be determined empirically. A suggested starting range can usually be found in the product information from the vendor. To lower background, add 0.1 - 0.2% Tween® 20 to the diluted antibody before incubation. The optimum Tween 20 concentration will depend on the antibody.
   NOTES:
   - *Two-color detection requires careful selection of primary and secondary antibodies. For details, see III. Guidelines for Two Color Western Detection.*
   - *The MPX™ Blotting System can be used to efficiently determine the optimum antibody concentration. For details, search for One Blot Western Optimization Using the MPX Blotting System at http://biosupport.licor.com.*

4. Incubate blot in primary antibody for 60 minutes or longer at room temperature with gentle shaking. Optimum incubation times vary for different primary antibodies. Use enough antibody solution to completely cover the membrane.

5. Wash membrane 4 times for 5 minutes each at room temperature in PBS + 0.1% Tween 20 with gentle shaking, using a generous amount of buffer.

6. Dilute the fluorescently-labeled secondary antibody in Odyssey Blocking Buffer. ***Recommended dilution can be found in the pack insert for the IRDye® conjugate***. Add the same amount of Tween 20 to the diluted secondary antibody as was added to the primary antibody.
   NOTES:
   - *Avoid prolonged exposure of the antibody vial to light.*
   - *Be careful not to introduce contamination into the antibody vial.*
   - *For best sensitivity and performance, use freshly diluted antibody solution.*
   - *Adding 0.01% - 0.02% SDS to the diluted secondary antibody (in addition to Tween 20) will substantially reduce membrane background, particularly when using PVDF. However, DO NOT add SDS during blocking or to the diluted primary antibody. See* ***V. Adapting Western Blotting Protocols for Odyssey Detection*** *for more information about how and why to use SDS in the secondary antibody incubation.*
   - *The MPX™ Blotting System can be used to efficiently determine the optimum antibody concentration. For details, search for One Blot Western Optimization Using the MPX Blotting System at http://biosupport.licor.com.*

7. Incubate blot in secondary antibody for 30-60 minutes at room temperature with gentle shaking. Protect from light during incubation.
   NOTES:
   - *Incubating more than 60 minutes may increase background.*

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

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

10. Image on Aerius, or Odyssey® Family of Imagers.
    - Scan in the appropriate channels.
    - Protect the membrane from light until it has been imaged.
    - Keep the membrane wet to strip and re-use it. Once a membrane has dried, stripping is ineffective.
    - Blots can be allowed to dry before scanning, if desired. Signal strength may be enhanced on a dry membrane. The membrane can also be re-wetted for imaging.
    - The fluorescent signal on the membrane will remain stable for several months, or longer, if protected from light. Membranes may be stored dry at 4°C.
    - If signal on membrane is too strong or too weak, re-image the membrane at a lower or higher scan intensity setting, respectively. Adjust image acquisition time for Odyssey Fc. AutoMode in Odyssey CLx may be used to improve the dynamic range of the image.

Molecular Weight Marker

If you loaded the Odyssey Prestained Molecular Weight Marker (LI-COR, P/N 928-40000), it will be visible in the 700 nm channel and also faintly visible in the 800 nm channel. If using IRDye (680/800) Protein Marker, it will be visible in both the 700 nm and 800 nm channels. Pre-stained blue molecular weight markers from other sources can also be used. Load 1/3 to 1/5 of the amount you would normally use for Western transfer. Too much marker can result in very strong marker bands that may interfere with visualization of sample lanes. If using multicolored markers, some bands may not be visualized.

Optimization Tips

1. ***Follow the protocol carefully.***

2. No single blocking reagent will be optimal for every antigen-antibody pair. Some primary antibodies may exhibit greatly reduced signal or different non-specific binding in different blocking solutions. If it is difficult to detect the target protein, changing the blocking solution may dramatically improve performance. If the primary antibody has worked well in the past using chemiluminescent detection, try that blocking solution for infrared detection.

3. Addition of detergent such as Tween® 20 can reduce membrane background and non-specific binding. Refer to **V. Adapting Western Blotting Protocols for Odyssey Detection** for details.

4. To avoid background speckles on blots, use ultrapure water for buffers and rinse plastic dishes well before and after use. Never perform Western incubations or washes in dishes that have been used for Coomassie staining.

5. Membranes should be handled only by their edges, with clean forceps.

6. After handling membranes that have been incubating in antibody solutions, clean forceps thoroughly with distilled water and/or ethanol. If forceps are not cleaned after being dipped in antibody solutions, they can cause spots or streaks of fluorescence on the membrane that are difficult to wash away.

7. Do not wrap the membrane in plastic when scanning.

8. If a Western blot will be stripped, do not allow the membrane to dry. Stripping is ineffective once a membrane has dried, or even partially dried.

III. Guidelines for Two-Color Detection

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

The following guidelines will help design two-color experiments:

1. If the two primary antibodies are derived from different host species (for example, primary antibodies from mouse and chicken), IRDye® whole IgG secondary antibodies derived from the same host and labeled with different IRDye fluorophores must be used (for example, IRDye 800CW Donkey anti-Mouse and IRDye 680LT or IRDye 680RD Donkey anti-Chicken).

2. If the two primary antibodies are monoclonals (mouse) and are IgG_1, IgG_{2a}, or IgG_{2b}, IRDye Subclass Specific secondary antibodies must be used. The same subclasses cannot be combined in a two-color Western blot (for example, two IgG_1 primary antibodies). For details, refer to *Western Blot and In-Cell Western™ Assay Detection Using IRDye Subclass Specific Antibodies.*

3. Before combining primary antibodies in a two-color experiment, always perform preliminary blots with each primary antibody alone to determine the expected banding pattern and possible background bands. Slight cross-reactivity may occur and can complicate interpretation of a blot, particularly if the antigen is very abundant. If cross-reactivity is a problem, load less protein or reduce the amount of antibody.

4. One secondary antibody must be labeled with a 700 nm channel dye, and the other with an 800 nm channel dye.

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

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

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

- Combine the two primary antibodies in the diluted antibody solution in step 3. Incubate simultaneously with the membrane (step 4).
- Combine the two dye-labeled secondary antibodies in the diluted antibody solution in step 6. Incubate simultaneously with the membrane (step 7).

IV. Stripping the Membrane

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

V. Adapting Western Blotting Protocols for Detection with Odyssey® Systems

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

Primary Antibody Concentration

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

Secondary Antibody Concentration

Optimal dilutions of dye-conjugated secondary antibodies should also be determined. Refer to the IRDye® conjugate pack insert for recommendations. The amount of secondary required varies depending on how much antigen is being detected – abundant proteins with strong signals require less secondary antibody. Use the MPX Blotting system to optimize (search for *One Blot Western Optimization Using the MPX Blotting System* at http://biosupport.licor.com).

Detergent Concentration

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

Tween® 20:

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

SDS:

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

VI. General Tips

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

2. Store the IRDye secondary antibody vial at 4°C in the dark. IRDye secondary antibodies may be aliquoted and frozen for long-term storage. Minimize exposure to light and take care not to introduce contamination into the vial. Dilute immediately prior to use. If particulates are seen in the antibody solution, centrifuge before use.

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

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

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

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

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

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

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

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

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

VII. Imaging of Coomassie-Stained Protein

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

1. Wash gels with ultrapure water for 15 minutes.

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

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

4. Scan on an Aerius or Odyssey imaging system (Odyssey Classic, Odyssey CLx, Odyssey Sa, or Odyssey Fc) in the 700 nm channel only. *Please refer to the Tutorial Manual of each instrument for further information.*

    - If using Odyssey 3.0 software, select the Protein Gel scan preset.
    - If using Aerius and/or Odyssey Sa software, set the focus offset to 3.0 plus one-half the thickness of the gel.
    - In Image Studio, select Protein Gel, using the Custom setting, under the setup tab.

VIII. Troubleshooting Guide

Problem	Possible Cause	Solution / Prevention
High background, uniformly distributed.	BSA used for blocking.	Blocking solutions containing BSA may cause high membrane back ground. Try adding SDS to reduce background, or switch to a different blocker.
	Not using optimal blocking reagent.	Compare different blocking buffers to find the most effective; try blocking longer.
	Background on nitrocellulose.	Add Tween® 20 to the diluted antibodies to reduce background. Try adding SDS to diluted secondary antibody.
	Background on PVDF.	Use low-fluorescent PVDF membrane. With IRDye® 680LT conjugates, always use SDS (0.01-0.02% final concentration) and Tween 20 (0.1-0.2% final) during the detection incubation step.
	Antibody concentrations too high.	Optimize primary and secondary antibody dilutions using MPX™ blotting system. For details, see One Blot Western Optimization Using the MPX Blotting System at http://biosupport.licor.com
	Insufficient washing.	Increase number of washes and buffer volume.
		Make sure that 0.1% Tween 20 is present in buffer and increase if needed. Note that excess Tween 20 (0.5-1%) may decrease signal.
	Cross-reactivity of antibody with contaminants in blocking	Use Odyssey Blocking Buffer instead of milk. Milk usually contains IgGs that cross-react with anti-goat secondary antibodies.
	Inadequate antibody volume used.	Increase antibody volume so entire membrane surface is sufficiently covered with liquid at all times (use heat-seal bags if volume is limiting). Do not allow any area of membrane to dry out.
		Use agitation for all antibody incubations.
	Membrane contamination.	Always handle membranes carefully and with clean forceps. Do not allow membrane to dry. Use clean dishes, bags, or trays for incubations.

Problem	Possible Cause	Solution / Prevention
Uneven blotchy or speckled background.	Blocking multiple membranes together in small volume.	If multiple membranes are being blocked in the same dish, ensure that blocker volume is adequate for all membranes to move freely and make contact with liquid.
	Membrane not fully wetted or allowed to partially dry.	Keep membrane completely wet at all times. This is particularly crucial if blot will be stripped and re-used.
		If using PVDF, remember to first pre-wet in 100% methanol.
	Contaminated forceps or dishes.	Always carefully clean forceps after they are dipped into an antibody solution, particularly dye-labeled secondary antibody. Dirty forceps can deposit dye on the membrane that will not wash away.
		Use clean dishes, bags or trays for incubations.
	Dirty scanning surface or silicone mat.	Clean scanning surface and mat carefully before each use. Dust, lint, and residue will cause speckles.
	Incompatible marker or pen used to mark membrane.	Use only pencil or Odyssey® pen (nitrocellulose only) to mark membranes.
Weak or no signal.	Not using optimal blocking reagent.	Primary antibody may perform substantially better with a different blocker.
	Insufficient antibody used.	Primary antibody may be of low affinity. Increase amount of antibody or try a different source.
		Extend primary antibody incubation time (try 4 - 8 hr at room temperature, or overnight at 4°C).
		Increase amount of primary or secondary antibody, optimizing for best performance.
		Try substituting a different dye-labeled secondary antibody.
		Primary or secondary antibody may have lost reactivity due to age or storage conditions.
	Too much detergent present; signal being washed away.	Decrease Tween® 20 and/or SDS in diluted antibodies. Recommended SDS concentration is 0.01 - 0.02%, but some antibodies may require an even lower concentration.

Problem	Possible Cause	Solution / Prevention
Weak or no signal. *(Continued)*	Insufficient antigen loaded.	Load more protein on the gel. Try using the narrowest possible well size to concentrate antigen.
	Protein did not transfer well.	Check transfer buffer choice and blotting procedure.
		Use pre-stained molecular weight marker to monitor transfer, and stain gel after transfer to make sure proteins are not retained in gel.
	Protein lost from membrane during detection.	Extended blocking times or high concentrations of detergent in diluted antibodies may cause loss of antigen from the blotted membrane.
	Proteins not retained on membrane during transfer.	Allow membrane to air dry completely (1 - 2 hr) after transfer. This helps make the binding irreversible.
		Addition of 20% methanol to transfer buffer may improve antigen binding. Note: Methanol decreases pore size of gel and can hamper transfer of large proteins.
		SDS in transfer buffer may interfere with binding of transferred proteins, especially for low molecular weight proteins. Try reducing or eliminating SDS. *Note: Presence of up to 0.05% SDS does improve transfer efficiency of some proteins.*
		Small proteins may pass through membrane during transfer ("blow-through"). Use membrane with smaller pore size or reduce transfer time.
Non-specific or unexpected bands.	Antibody concentrations too high.	Reduce the amount of antibody used. Reduce antibody incubation times. Increase Tween® 20 in diluted antibodies.
		Add or increase SDS in diluted secondary antibodies.
	Not using optimal blocking reagent.	Choice of blocker may affect background bands. Try a different blocker.
	Cross-reactivity between antibodies in a two-color experiment.	Double-check the sources and specificities of the primary and secondary antibodies used (see III. Guidelines for Two-Color Detection).
		Use only highly cross-adsorbed secondary antibodies.

Cause	Possible Cause	Solution / Prevention
Non-specific or unexpected bands. *(Continued)*		There is always potential for cross-reactivity in two-color experiments. Use less secondary antibody to minimize.
		Always test the two colors on separate blots first so you know what bands to expect and where.
		Avoid using mouse and rat antibodies together, if possible. Because the species are so closely related, anti-mouse will react with rat IgG to some extent, and anti-rat with mouse IgG. Sheep and goat antibodies may exhibit the same behavior.
	Bleed through of signal from one channel into other channel.	Check the fluorescent dye used. Fluorophores such as Alexa Fluor® 750 may appear in both channels and are not recommended for use with the Odyssey® Imaging Systems.
		If signal in one channel is very strong (near or at saturation), it may generate a small amount of bleedthrough signal in the other channel. Minimize this by using a lower scan intensity setting in the problem channel. Try AutoMode on Odyssey CLx.
		Reduce signal by reducing the amount of protein loaded or antibody.

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All other countries, contact LI-COR Biosciences or a local LI-COR distributor:
http://www.licor.com/distributors

www.licor.com/bio

Doc# 988-12622
0112

Odyssey Western Blot Blocker Optimization

Developed for:

Aerius and Odyssey® Family of Imagers

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

Part Numbers:

927-40000
927-40003
927-40010
927-40050
927-40100
927-40125
927-40150
927-40200
927-40300

LI-COR®

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I. Required Reagents

- Odyssey Protein Molecular Weight Marker (LI-COR P/N 928-40000)
- IRDye® Secondary Antibodies (LI-COR)
- Blocking Buffer
  - Blocking Buffer Sample Pack (LI-COR P/N 927-40050)
    - Odyssey Blocking Buffer
    - Casein Blocking Buffer
  - Odyssey Blocking Buffer (LI-COR P/N 927-40100 and 927-40000)
  - Casein Blocking Buffer, (LI-COR P/N 927-40300 and 927-40200)
- Membrane
  - Odyssey Nitrocellulose (0.22 µm), 10 pack (LI-COR P/N 926-31090)
  - Odyssey Nitrocellulose (0.22 µm), roll (LI-COR P/N 926-31092)
  - Millipore Immobilon®-FL (0.45 µm)
    - Blocking Buffer & PVDF Membrane Kit (LI-COR P/N 926-31098)
    - 4X Sample Loading Buffer and PVDF Membrane Kit (LI-COR P/N 926-31097)
- Primary antibodies (primary antibodies must be from host species compatible with the secondary antibodies being used -- if using subclass specific antibodies, please refer to Technical Note "Western Blot and In-Cell Western™ Assay Detection Using IRDye® Subclass Specific Antibodies").
- Tween® 20
- PBS Buffer (LI-COR P/N 928-40020)
- Methanol (when using Immobilon®-FL PVDF membrane)

- SDS (when using Immobilon-FL PVDF membrane)

- Western Blot Incubation Box
  – Medium (8.9 x 6.6 x 2.9 cm), LI-COR P/Ns 929-97201 (1 pack), 929-97205 (5 pack), and 929-97210 (10 pack)

II. Gel Preparation for Blocker Optimization

Standard protein electrophoresis conditions and reagents can be used for gel and sample preparation. Following is a suggested template for sample electrophoresis to maximize blocker optimization and efficiently choose the best blocking conditions for a given primary antibody.

Using a 15-well gel, load the following samples in order indicated:

Lane	Sample	Amount
1	Protein Marker	1-3 µL
2	Primary Antibody	5 µL of a 1:1000* dilution in PBS
3	Sample Lysate	10 µg
4	Sample Lysate	5 µg
5	Sample Lysate	2.5 µg
6	Protein Marker	1-3 µL
7	Primary Antibody	5 µL of a 1:1000* dilution in PBS
8	Sample Lysate	10 µg
9	Sample Lysate	5 µg
10	Sample Lysate	2.5 µg
11	Protein Marker	1-3 µL
12	Primary Antibody	5 µL of a 1:1000* dilution in PBS
13	Sample Lysate	10 µg
14	Sample Lysate	5 µg
15	Sample Lysate	2.5 µg

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

III. Western Blocker Optimization Method

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

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

NOTE: Do not write on membrane with an ink pen or marker, as the ink will fluoresce on the Odyssey Imager. Mark with pencil or Odyssey Pen (P/N 926-71804) to avoid this problem. Use pencil only for PVDF membrane, as wetting in methanol will cause ink to run.

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

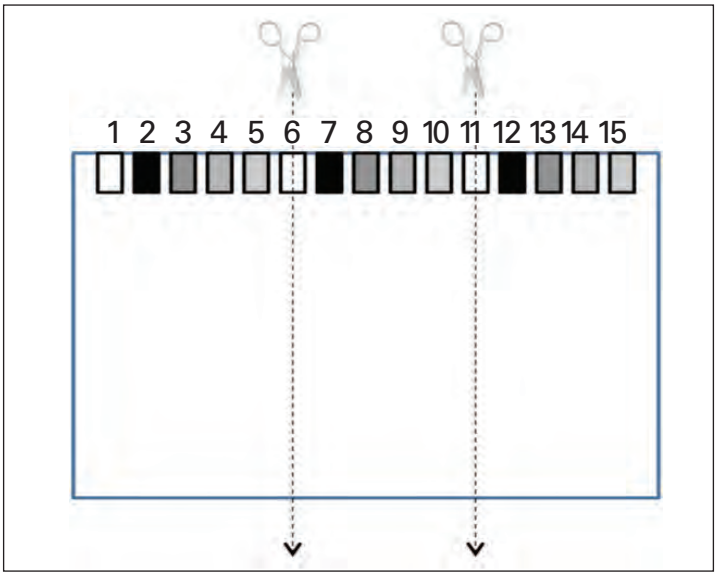

Figure 1. Cut Western blot along the Marker lanes into three individual optimization blots.

After cutting membrane, perform the following steps:

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

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

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

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

    * *The correct working range for antibody dilution depends on the characteristics of your primary antibody. Start with the dilution recommended by the primary antibody vendor for Western blot applications.*

4. Incubate blots in diluted primary antibody for 1 to 4 hours* at room temperature, or overnight at 4 °C while gently shaking.

    * *Incubation times vary for different primary antibodies.*

5. Wash membranes:

    - Pour off primary antibody solution.
    - Rinse membrane with 1X PBS-T (0.1% Tween® 20).
    - Cover blot with 1X PBS-T (0.1% Tween 20).
    - Shake vigorously on platform shaker at room temperature for 5 minutes.
    - Pour off wash solution.
    - Repeat 3 additional times.

6. Dilute secondary antibody* in 10 mL of appropriate diluent listed below:

    Secondary antibody diluent for Immobilon®-FL PVDF membrane
    - Box 1 – Odyssey Blocking Buffer + 0.2% Tween 20 + 0.01% SDS + Secondary Antibody
    - Box 2 – Casein Blocking Buffer + 0.2% Tween 20 + 0.01% SDS + Secondary Antibody
    - Box 3 – Blocking Buffer of your choice + Secondary Antibody

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

    * *For IRDye® 800CW and IRDye 680RD conjugates, suggested dilution range is 1:5,000 to 1:25,000 and may require optimization. For IRDye 680LT conjugates, suggested dilution range is 1:20,000 to 1:50,000. Please consult pack insert.*

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

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

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

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

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

Tips

- Follow the protocol carefully.

- For additional Odyssey Western detection tips, see www.licor.com/WesternBlotTips

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Normalization Accuracy for Western Blotting

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1. Introduction

Internal controls are essential for accurate, quantitative measurement of target protein expression on Western blots. These controls are used to correct for errors introduced by sampling irregularities, unequal loading, and uneven protein transfer across a membrane. These errors are inevitable and arise from technical variation rather than biological differences in the amount of protein expression. When a loading control is used for normalization, the data are typically rescaled to a smaller range by expressing each data point relative to the strongest signal obtained on the blot (generating data that range from 0 – 1, as shown in Fig. 1 and Table 1). This eliminates variation introduced by sample handling and allows the researcher to compare data that may exhibit small but meaningful changes in values. The process of data normalization is described in Section 2.

The protein products of housekeeping genes (HKGs) are often used as loading controls, because they are generally thought to be expressed at consistent levels across nearly all tissue types and experimental conditions; however, some treatments and conditions may cause variability in HKG expression (discussed in detail in Section 4). If such variability occurs, it will likely affect the outcome of data analysis when normalization is performed. Your choice of loading control is a very important parameter, and must be carefully considered to ensure accurate, quantitative measurement of target protein expression. See Section 4 for more information about choosing an appropriate HKG as a loading control.

2. Normalization of RNA silencing data from a Western blot

2.1 Western blot data

RNAi targeted gene silencing is now a well-established method used to answer critical biological questions. The method requires very rigorous controls and careful selection of siRNAs in order to limit off-target effects. RNA silencing using qRT-PCR only measures the mRNA levels of a target gene, and not protein levels. Concomitant validation of reduction of target protein expression is essential to confirm that protein levels reflect observed changes in gene expression, and mRNA levels confirm that the transcript half-life is not of an extended nature. Figure 1 presents data from a gene silencing experiment where HeLa cells were transfected in triplicate with either nonsense control siRNA (NS1, 2 and 3), or AKT siRNA (AKT1, 2, and 3). The Western blot utilized two spectrally different near-infrared (NIR) labeled secondary antibodies to facilitate normalization of AKT (700 nm, red) to the housekeeping gene, actin, in the 800 nm channel (green).

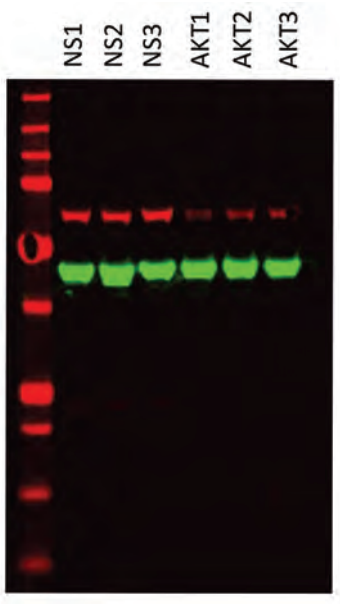


Figure 1. Two-color Western blot of HeLa cell lysates transfected with nonsense control siRNA (NS1-3) or AKT siRNA (AKT1-3). The proteins were detected with either mouse anti-pan actin and IRDye® 800CW Goat anti-Mouse IgG (LI-COR® P/N 926-32210) or rabbit anti-AKT and IRDye 680LT Goat anti-Rabbit IgG (LI-COR P/N 926-68021).

Table 1. Data analysis of AKT silencing in HeLa cells

		NS1	NS2	NS3	AKT1	AKT2	AKT3
Raw Data	800 channel Integrated Intensities (I.I.)	**55379**	53468	52784	49352	50744	48382
	700 channel Integrated Intensities (I.I.)	16196	15155	19491	4210	6468	5157
Normalized Data	Relative 800 I.I.= All I.I./greatest I.I. value	**1**	0.97	0.95	0.89	0.92	0.87
	700 channel I.I./Relative 800 I.I. for that lane	16196	15697	20450	4724	7059	5903
Data Analysis	GM* of adjusted NS1-3 values	17323					
	GM of adjusted AKT1-3 values	5817					
	% Silencing **	66%					

*GM=Geometric mean
** % silencing calculated using: 100-(AKT GM/NS GM) x 100

2.2 Steps for normalization:

1. Determine which sample has the highest value for the normalization control. In this example, the standard is actin (detected in the 800 nm channel with IRDye® 800CW) and the highest value is 55379. Divide each value for actin by 55379 to get a relative value (third row of Table I). All of the values will be between 0 and 1.00.

2. Divide the target protein values (AKT, detected in the 700 nm channel with IRDye 680LT) by the calculated relative 800 nm value for the matching sample. For example, the NS1 700 nm value is 16196 and should be divided by the NS1 calculated relative 800 nm value of 1.00. NS2 is 15155 and is divided by 0.97, etc. (fourth row of Table I).

3. The adjusted values are then used in the calculation of the geometric mean for both the standard and the target protein (see Section 2.3).

4. In this example, the numbers were then used to calculate the percent of RNA silencing using the equation 100-(AKT GM*/NS GM) X 100 = 66% silencing.
   **GM = Geometric mean.*

2.3 Using the geometric mean

For normalization of data, it is often helpful to calculate the geometric mean rather than the arithmetic mean. The geometric mean is much less affected by outliers, because the formula employs the *n*th root of the product of *n* values in a data set.

Example: Data set values = 9, 4, 1

- The geometric mean of this data set is **3√ (9 x 4 x 1) = 3.3**.
- The arithmetic mean for the same data set is the sum of all values in the data set, divided by the number of values, *n*, in that set. Therefore, the arithmetic mean is **(9 + 4 + 1) / 3 = 4.7**.

The geometric mean compensates for the very high and very low values, and is therefore a more robust and appropriate method for normalization of protein levels (Vandesompele, *et al*. 2002)

3. Housekeeping genes as normalization controls

The protein products of housekeeping genes (HKGs) are particularly applicable as controls because of their involvement in cell maintenance and critical cell functions such as transcription initiation control, ribosomal or cytoskeletal structure, and in the regulation of metabolic pathways and protein synthesis. In short, they are chosen because their expression is indispensable for cell survival. HKGs were originally defined as highly expressed genes that were consistently expressed across various tissue types and under all experimental conditions. Several benchmark studies employing high density nucleotide arrays and using various tissue types have provided a compendium of HKGs that are expressed in all tissue types, and also identified tissue-selective genes (Warrington et al, 2000; Hsiao *et al*, 2001). The tissue-selective genes may be expressed in a number of tissues, but are predominantly expressed in only a few. This type of gene expression may be indicative of tissue function and could serve as a marker and/or drug target for a disease state.

4. Housekeeping genes and expression variability

4.1 Variability in tissue types

Many studies have addressed possible variability of HKG expression, especially for popular controls such as glyceraldehyde-3-phosphate dehydrogenase (GAPDH), hypozanthine phosphoribosyltransferase 1 (HPRT1), and ribosomal protein large P1 (RPLP1). In one study, GAPDH expression was evaluated by qRT-PCR in 72 pathologically normal tissues, and was found to be higher in tissues that require greater energy demands (such as skeletal muscle, brain and heart) than in tissues such as the pancreas, ovary, and esophagus (Barber, *et al*, 2005). However, GAPDH expression was quite similar within the same tissue and even within clusters of related tissues, such as stomach antrum, body, and fundus, or the kidney cortex, medulla, and pelvis. However, in another study that ranked HKGs by average expression intensity in 42 tissues, GAPDH was listed as one of the top 20 HKGs with the highest and most consistent average expression (She *et al*, 2009). A study that examined HPRT1 and RPLP1 expression using qRT-PCR reported age-specific differential expression in adult and neonatal cardiac cells (Tan *et al*, 2011).

4.2 Variability caused by cell treatments

The tendency of HKGs to produce their protein products at a steady rate is the basis for their popularity as controls; however, HKG expression may vary not only between tissue types (as described above), but also within a single tissue type or cell type. Chemical or pharmacological treatments and environmental changes can also have wide-ranging effects. Conversely, some HKGs are not affected by cell treatments. HPRT1 has been shown to be an excellent internal control for estrogen studies in fathead minnows as well as in mammals, because it has been shown to be estrogen independent (Filby and Tyler, 2007; Rey *et al*, 2000).

4.3 Variability caused by environmental changes

Environmental changes can also affect expression of HKGs. Hypoxia is known to affect levels of GAPDH. In non-proliferating cells or cells treated with anti-proliferation agents, proliferating cell nuclear antigen (PCNA) is useless as a control. T cell activating agents such as PHA and PMA have no effect on 18S rRNA

levels or on beta-2-microglobulin expression in human lymphoblastoid cells, but do affect transcription binding protein (TBP) (Anis *et al*, 2005; Banda *et al*, 2008).

Overexpression of proteins is a hallmark of cancer, and HKGs are no exception. For example, ribosomal proteins L7a and L37 were recently found to be overexpressed in prostate cancer tissues, compared to a normal prostate epithelial cell line (Blanquicett *et al*, 2002). HKGs encoding metabolic enzymes have also demonstrated considerable change in expression in cancerous colon tissues (Blanquicett *et al*, 2002).

5. Choosing the correct normalization control

All of the factors that can affect HKG expression must be kept in mind when choosing normalization standard(s) for a given experiment. These important points should be considered:

1. Does your experimental protocol make comparisons between different types of tissues?

2. Does your experimental protocol make comparisons between treated and untreated cells within the same cell line?

3. Does your experimental protocol make comparisons between normal and cancerous tissue of the same type?

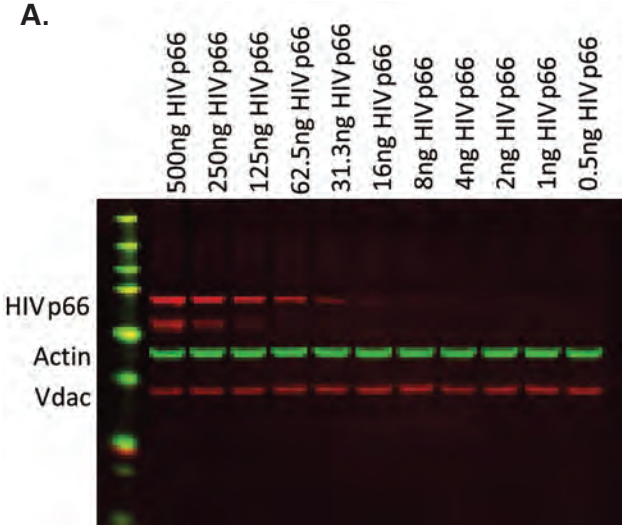
In all of these cases, it is important to run a pilot experiment under the same conditions you plan to use for the full experiment, to make sure that the expression of your normalization standard is NOT affected by the experimental protocol (i.e., chemical treatment, environmental changes, tissue choice). The pilot experiment is especially critical when making comparisons across tissue types.

Once the experimental factors that affect the expression of your internal standard have been accounted for, you must be certain that the detection limit and linear range of detection for the normalization standard fall within the same parameters as your target protein. The detection limit and linear range defines the useful scope of the assay. The limit of detection is the lowest concentration of analyte that can be reliably detected by your instrumentation above the blank, while the linear range is the extent to which quantification can be made with a known level of confidence (Armbruster and Pry, 2008). Therefore, not only must you be at or above the level of detection, but you must be able to precisely measure differences in analyte amounts where predefined goals for bias and imprecision, such as a coefficient of variation (CV), are met (ie., CV = 20% for 3 replicates). If your unknown target protein falls outside of the defined standards, then you will be unable to assign a quantitative value because the level of protein will either be too low to detect, or the level will be so high the protein level will reach saturation.

The example given in Figure 2A shows two-fold dilutions of purified HIV p66 spiked into wells loaded with 5 µg of C32 cell lysate. After SDS-PAGE and transfer to nitrocellulose, a Western blot was performed using anti-HIV p66, anti-actin, and anti-Vdac antibodies. The membrane was then scanned on an Odyssey® imager (Fig. 2A), and the HIV p66, actin, and Vdac proteins were assigned a signal intensity (Fig. 2B, 2D). The intensity values for HIV p66 and Vdac were normalized against the actin loading control (Fig. 2B, 2D). The HIV p66 values were then used to create a standard curve (Fig. 2C) to interpolate values for the unknown amounts of Vdac protein in each lane (Fig. 2D).
In this example, the unknown concentrations of Vdac were within the actin-normalized HIV p66 values, and interpolated values were assigned. If the unknowns had fallen outside of the actin-normalized HIV p66 values, the values would be subject to considerable error. If the values were above the normalized value

for HIV p66 500 ng, there is a possibility of saturation of the signal and underestimation of the true signal intensity of the protein, based on the inability of the instrument to measure differences in protein concentration at that level. The same type of inaccuracy can occur if the signal intensity falls below the lowest normalized HIV p66 value. If the signal intensity is also below the detection limit, concentration of the protein would be indistinguishable from the blank control.

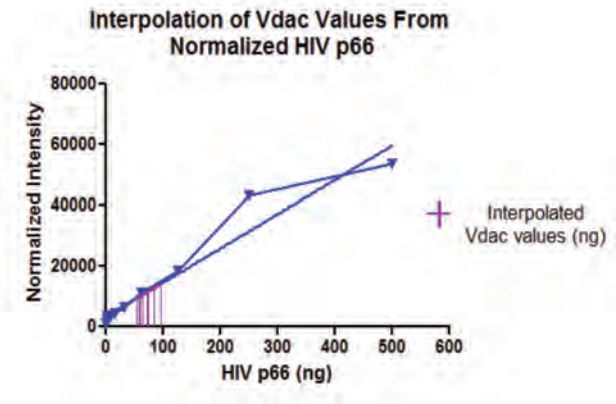
A.



B.

HIV p66	Intensity	Actin Intensity	Normalized HIV p66 Values
500 ng	48763	25782	53681
250 ng	40213	26362	43294
125 ng	16785	25934	18369
62.5 ng	10519	26808	11137
31.3 ng	6028	26799	6384
16 ng	4331	28382	4331
8 ng	2925	25823	3215
4 ng	2069	24597	2387
2 ng	2503	28195	2520
1 ng	3441	27224	3587
0.5 ng	1188	27467	1228

C.



D.

Vdac	Intensity	Normalized to Actin	Interpolated Values
Lane 1	8675	9550	55
Lane 2	9293	10005	59
Lane 3	9271	10146	60
Lane 4	10934	11576	73
Lane 5	11052	11705	74
Lane 6	11672	11672	73
Lane 7	12873	14149	95
Lane 8	9271	10698	65
Lane 9	11591	11668	73
Lane 10	12289	12812	84
Lane 11	11272	11648	73

Figure 2.

A. Two-fold dilutions of purified HIV p66 spiked into 5 µg of C32 cell lysates used as a standard curve to interpolate unknown Vdac values in a Western blot. HIV p66 was detected using mouse anti-p66 (Immunodiagnostics, Woburn, MA), and Vdac was detected using mouse anti-Vdac (Mitosciences, Eugene, OR). Both target proteins were detected with IRDye® 680LT Goat anti-Mouse IgG (LI-COR P/N 926-68020) and an Odyssey® Imager. Actin was detected using Beta-Actin Rabbit Monoclonal antibody (LI-COR® P/N 926-42210) with IRDye 800CW Goat anti-Rabbit IgG (LI-COR P/N 926-32211).

B. Table showing HIV p66 standard values in ng, HIV p66 signal intensity, actin signal intensity in the corresponding lane, and the HIV p66 signal intensity values normalized to actin.

C. Standard curve of HIV p66 normalized signal intensity values vs HIV p66 in ng used to interpolate unknown normalized Vdac values in corresponding lanes.

D. Table showing Vdac signal intensities, Vdac signal intensities normalized to actin, and interpolated values from the HIV p66 standard curve.

It is important to keep in mind that high-abundance target proteins should be normalized against highly expressed HKGs (such as actin or tubulin), while low-abundance proteins should be normalized against lower expressed HKGs (such as COX IV or HPRT1; see Figure 3) to ensure that detection limits and linear ranges are similar. The COX IV antibody is an excellent loading control for normalization of low-abundance target proteins. For highly-expressed proteins like actin, heavy loading (> 15 µg lysate/lane) may affect linearity and accuracy of detection.

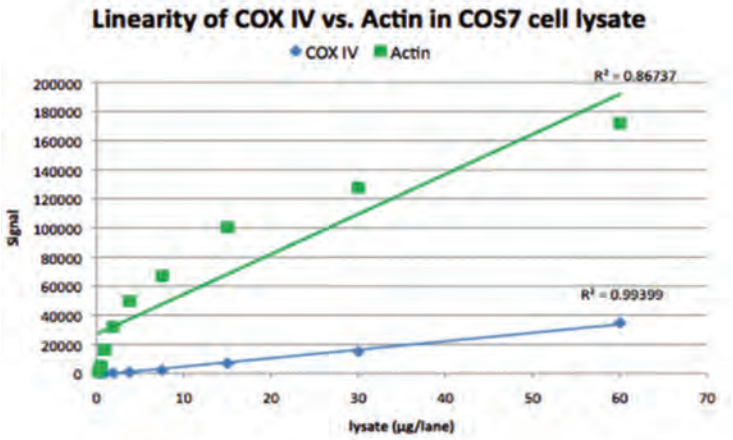


Figure 3. Detection of COX IV with COX IV Rabbit Monoclonal primary antibody (LI-COR® P/N 926-42214) is linear across a wide range, even when lysate is heavily loaded. COX IV and actin (Beta-Actin Rabbit Monoclonal; LI-COR P/N 926-42210) were detected in COS7 cell lysates, using IRDye® 800CW Goat anti-Rabbit (LI-COR P/N 926-32211) and the Odyssey® Imager.

6. Choosing the correct method of detection for normalization

6.1 Chemiluminescent detection (ECL)

The method used for Western blot detection can also be critical for obtaining accurate quantitative data. Chemiluminescent reagents are commonly used, and signal is captured either by film or by digital imaging. In chemiluminescent detection, light is generated by a dynamic enzymatic reaction, producing qualitative or semi-quantitative results (depending on the substrate, imaging system, and protocol used).

Exposure time can have a dramatic effect, and requires optimization. This can be especially problematic when the protein of interest is of low abundance, relative to the chosen loading control. Saturation and spreading of the strong control bands (blowout) can obscure variations in sample loading and make it impossible to accurately quantify the loading controls. Saturation can also limit the linear dynamic range, particularly when film is used. Densitometric analysis of film adds another layer of inaccuracy to quantitation of proteins. Densitometry is a measure of optical density and is therefore an indirect function of the light generated by the chemiluminescent substrate (Baskin and Stahl, 1993).

The use of digital imaging avoids some of the undesirable aspects of film capture of chemiluminescence. In digital imaging, the dynamic range is influenced by the type of chemiluminescent substrate used. Long-duration substrates provide better dynamic range but are more expensive. The distance of the membrane from the camera requires long integration times and capturing multiple images of the same blot may not be possible; however, the image is digitally archived and densitometric scanning is not an issue.

Both film and digital imaging require that the linear range is defined with standards and that the unknown samples must fall within the detectable range delineated by the standards. Normalization with chemiluminescence is complicated by the one-color, single-plex nature of the method. The blot must be stripped and reprobed with a normalization antibody, or membranes prepared in duplicate and probed separately. The stripping process can vary widely, causing inconsistent and undetermined protein loss from the membrane that is likely influenced by the amino acid composition and hydrophobic side chains of each protein sequence (Matsudaira, 1987). Quantitation of a stripped blot is therefore compromised, and this limitation should always be kept in mind. Normalization on separate blots is inaccurate due to blot-to blot variations resulting from loading errors and variable protein transfer. Normalization can be performed on a single blot if the normalization protein and target protein are sufficiently different in size; however, appropriate controls to measure antibody cross-reactivity must be in place to ensure accuracy.

6.2 NIR fluorescent detection

Near-infrared (NIR) fluorescence detection is also used for Western blot analysis. This method employs fluorophore-labeled antibodies to generate a stable, reproducible fluorescent signal that is detected with a laser-based imager. No enzyme or substrate is required. Fluorescent detection does not require optimization of exposure times, and allows both strong and weak bands to be imaged clearly. The fluorescent signal is directly proportional to the amount of target protein.

Multiplex detection is easily achieved using secondary antibodies labeled with two spectrally-distinct NIR fluorophores (Fig. 4). This allows simultaneous detection of the normalization standard and the protein target, even if they are similar in molecular weight, without stripping and reprobing. An example of this concept is the ratiometric analysis of total Epidermal Growth Factor Receptor (EGFR) and phosphorylated EGFR in unstimulated and EGF-stimulated A431 cells. EGFR is recognized by the pan-EGFR antibody and the IRDye® 800CW (green) secondary antibody, while phosphorylated EGFR is recognized by the anti-phospho-EGFR antibody and the IRDye 680 (red) secondary antibody. The two channels (red and green) can be overlaid to show the total protein (yellow) in the EGF stimulated cells. This type of multiplexing is not possible with chemiluminescence, and it greatly increases the accuracy of quantitative immunoblotting.

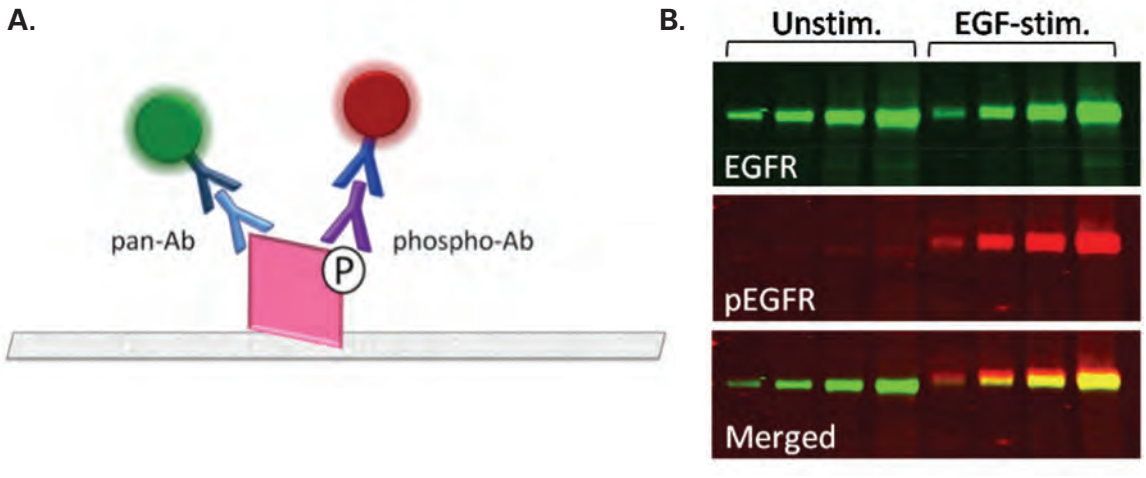


Figure 4.
A) Multiplex phosphorylation analysis combines a phospho-antibody with an antibody that recognizes the target protein regardless of its phosphorylation state (pan-antibody).
B) Multiplex phosphorylation analysis used to detect EGFR phosphorylation in EGF-stimulated A431 cells. This type of normalization corrects for both loading variation and changes in levels of the target protein.

Multiplex detection for normalization using an HKG protein unrelated to the target was illustrated in Section 2 in an RNAi gene silencing experiment (Fig. 1). If your experiment requires more than one normalization control, separate lanes can be assigned various antigen/antibody combinations, the membrane cut and incubated with appropriate antibodies separately, and then realigned for imaging. It is very important to include single antibody incubations to control for cross-reactivity if you use multiplex detection.

The benefits of NIR imaging for Western blot normalization were clearly shown in a study published in 2008, where the linearity of beta-actin and GAPDH signals was evaluated at various times and sample concentrations. A load-dependent response in signal intensity was observed over a 250-fold range of sample concentrations, with R^2 values as high as 0.9939 (Weldon *et al,* 2008). Longer antibody incubations continued to detect differences in protein levels, and load-dependent responses became more linear. These findings were in direct contrast to a previous study that examined the same controls using chemiluminescent Western blot analysis (Dittmer and Dittmer, 2006). That study reported failure to distinguish load-dependent differences in beta-actin signals, especially with longer incubation times.

7. Conclusions

The normalization of data to an internal control is critical for meaningful quantitative analysis. Small changes in protein expression can have a huge impact on data interpretation. Your choice of HKG can significantly affect the outcome, and it is well worth the time and attention to carefully select the most appropriate HKG(s) for your experiment. Pilot experiments should be performed to be certain your choice is appropriate for the experimental protocol, to ensure the best outcome. Detection method also is an important factor and should be a significant part of the pre-experimental thought process. A single HKG may not be the best for your experimental protocol, and careful consideration of all factors affecting HKG expression must be taken into account to achieve the best result.

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Doc # 988-13547
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Chemiluminescent Western Blots

Frequently Asked Questions

Developed for:

Odyssey® Fc Imaging System

LI-COR® Biosciences

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

1. Blocking Buffer

1.1 Can I dilute the HRP-conjugated secondary antibodies in the Odyssey® blocking buffer?

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

1.2 Can I use the Odyssey blocking buffer to block my blot?

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

1.3 Can I use milk-based blockers?

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

1.4 What is the best blocker for chemiluminescent Western blots?

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

2. Primary and Secondary Antibodies

2.1 Why is the signal missing in the middle of the bands?

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

2.2 Does it matter where I purchased the HRP-conjugated secondary antibodies?

The reactivity of secondary antibodies ranges widely between vendors. As well, the ratio of HRP enzyme to antibody varies, and may affect the detection of the target. If the secondary antibodies from one vendor are not working, trying antibodies from other vendors may be helpful.

2.3 Should the HRP-conjugated secondary antibodies be highly cross-adsorbed?

Although highly cross-adsorbed antibodies are essential for two-channel, multiplex detection, it is not always necessary with chemiluminescent blotting for a single target.

3. Washing Buffer

3.1 Does it matter how I wash the membranes after antibody incubation?

Yes. Adequately washing the membranes will greatly improve the appearance of the chemiluminescent Western blot. Wash the membranes with a saline-buffered solution con-

taining 0.05 to 0.1% of a non-ionic detergent such as Tween® 20. Wash four times for five minutes each time with ample wash solution on a shaker or rotator.

4. Substrate

4.1 Which substrate do I use?

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

4.2 How do I apply the substrate?

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

4.3 The membrane dried during imaging. Can I apply more substrate and image again?

No. Applying more substrate to a dried blot will likely result in high background.

4.4 How do I keep the membrane from drying out?

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

5. Imaging

5.1 Can I use the Odyssey Fc Imaging Tray multiple times?

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

5.2 Can I wrap the blot in plastic wrap before imaging?

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

5.3 Why are the bands on my blot so light?

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

continued

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

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

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

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Optimizing Chemiluminescent Western Blots

Developed for:

Odyssey® Fc Imaging System

LI-COR®
Experience Excellence

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

Contents

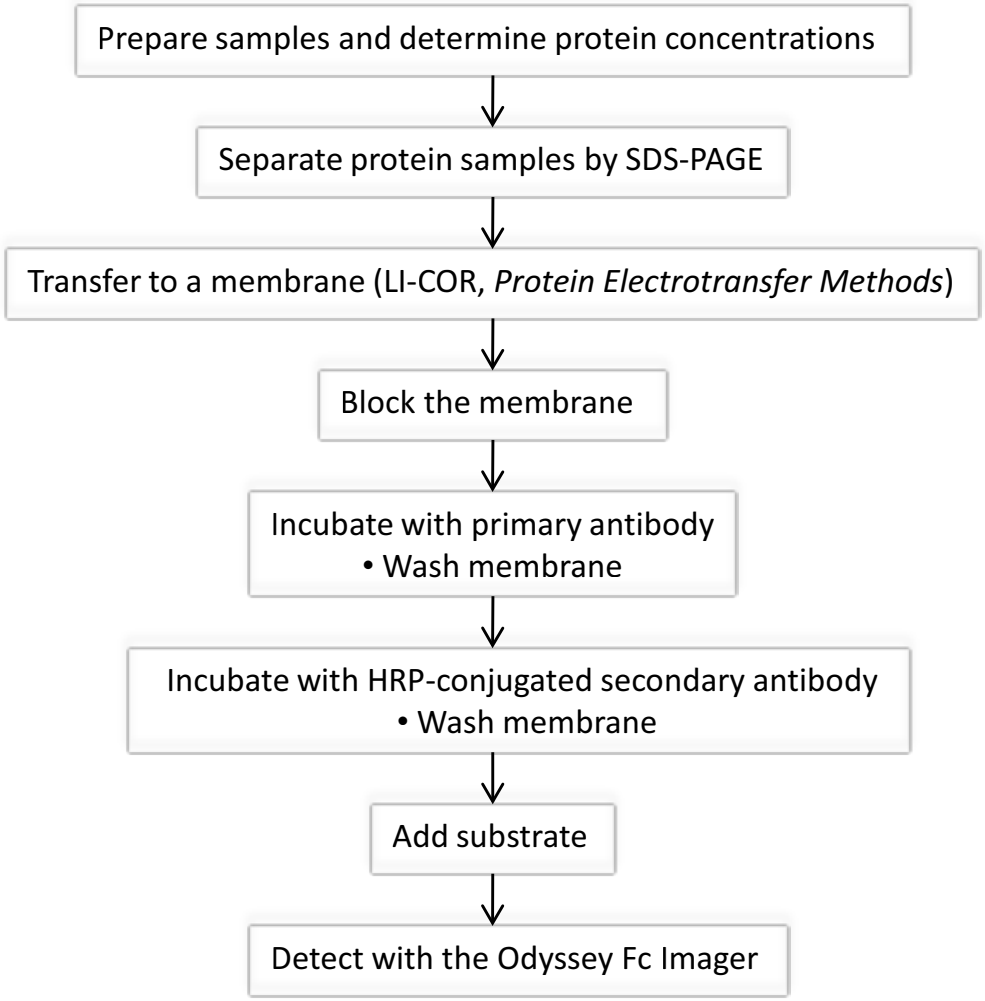
I. Introduction to Chemiluminescent Western Blotting on the Odyssey Fc Imager

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.

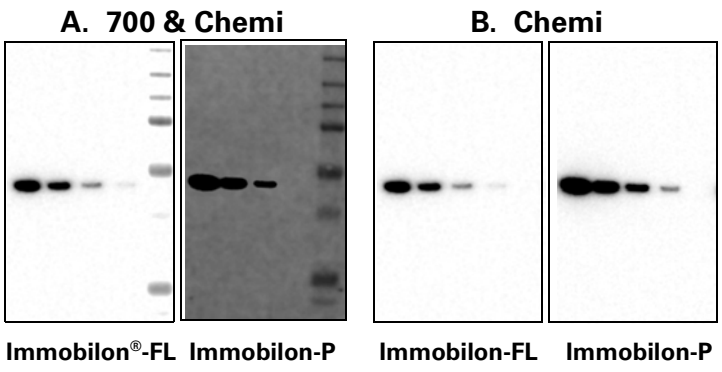


Figure 1. Two-fold dilutions of C32 lysate (1.25 µg - 156 µg) were resolved on a 10% Bis Tris gel and transferred to the indicated PVDF membranes. The membranes were blocked with skim milk, probed with rabbit ß-actin antibody (LI-COR, P/N 926-42211), and detected with Goat Anti-Rabbit-HRP. The blots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

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 characterisitics 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.

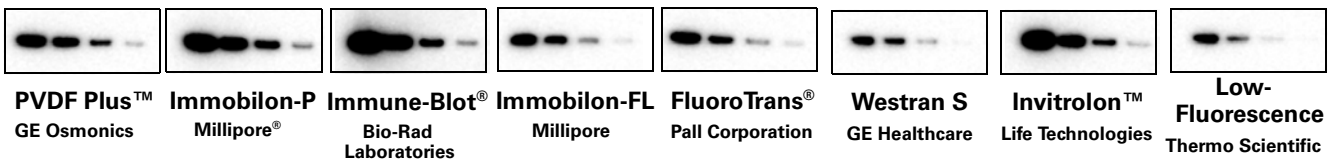


Figure 2. Odyssey Fc (2 minute) images shown in the Chemi channel. The settings for all of the images are normalized by linking the Lookup Tables in Image Studio software. Experimental details are identical to those provided in Figure 1 above.

B. Blocking Buffer

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

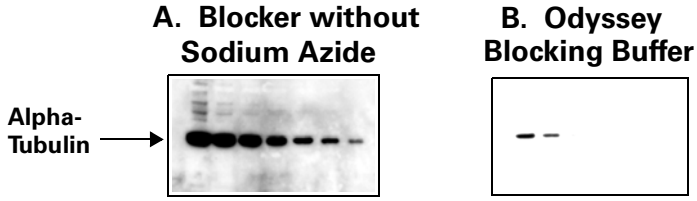


Figure 3. Serial dilutions of NIH/3T3 cell lysate were probed with Alpha-Tubulin Mouse mAb (LI-COR, P/N 926-42213). Substrate applied was SuperSignal® West Dura (Thermo Scientific). Images were acquired on the Odyssey Fc Chemi channel for 2 minutes, shown with normalized image display settings. The blot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

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

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

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

C. Primary Antibody Selection

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

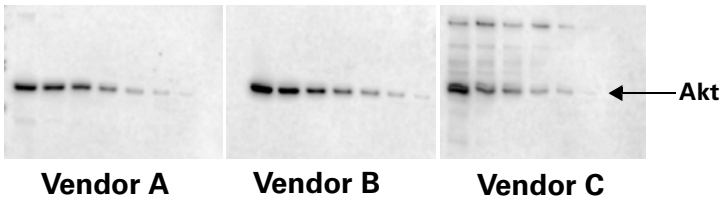


Figure 4. Serial dilutions of NIH/3T3 lysate were probed with Akt mAb primary antibodies from different vendors. All blots were blocked with 5% skim milk and detected with HRP-conjugated Goat Anti-Mouse and SuperSignal® West Dura substrate. Western blots were imaged on the Odyssey Fc Chemi channel for 2 minutes, shown with normalized image display settings.

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.*

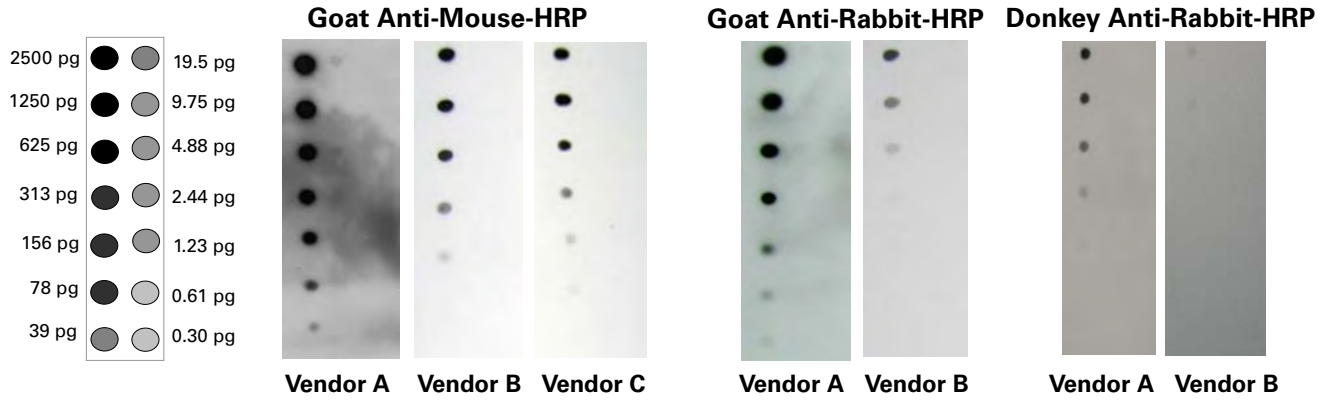


Figure 5. Serial dilutions of mouse or rabbit IgG spotted onto nitrocellulose (2500 pg to 0.3 pg) and probed with HRP-conjugated secondary antibodies from various vendors. Blots were detected with SuperSignal® West Dura chemiluminescent substrate (Thermo Scientific) and exposed to film for 15 seconds. Secondary antibodies from Vendor A consistently demonstrated an improved limit of detection.

E. Concentrations of Primary and Secondary Antibodies

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

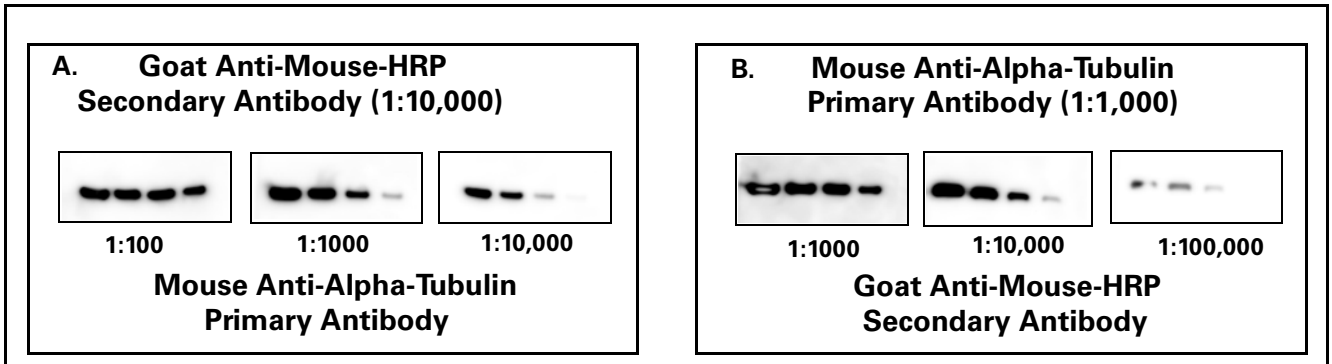


Figure 6. Two-fold serial dilutions of C32 lysate (10µg-1.25µg/lane) were resolved by SDS-PAGE and transferred to nitrocellulose. In Panel A, blots were detected with varying dilutions of primary antibody and a constant dilution of secondary antibody. In Panel B, a constant dilution of primary antibody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

F. Washing the Membranes

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

G. Chemiluminescent Substrates

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

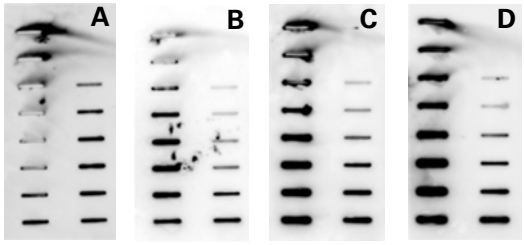


Figure 7. Two-fold serial dilutions of HRP-conjugated secondary antibody (1 ng-1.25 fg) were spotted onto nitrocellulose using a slot blot apparatus. Blots were detected with various chemiluminescent substrates (A-D).

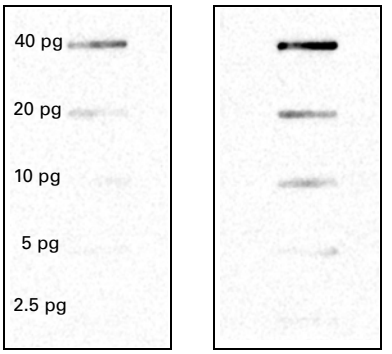


Figure 8. Effect of temperature on enzymatic activity. HRP-conjugated secondary antibody (40-2.5 pg) was applied to a membrane using a slot blot apparatus.

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.

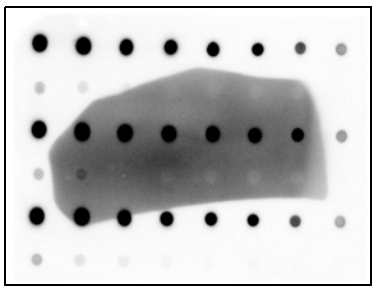


Figure 9. High background due to excess substrate (A) and addition of substrate to a dried blot (B).

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

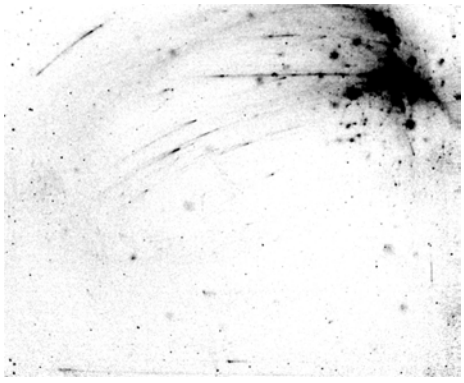


Figure 10. Contamination of the Odyssey Fc Imaging Tray.

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.

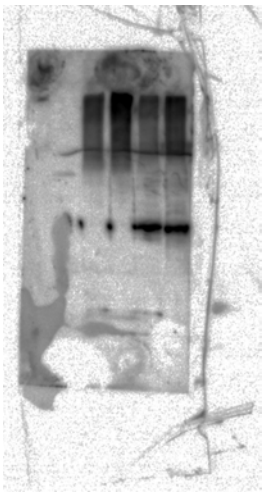


Figure 11. Unwanted background caused by plastic wrap.

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.

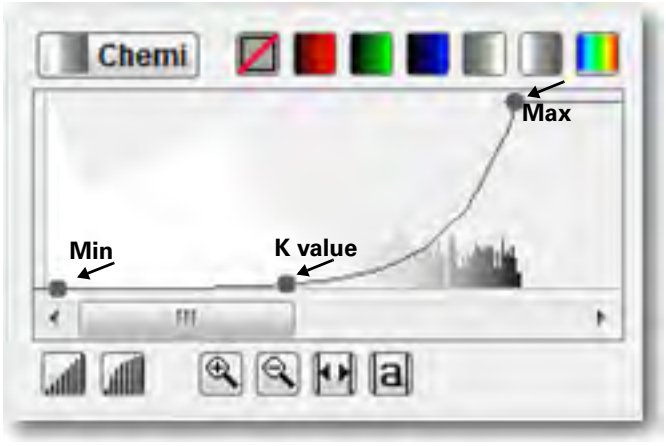


Figure 12. Use the three adjustable points to optimize the displayed image.

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.

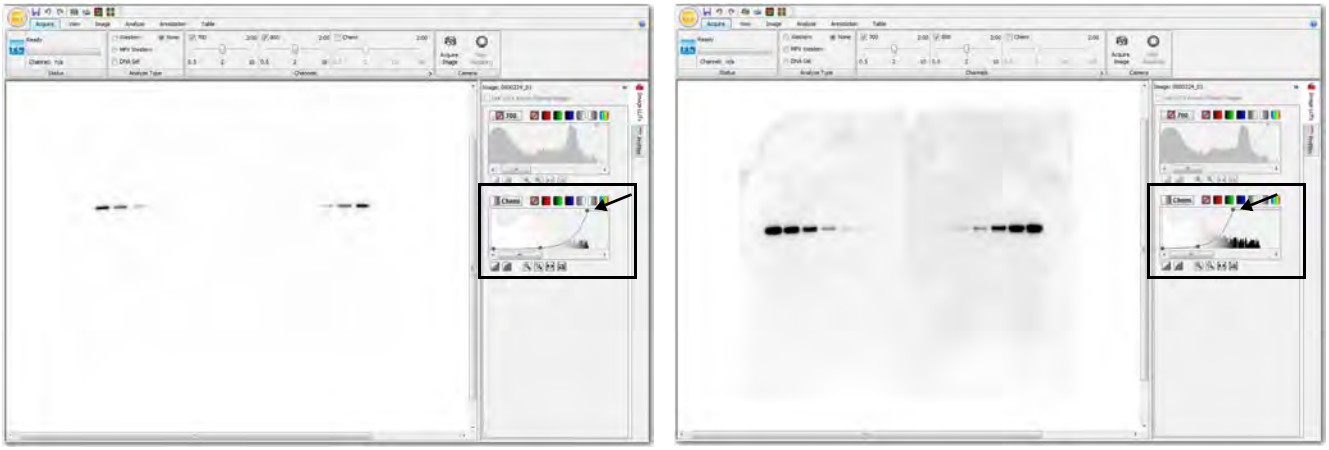


Figure 13. The curve overlaying the histogram in the Lookup Table was adjusted by moving the Max point to the left to make the bands appear darker in the second image.

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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Imaging Nucleic Acid Gels on the Odyssey® Fc Imager

Developed for:

Odyssey Fc Imaging System

LI-COR®
Experience Excellence

Published September 2011. The most recent version of this protocol is posted at: *http://biosupport.licor.com*

Contents

I. Introduction

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

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

Specific instructions are given in this technical note for ethidium bromide and SYBR Safe use. There are a variety of commercial DNA stains that may be appropriate for fluorescent imaging with the Odyssey Fc 600 channel. SYBR Green I (Life Technologies), GelStar (FMC), Gel Red™ (Biotium), Gel Green™ (Biotium) and Nancy-520 (Sigma) stains have also been tested at LI-COR (see example images on page 7). Other nucleic acid binding stains may also be compatible with the Odyssey Fc Imager. Please check the excitation and emission spectra of each stain.

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

Note: *Any questions regarding specific properties of the DNA binding stains should be directed to the representative vendors listed in this technical guide.*

II. DNA Separation and Detection on Agarose Gels

A. Suggested Materials

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

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

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

B. In-Gel Pre-Staining Protocol

Gel Preparation

1. Prepare desired agarose (0.8%, 1.0%, 1.2%, etc.) in 1X TAE or 1X TBE buffer.
2. Heat to dissolve agarose.
3. Cool solution until warm to the touch (60°F) prior to adding DNA stain.
   - **Ethidium Bromide**— Stock solutions are typically 10 mg/mL. Add ethidium bromide to give a final concentration of 0.5 µg/mL.
   - **SYBR Safe**— Stock solutions are typically 10,000X. Add SYBR Safe to a final concentration of 1X.
4. Pour molten agarose solution into casting tray and set comb into place. Allow gel to solidify.
5. Remove comb and place gel in buffer tank.

   Note: *Both Ethidium Bromide and SYBR Safe are positively charged stains and will migrate in the opposite direction of the DNA. If the stain is included only in the gel, but not the buffer, there will be an area of high background indicating the stain has not migrated out of the gel.*

Buffer Preparation

6. Prepare enough buffer (1X TAE or 1X TBE) to fill the apparatus.
7. Add DNA stain to buffer.
   - **Ethidium Bromide**— Add 5 µL of 10 mg/mL EtBr stock solution to 100 mL of buffer (final concentration: 0.5 µg/mL).

- **SYBR® Safe—** Add 10 µL of 10,000X SYBR Safe stock solution to 100 mL of buffer (final concentration: 1X).

Electrophoresis and Destaining

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

C. Post-Electrophoresis Staining Protocol

Gel Preparation

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

Buffer Preparation

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

Electrophoresis and Destaining

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

Staining Procedure

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

D. E-Gel® Pre-Cast Agarose Gels

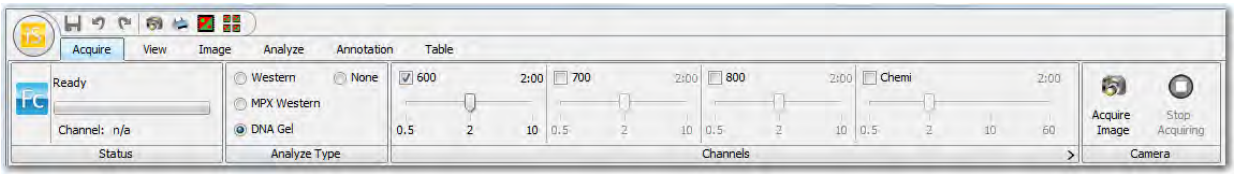
The E-Gel pre-cast agarose gels containing Ethidium Bromide or SYBR Safe are compatible with digital imaging on the Odyssey Fc Imager using the 600 channel. The clear versions of the E-Gel gels allow for post-staining with a DNA binding stain of your choice. Follow the manufacturer's protocols for sample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

III. Image Acquisition on the Odyssey Fc Imager

1. Place gel face-up on an Odyssey Fc Imaging Tray.

   Note: *E-Gel® gel cassettes can be placed directly on the tray without removing the gel. The cassette has low background in the sample imaging area.*

2. Open the imaging drawer by pressing the imaging drawer open/close button.
3. Place the Odyssey Fc Imaging Tray containing the gel in the imaging drawer. Close the drawer by pressing the imaging drawer open/close button again.
4. Open Image Studio software and connect to the Odyssey Fc Imager.
5. Click on the **Acquire** tab to show the Acquire ribbon.



6. In the Analyze Type group, select **DNA Gel** for automatic analysis or select **None**.
7. In the Channels group, select the **600** channel and deselect the other channels.
8. Select the acquisition time by dragging the slider in the 600 box. Typical acquisition times for agarose gels are from 0.5 to 2 minutes.
9. Once the parameters have been set, click on **Acquire Image** to start the acquisition. The Status group provides information on the imaging process.

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

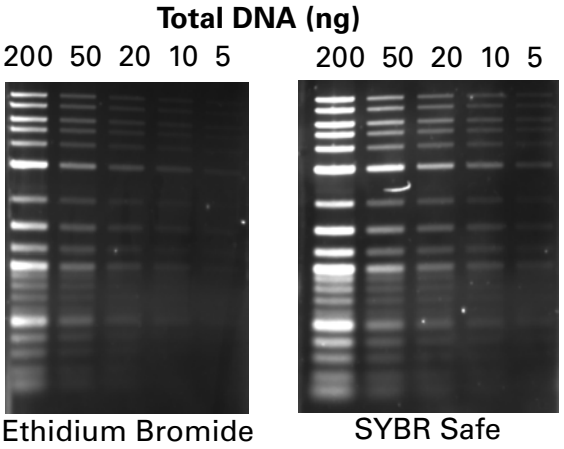
10. Adjust the Lookup Table for the 600 channel to optimize the image display. Refer to *How to Adjust the Lookup Tables in Image Studio for an Optimal Image Display* (LI-COR Biosciences) for more information.

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

IV. Results — Ethidium Bromide and SYBR Safe

A. Sensitivity of the Odyssey Fc Imager, 600 Channel

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



Sensitivity of the Odyssey Fc Imager

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

B. DNA Samples — Plasmid Digests and PCR Products

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

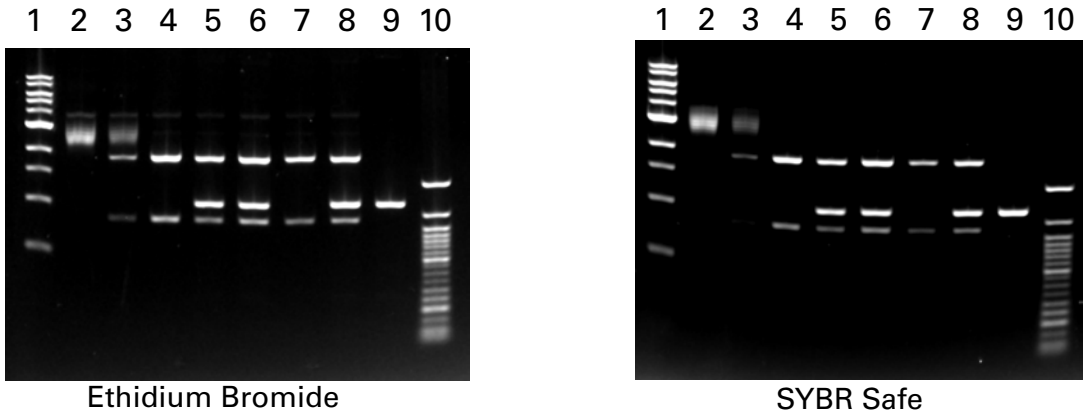


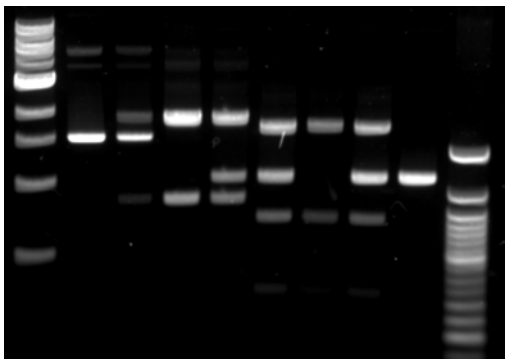
Figure 2. 1.2% E-Gel gels (Ethidium Bromide and SYBR Safe) run for 30 minutes and then imaged on the Odyssey Fc Imager (2 minutes) in E-Gel cassettes. Lane contents shown in the following guide.

Lane 1: 380 ng 1 kb Ladder
Lane 2: 100 ng pUC19
Lane 3: 500 ng pUC19 + pUC19/XmnI/HindIII
Lane 4: 150 ng pUC19/XmnI/HindIII
Lane 5: 380 ng pUC19/XmnI/HindIII + 50 ng PCR product
Lane 6: 380 ng pUC19/XmnI/HindIII + 75 ng PCR product
Lane 7: 80 ng pUC19/XmnI/HindIII
Lane 8: 100 ng pUC19/XmnI/HindIII + 100 ng PCR product
Lane 9: 125 ng PCR product
Lane 10: 400 ng 50 bp Ladder

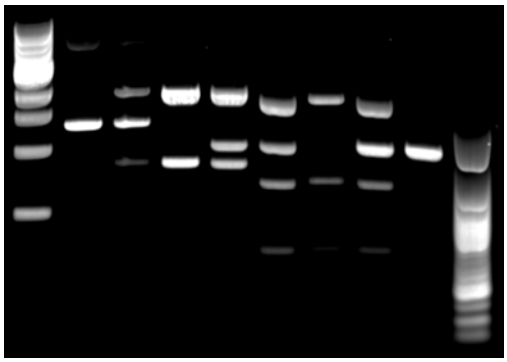
C. Examples of Other DNA Stains

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

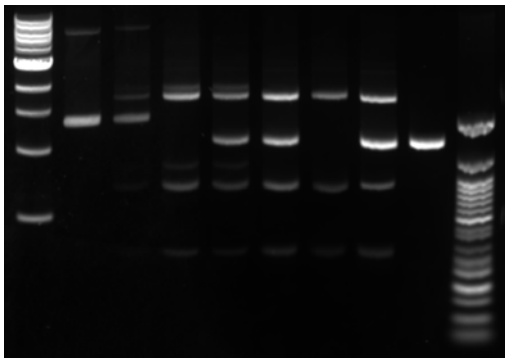
Nancy-520



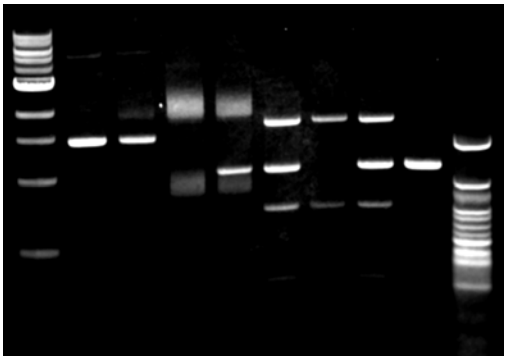
GelStar



GelGreen™



GelRed™



SYBR® Green I

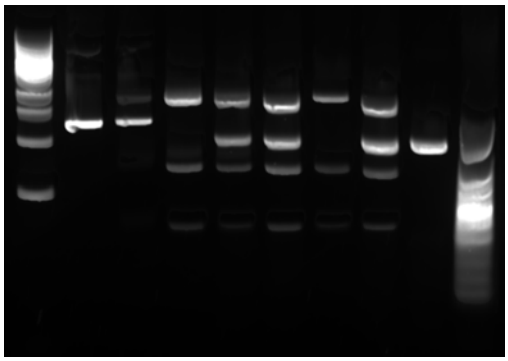


Figure 3: Gels were pre-stained using these DNA stains according to the manufacturers' recommendations. Images were acquired on the Odyssey Fc Imager in the 600 channel (2 minutes). Lane contents are identical to those described in Figure 2.

V. References

1. Waring MJ., (1965) *J Mol Biol.* 13(1):269-82 *Complex formation between ethidium bromide and nucleic acids*

2. LePecq JB, Paoletti C., (1967) *J Mol Biol.* 27(1):87-106 *A fluorescent complex between ethidium bromide and nucleic acids. Physical-chemical characterization*

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

LI-COR Biosciences, (2011) *How to Adjust the Lookup Tables in Image Studio for an Optimal Image Display*

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Syto® 60 Staining of Nucleic Acids in Gels

Developed for:

Aerius, and Odyssey® Family of Imagers

LI-COR®
Experience Excellence

Published June 2010. Revised October 2011. The most recent version of this pack insert is posted at http://biosupport.licor.com/support

Table of Contents

The Syto® 60 stain is a red fluorescent nucleic acid stain supplied as a 5 mM solution in DMSO by Invitrogen, P/N S-11342. Any questions regarding the Syto 60 stain should be directed to Invitrogen (www.invitrogen.com).

I. INTRODUCTION

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

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

II. METHODS

Method I. Electrophoretic Staining

Purpose: To obtain an archivable, digital image of a DNA agarose gel using an Odyssey Imaging System.
NOTE: This method may not be optimal for visualizing bands smaller than 100 bp.

Method:

1. Dilute the Syto 60 stain 1:1000 in TE buffer, mix well.
   NOTE: Syto 60 stain is stable for up to 1 week at 4°C when diluted.
2. Prepare DNA samples in loading dye and reserve an additional 1 µL in the final volume to accommodate the 1 µL of Syto 60 stain for loading.
3. To each sample, add 1 µL of the diluted Syto 60 stain and mix well with a pipettor.
4. Incubate at room temperature for 5 minutes.
5. Load the samples on the gel.
6. Run the gel at ~5-10 V/cm for ~1 hour or less.

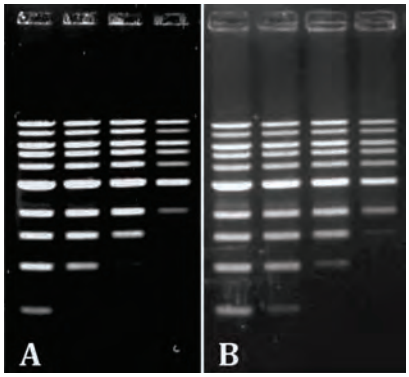

Figure 1. Two-fold dilutions of 1 kb ladder, from 1 µg to 0.125 µg, separated on a 1.2% agarose gel at 8V/cm in 1X TAE buffer for 1 hour. Panel A is the image of the gel obtained from the Odyssey Infrared Imaging System using an intensity of 5.0, gel face down. Panel B is the image of the gel acquired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

7. Use the Odyssey®, Odyssey CLx, Odyssey Sa, Odyssey Fc, or Aerius Imaging Systems to obtain a digital image of the Syto 60-stained DNA.

   Odyssey or Odyssey CLx System Settings:
   - Gel face down on scan bed
   - 700 nm channel intensity: 5-8
   - Focus offset: 0.5 mm

   Odyssey Sa or Aerius System Settings:
   - Gel face down on scan bed
   - 700 nm channel intensity: 5-8
   - Focus offset: 1.7 - 2.0 mm

   Odyssey Fc System Settings:
   - Gel face up on imaging tray
   - Acquisition time: 2 min.

Method II. Dual Electrophoretic Staining

Purpose: To obtain a digital image using an Odyssey® Imaging System and then visualize DNA bands on a UV transilluminator for excision.
NOTE: This method may not be optimal for visualizing bands smaller than 100 bp.

Method:

1. Dilute the Syto 60 stain 1:1000 in TE buffer, mix well.
   NOTE: The Syto 60 stain is stable for up to 1 week at 4°C when diluted.
2. Dilute EtBr (10 mg/mL solution) 1:500 in TE buffer, mix well (made fresh).
3. Prepare DNA samples in loading dye and reserve an additional volume of 2 µL to accommodate the volume of Syto 60 stain and EtBr for loading.
4. To each sample, add 1 µL of the diluted EtBr and mix with a pipettor.
5. To each sample, add 1 µL of the diluted Syto 60 stain and mix with pipettor.
6. Incubate at room temperature for 5 minutes.
7. Load the samples.
8. Run the gel at ~5-10 V/cm for ~1 hour or less.
   NOTE: Longer run times result in fading of the Syto 60 intensity.
9. Image on an Odyssey Imaging System in the 700 nm channel to obtain a digital image of Syto 60-stained DNA.

   Odyssey or Odyssey CLx Imaging System Settings:
   - Gel face down on scan bed
   - 700 nm channel intensity: 5-8
   - Focus offset: 0.5 mm

   Odyssey Sa or Aerius System Settings:
   - Gel face down on scan bed
   - 700 nm channel intensity: 5-8
   - Focus offset: 1.7 - 2.0 mm

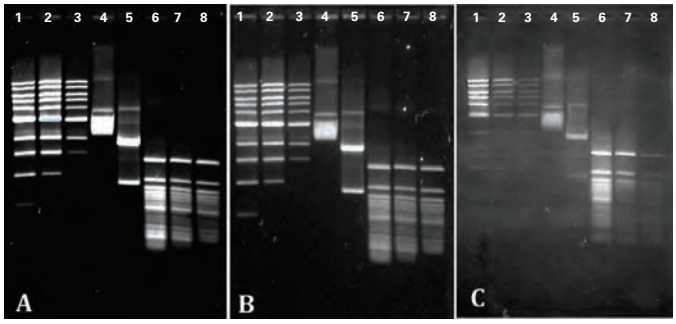

Figure 2. A 1.2% agarose gel was imaged using the Odyssey® Infrared Imaging System (panel A), Odyssey Fc Imaging System (panel B) or a UV transilluminator and the image captured using Polaroid 667 film (panel C). Lane 1) 1 µg 1 kb ladder; Lane 2) 0.5 µg 1 kb ladder; Lane 3) 0.25 µg 1 kb ladder; Lane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

Odyssey Fc System Settings:
- Gel face up on imaging tray
- Acquisition time: 2 min.

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

Hints and Tips for Methods I and II

1. The range of dilution for the Syto 60 stain is 1:500 to 1:20,000. The dilution to use is dependent on the DNA size, concentration, and whether the Syto 60 stain will be used in combination with EtBr.
   NOTE: This method may not be optimal for visualizing bands smaller than 100 bp.
2. The Syto 60 stain, diluted within the recommended range in TE buffer, is stable for 1 week at 4°C.
3. EtBr is not stable in TE and should be diluted fresh each time.
4. The grade of agarose is important. High grade or Molecular Biology grade agarose is less likely to cause "speckling" on Odyssey® images.
5. When using the Odyssey, Odyssey CLx, Odyssey Sa, and Aerius to image DNA gels stained with Syto 60 stain, it may be necessary to scan the gel with the front side on the glass and/or adjust the focus offset, depending on the gel thickness. A 5 mm - 7 mm thick gel is optimum.
6. Addition of EtBr to the gel and running buffer with the Syto 60 stain added in the sample is not recommended.

Method III. Post-electrophoretic staining

Purpose: To obtain an archivable, digital image of a DNA agarose gel using an Odyssey Imaging System.
NOTE: This method IS recommended for visualizing <100 bp.

Method:
1. Two parallel 1.3% agarose/TBE gels were loaded with serial two-fold dilutions of 100 bp DNA ladder (New England Biolabs) from 1 µg to 0.3 µg per lane.
2. The gels were electrophoresed in 1X TBE running buffer at approximately 5 V/cm.

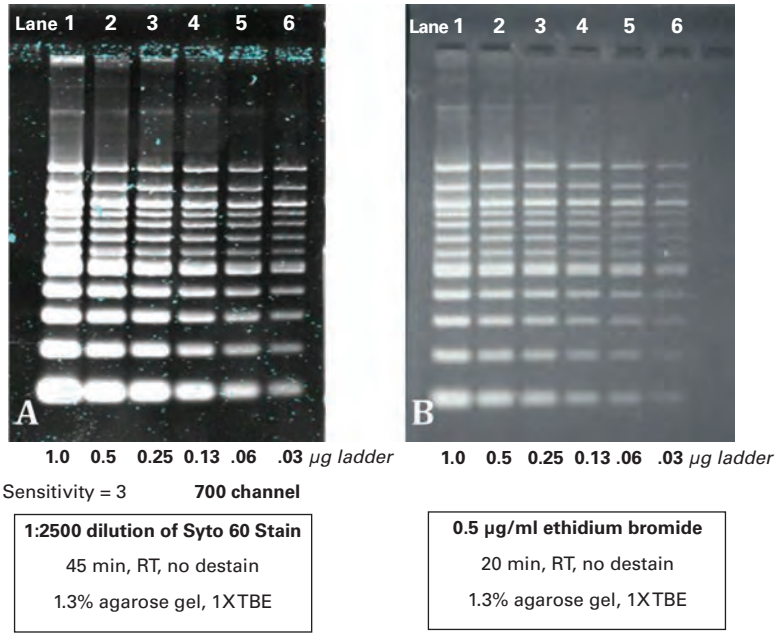

Figure 3. Panel A: Image of a Syto 60 stained gel using the Odyssey Infrared Imaging System. Panel B: Image of ethidium bromide-stained gel using a Polaroid camera.

3. One gel was stained with Syto 60 dye diluted 1:2500 in water for 45 minutes at room temperature, rinsed briefly with double distilled water and then imaged in the 700 nm channel using an Odyssey Family Imager, or Aerius. Use the instrument settings provided in Methods I and II.

4. The other gel was stained in 0.5 µg/mL ethidium bromide for 20 minutes at room temperature, rinsed briefly in water, and imaged using a UV transilluminator and a standard CCD camera. The Odyssey Fc with 600-channel capabilities can also be used to image ethidium bromide gels. See *Imaging Nucleic Acid Gels on the Odyssey Fc Imager* for additional information.

Recommended Dilutions and Time Requirements for Method III.

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

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

Syto® 60 Nucleic Acid Stain Dilution	Minimum Staining Time
1:2000	5-15 min
1:2500	15-30 min
1:5000	30-45 min
1:10000	45 min
1:15000	45 min
1:20000	45 min

Speckle Reduction

The appearance of speckles on the gel may be present after post-electrophoretic staining. Use the Odyssey® Application software's "FILTER" then "Noise Removal" function, or Image Studio's "NOISE REDUCTION" function, to improve the appearance

of the images (see Figure 4). To reduce the appearance of speckles on the gel, cut off the wells before post-electrophoretic staining and rinse the gel in water.
NOTE: The type and concentration of agarose will affect the degree of speckling. For example, low melting-point agarose tends to be highly prone to speckling.

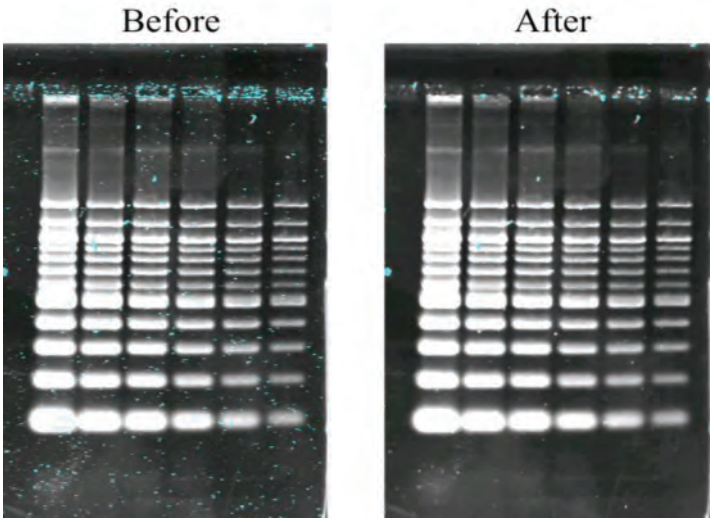

Figure 4. Image of agarose gel showing before and after using Odyssey Infrared Imaging System software's "FILTER" then "Noise Removal" function.

III. CONCLUSIONS

A table of cost comparisons for the Syto 60 stain and the ethidium bromide staining reagents used for each method is provided below. The recommended dilution of the Syto 60 staining reagent makes it more competitive with ethidium bromide on a cost basis, and the small amount of Syto 60 stain used in the sample is environmentally friendly.

Cost Comparisons

	Dilution	Staining Method	Cost
Syto 60 stain	1:1000	Method I or II	$0.006 (1 µL/well, 8 wells)
Syto 60 stain	1:20000	Method I	$0.0003 (1 µL/well, 8 wells)
Syto 60 stain	1:2500	Method III	$7.56 (25 mL)
EtBr	1:500	Method II	$0.00006 (1 µL/well, 8 wells)
EtBr	1:2000	Method III	$0.049 (25 mL)

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