ODYSSEY® CLx

Application Protocols Manual

LI-COR®

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988-16230
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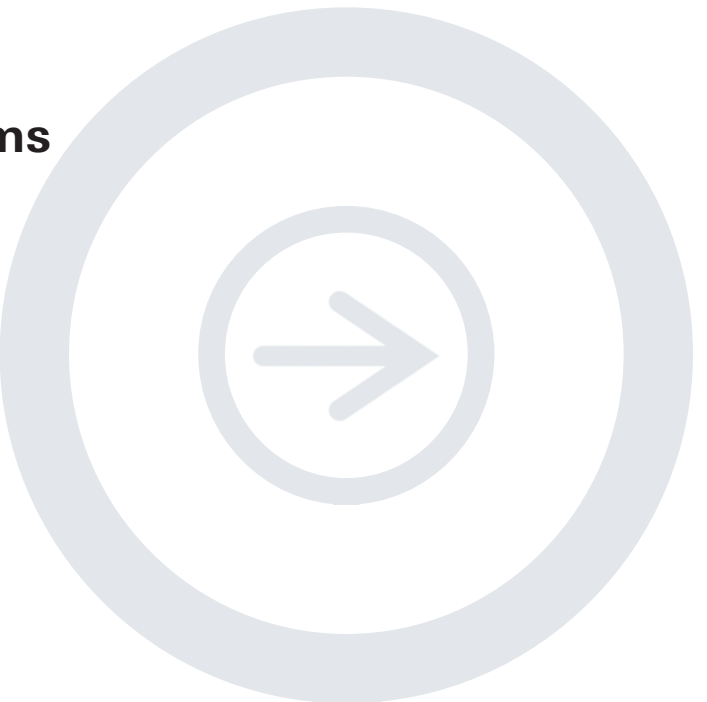
Near-Infrared (NIR) Western Blot Detection

Developed for:

Odyssey® Classic,
Odyssey CLx,
Odyssey Fc, and
Odyssey Sa
Infrared Imaging Systems



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I. Required Reagents

- Blotted nitrocellulose (LI-COR, P/N 926-31090/926-31092) or Immobilon®-FL PVDF membrane (LI-COR, P/N 926-31099 or 926-31100)
- Odyssey® Blocking Buffer (PBS) (LI-COR, P/N 927-40000, 927-40100) or Odyssey Blocking Buffer (TBS) (LI-COR, P/N 927-50000, 927-50100)**
- Primary antibodies
- IRDye® 800CW, 680RD, or 680LT secondary antibodies (LI-COR)
- Tween® 20
- PBS/TBS**
- Ultrapure water
- Methanol for wetting of PVDF
- SDS

** *IMPORTANT! PBS-based and TBS-based blocking buffers may be used with this protocol. Odyssey Blocking Buffer is available in both formulations.*
Be sure to keep your buffer system consistent throughout the protocol for blocking, antibody dilutions, and washes*. For example, if you use a TBS-based buffer system, choose Odyssey Blocking Buffer (TBS). If you use a PBS-based buffer system, choose Odyssey Blocking Buffer (PBS).*

II. Quick Start Hints and Tips

Infrared fluorescence detection with Odyssey Family Imaging Systems provides a quantitative two-color detection method for Western blots. Following, you will find some basic Hints and Tips to help you get started. For a more comprehensive guide, see *Good Westerns Gone Bad: Tips to Make Your NIR Western Blot Great* at www.licor.com/GWGBIR

1. Store the IRDye secondary antibody vial in darkness at 4 °C. 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.

2. The best transfer conditions, membrane, and blocking agent for experiments will vary, depending on the antigen and antibody.

3. Do not write on blot with a pen or Sharpie® marker. Ink from most pens and markers will fluoresce in the 700 nm channel of all Odyssey® Family Imaging Systems. The ink may wash off and re-deposit elsewhere on the membrane, causing increased background. Use a pencil to mark membranes. Ink from the Odyssey Pen does not fluoresce and can be used to mark nitrocellulose membranes. If the Odyssey Pen is used for PVDF membranes, the ink will dissolve and wash off when the blot is wetted in methanol.

4. Let the membrane dry after transfer for 1 hour or overnight, to maximize protein retention on the membrane.

5. Handle blot with clean forceps only.

6. Before using forceps, incubation trays, and the Odyssey scanning surface or sample tray (if applicable), clean with 100% methanol to remove any residual dye signal from previous use. Rinse with a small volume of distilled water, followed by isopropanol. Dry with a lint-free wipe.

7. When processing Western blots, do not use dishes/boxes that have ever been used for Coomassie staining. The Odyssey imagers are very sensitive to Coomassie (which is a strongly-fluorescent dye), and use of dishes with small traces of Coomassie will add a tremendous amount of background in the 700 nm channel.

   Maintain the same buffer system throughout the Western blot process. For example, if you block your blot in Odyssey Blocking Buffer (PBS), use PBS-based buffers throughout the protocol.

8. Do not include detergents during the blocking step.

9. For 1-color blots, use IRDye® 800CW secondary antibody for detection of the protein for best sensitivity.

10. For 2-color blots:
    - Make sure primary antibodies are from different species (for example, Rabbit and Mouse)
    - Use the IRDye 800CW secondary antibody to detect the protein target with lowest abundance, and IRDye 680RD secondary antibody to detect the more abundant protein.

11. If you are using PVDF, add 0.01% SDS to the diluted secondary antibody. Do not add SDS if using nitrocellulose membrane.

12. Incubate with secondary antibodies in the dark for one hour with gentle shaking. The incubation box can be covered with aluminum foil.

III. Western Blot Detection Methods

This protocol is designed to help you achieve success with NIR Western blot detection methods. ***Read the entire protocol carefully before beginning your optimization experiments.***

Membrane Guidelines

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

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

To learn more about optimizing your Western blots, see *Good Westerns Gone Bad: Tips to Make Your NIR Western Blot Great* (www.licor.com/GWGBIR)

For detailed gel transfer information, see *Protein Electrotransfer Methods and the Odyssey Infrared Imaging Systems* (www.licor.com/proteintransfer)

Western Blot Detection Protocol

After membrane transfer, perform the following steps:

1. For **PVDF** membrane:
   a. Pre-wet 1 minute in 100% methanol.
   b. Rinse with ultra pure water.
   c. Wet in 1X PBS or TBS for 2 minutes (using the appropriate buffer system).

   For **nitrocellulose** membrane:
   a. Wet in 1X PBS or TBS for 2 minutes, or until fully hydrated (using the appropriate buffer system).

2. Place membrane in incubation box and block the membrane in Odyssey Blocking Buffer (PBS or TBS) for 1 hour with gentle shaking. Be sure to use sufficient blocking buffer to cover the membrane (a minimum of 0.4 mL/cm^2 is suggested). For a detailed Western blot blocker optimization protocol, see *Odyssey Western Blot Blocker Optimization* (www.licor.com/optimize)

3. Prepare primary antibody:
   a. Primary antibody diluent: Odyssey Blocking Buffer (PBS or TBS) +0.2% Tween® 20 (final concentration).
   b. Dilute primary antibody in Odyssey Blocking Buffer + 0.2% Tween 20, using the vendor's recommended dilution for Western blot applications. Dilutions typically range from 1:200 - 1:5,000, depending on the primary antibody.
   c. Use enough antibody solution to completely cover the membrane.

For further details about primary antibody optimization, see *One Blot Western Optimization Using the MPX™ Blotting System* (www.licor.com/oneblot)

4. Incubate blot in diluted primary antibody for 1 to 4 hours* at room temperature with gentle shaking, or overnight at 4 °C.
   *Optimal incubation times vary for different primary antibodies.

If the procedure cannot be completed in full, this is a good place to stop until the following day. Incubate the blot in primary antibody overnight at 4 °C with gentle shaking, and resume the protocol the next day.

5. Wash membranes:
   - Pour off primary antibody solution.
   - Rinse membrane with appropriate buffer – 1X TBS-T (0.1% Tween® 20) or 1X PBS-T (0.1% Tween 20).
   - Cover blot with 1X TBS-T (0.1% Tween 20) or 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 IRDye® secondary antibody in the appropriate diluent listed below:
   a. **Secondary antibody diluent: PVDF membrane**
      - To blocking buffer, add Tween 20 to a final concentration of 0.2% and SDS to a final concentration of 0.01%.
   b. **Secondary antibody diluent: nitrocellulose membrane**
      - To blocking buffer, add Tween 20 to a final concentration of 0.2%. ***Do not*** add SDS.

   NOTE: A *Suggested dilution range for secondary antibodies is typically 1:5,000 to 1:25,000. Recommended dilutions can be found on the secondary antibody pack insert. Use 1:20,000 as a suggested starting point if using LI-COR secondary antibodies.*

7. **Protect membrane from light during incubation.** Incubate blot in diluted secondary antibody for one hour at room temperature with gentle shaking.

8. **Protect membrane from light during washes.**
   Wash membranes:
   - Pour off secondary antibody solution.
   - Rinse membrane with the appropriate buffer – 1X TBS-T (0.1% Tween 20) or 1X PBS-T (0.1% Tween 20).
   - Cover blot with 1X TBS-T (0.1% Tween 20) or 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.

9. Rinse membrane with 1X TBS/PBS (as appropriate) to remove residual Tween 20.

10. The Western blot is now ready to image.
    - The membrane can be stored in 1X TBS or 1X PBS for up to 48 hours in the dark at 4 °C.

If the blot is prepared more than 48 hours in advance, air-dry the blot and store in the dark at room temperature until ready to image.

11. Image with an Odyssey® Family Imaging System.
    - If signal on membrane is too strong or too weak, adjust the imaging parameters to optimize the image.
      – Odyssey Classic: Re-image the membrane at a lower or higher scan intensity setting, respectively.
      – Odyssey Fc: Adjust image acquisition time.
      – Odyssey CLx: Use the AutoScan function to improve the dynamic range of the image.

IV. Guidelines for Two-Color Detection

Two different antigens can be detected simultaneously on the same blot using IRDye® secondary antibodies. 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 (Step 3, Section III). Incubate simultaneously with membrane (Step 4, Section III). The primary antibodies must be from two different host species.
- Combine the two IRDye® secondary antibodies in the diluted antibody solution (Step 6, Section III). Incubate simultaneously with membrane (Step 7, Section III).

Two-color detection requires careful selection of primary and secondary antibodies. The following guidelines will help you successfully design two-color experiments:

- The two primary antibodies must be derived from different host species so that they can be discriminated by secondary antibodies of different specificities (for example, primary antibodies from rabbit and mouse will be discriminated by anti-rabbit IgG and anti-mouse IgG secondary antibodies, respectively).
- If the two primary antibodies are mouse monoclonals from different IgG subclasses (IgG_1, IgG_{2a}, or IgG_{2b}), IRDye Subclass-Specific secondary antibodies can be used for multiplex detection. 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* (www.licor.com/subclass)
- Anti-Goat secondary antibodies cannot be multiplexed with Goat-derived secondary antibodies (Example: Donkey anti-Goat and Goat anti-Rabbit). The secondary antibodies will cross-react.
- One secondary antibody must be labeled with a 700 nm channel dye and the other with 800 nm channel dye.
- In general, it is recommended that the IRDye® 800CW secondary antibody (800 nm channel) be used to detect the lower-abundance protein target and IRDye 680RD secondary antibody (700 nm) to detect the more abundant protein.

- Always use highly cross-adsorbed secondary antibodies for two-color detection. Failure to use cross-adsorbed antibodies may result in increased cross-reactivity of the secondary antibodies.
- For best results, avoid using primary antibodies from mouse and rat together in a two-color experiment. The two species are so closely related that it is not possible to completely adsorb away all cross-reactivity. If there is no other option, it is crucial to run single-color blots first with each individual antibody to be certain of expected band sizes.

V. Adapting Western Blot Protocols for Odyssey® Imaging 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, while minimizing background. Three parameters should be optimized: primary antibody concentration, IRDye 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. Start with the vendor's dilution recommendation for Western blotting or with the dilution normally used for chemiluminescent detection.

Secondary Antibody Concentration

Optimal dilutions of IRDye secondary antibodies should also be determined. Refer to the appropriate pack insert for recommendations at http://www.licor.com/packinsert. The amount of secondary antibody required varies depending on how much antigen is being detected. Abundant proteins with strong signals may require less secondary antibody. Using too much secondary antibody may increase membrane background and/or non-specific banding.

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 primary antibodies do not bind as tightly as others and may be washed away by too much detergent. Never expose the membrane to detergent until blocking is complete, as this may cause high membrane background.

Tween® 20:

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

SDS:

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

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

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Good Westerns Gone Bad:

Tips to Make Your NIR Western Blot Great

Developed for:

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

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

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I. Introduction to Western Blotting

Western blotting is used to positively identify a protein from a complex mixture. It was first introduced by Towbin, et al. in 1979, as a simple method of electrophoretic blotting of proteins to nitrocellulose sheets.[1] Since then, Western blotting methods for immobilizing proteins onto a membrane have become a common laboratory technique. Although many alterations to the original protocol have also been made, the general premise still exists. Macromolecules are separated using gel electrophoresis and transferred to a membrane, typically nitrocellulose or polyvinylidene fluoride (PVDF). The membrane is blocked to prevent non-specific binding of antibodies and probed with some form of detection antibody or conjugate.

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

II. Important Factors That Affect Western Blot Results

A. Membrane

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

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

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

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

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

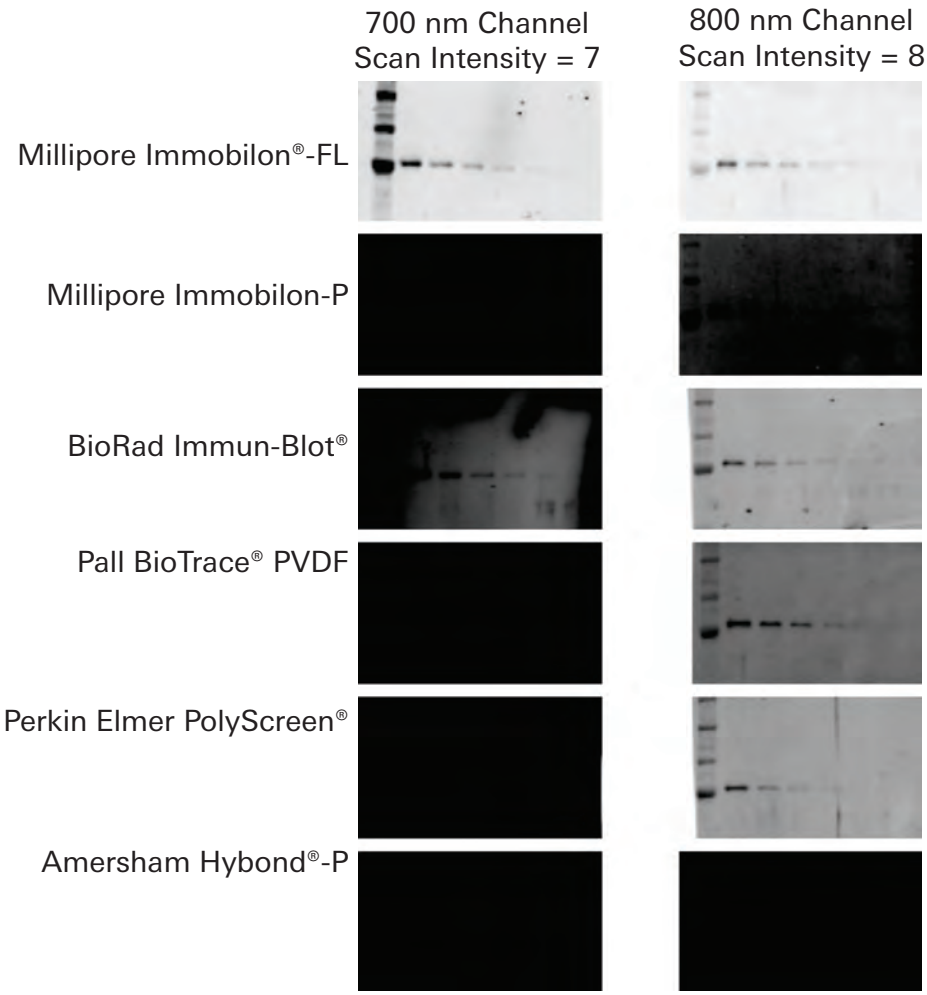


Figure 1. **Membrane autofluorescence from PVDF affects Western blot performance.** Transferrin was detected by Western blotting, using various vendors and brands of PVDF membrane. Blots were imaged with the Odyssey Classic Infrared Imaging System in both 700 and 800 nm channels.

B. Blocking Reagent

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

Detection reagents may cross-react with certain blocking buffers. Milk-based blockers may contain IgG that can cross-react with anti-goat antibodies. This can significantly increase background and reduce sensitivity. Milk-based blockers may also contain endogenous biotin or phospho-epitopes that can cause higher background.

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

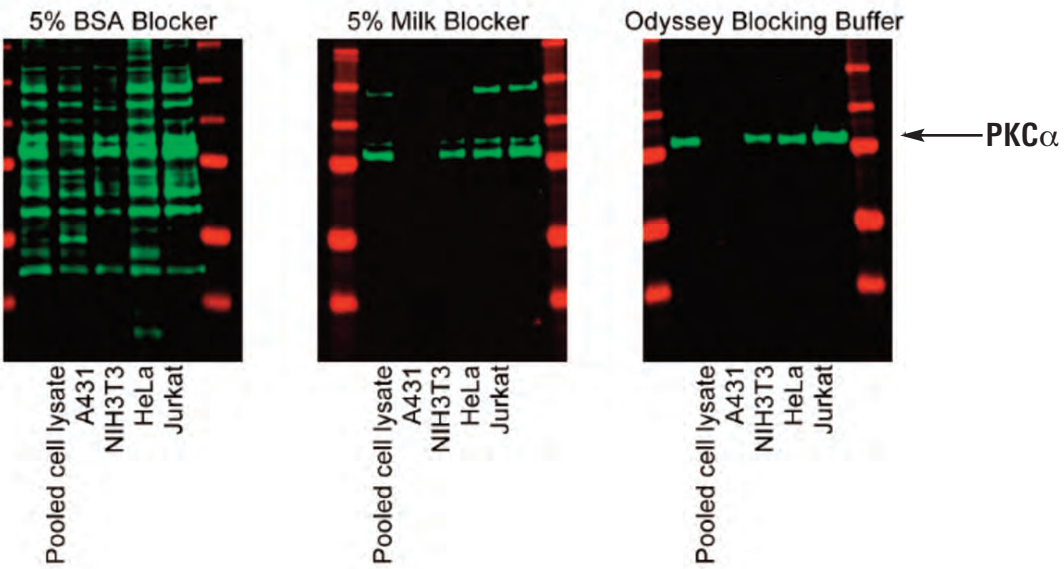


Figure 2. Effects of blocking buffer on detection of PKCα. Western blots were detected with anti-PKCα primary antibody and IRDye® 800CW Goat anti-Mouse IgG, using different blocking buffers. All blots were processed identically, with the exception of blocking reagent. Images were generated with an Odyssey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

C. TBS- vs. PBS-Based Blocking Buffers

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

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

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

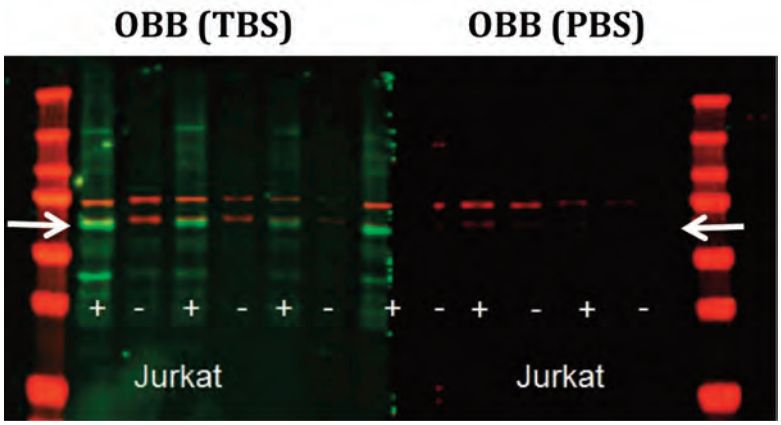


Figure 3. **Phospho-Akt detection is affected by TBS and PBS blocking buffers systems**. Jurkat cells were treated with Calyculin A (+) or left untreated (-), and lysates were loaded at 10 µg, 5 µg, and 2.5 µg/well. Odyssey Blocking Buffer (TBS) and Odyssey Blocking Buffer (PBS) were used for blocking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

Figure 4 shows detection of phospho-EGFR in A431 cell lysate stimulated with EGF (+) and in a nonstimulated (-) control. Both the PBS and TBS Odyssey Blocking Buffer systems enabled detection of phospho-EGFR. Because no single blocking reagent works well with all antigen-antibody pairs, it is important to test various buffer systems to ensure optimal detection of the protein target.

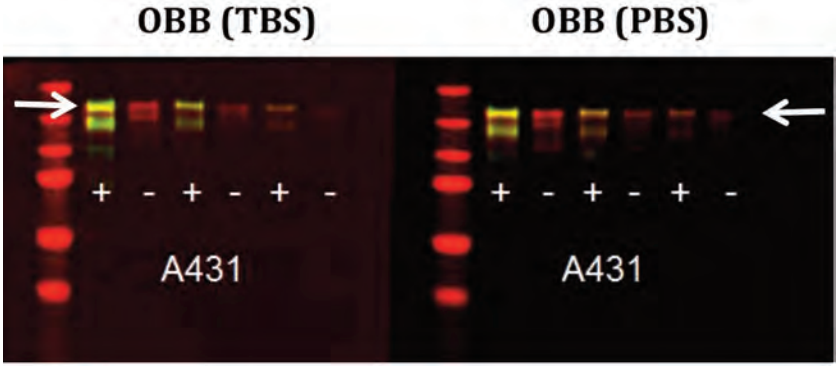


Figure 4. Phospho-EGFR is efficiently detected with both TBS and PBS blocking buffer systems. A431cells were treated with EGF (+) or left untreated (-), and lysates were loaded at 10 µg, 5 µg, and 2.5 µg/well. Odyssey® Blocking Buffer (TBS) and Odyssey Blocking Buffer (PBS) were used for blocking and antibody dilution, as indicated; appropriate TBS and PBS buffers were used for washes. Blot was probed with anti-phospho-EGFR mAb (rabbit; Epitomics P/N 1139-1) and anti-EGFR mAb (mouse; Life Technologies P/N AHR5062). IRDye® 800CW Goat anti-Rabbit IgG (LI-COR P/N 926-32211) and IRDye 680RD Goat anti-Mouse IgG (LI-COR P/N 926-68070) were used for detection. Blot was imaged with Odyssey CLx Imager (auto-scan settings, 700 and 800 nm channels). EGFR bands are shown in red (680 nm), and phospho-EGFR bands in green (800 nm). Phospho-EGFR bands appear yellow because 800 nm and 700 nm signals overlap. White arrows indicate position of EGFR band.

D. Detergents

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

Tween® 20

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

SDS

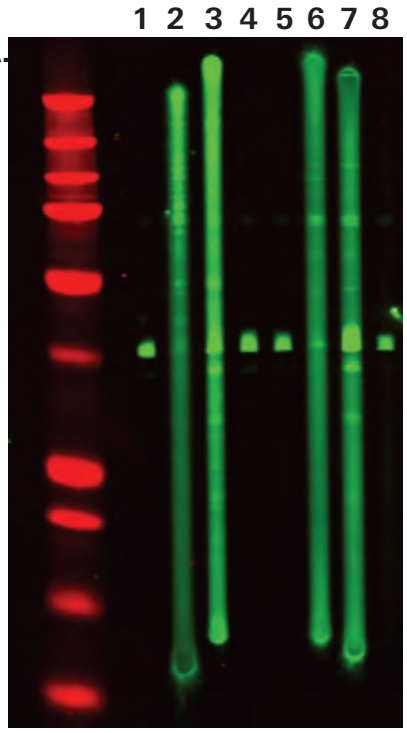
When using PVDF membrane, addition of SDS will dramatically reduce overall membrane background, especially in the 700 nm channel. It is critical to use only a very small amount, because SDS is an ionic detergent and can disrupt antibody-antigen interactions if too much is present at any time during the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

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

E. Primary Antibody

An antibody produced to detect a specific antigen is called the primary antibody, and it binds directly to the molecule of interest. Primary antibodies can be produced in a wide variety of species, such as mouse, rabbit, goat, chicken, rat, guinea pig, human, and many others. Primary antibodies from different vendors and sources for the same antigen may perform very differently. It may be necessary to test more than one primary antibody for optimal detection of the target protein in your Western blot system. Figure 5 is an example of how different primary antibodies may react.

A.



B.

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

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

F. Secondary Antibody Quality

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

Always use highly cross-adsorbed secondary antibodies for two-color detection. Failure to use cross-adsorbed antibodies may result in increased cross-reactivity (spurious bands that appear in the other detection channel; see Figure 6). LI-COR IRDye conjugated secondary antibodies are highly cross-adsorbed and optimized for two-color Western blot detection.

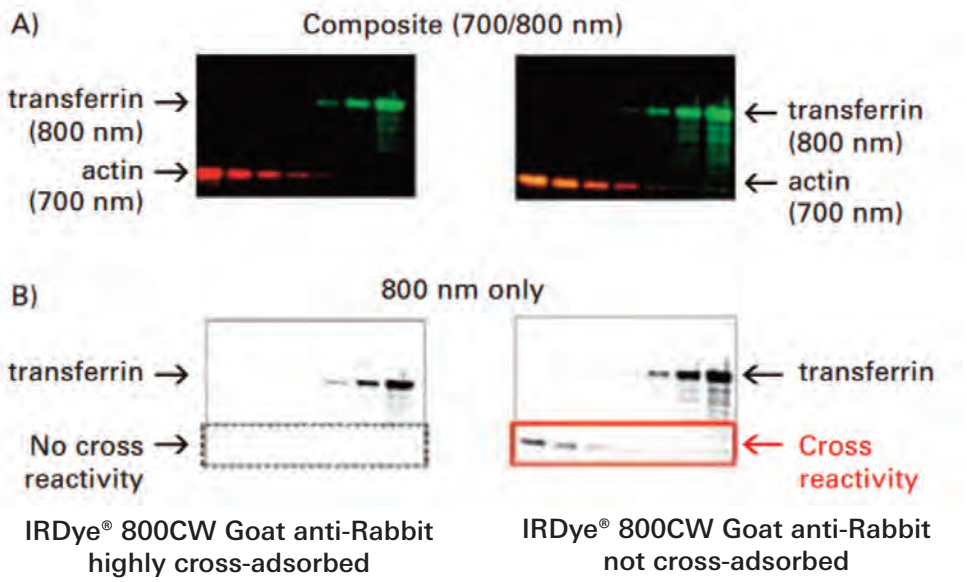
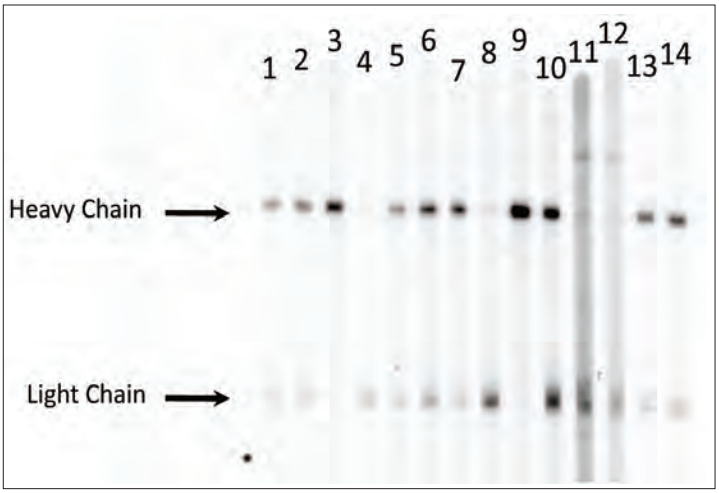


Figure 6. Highly cross-adsorbed secondary antibodies eliminate cross-reactive bands on multiplex Western blots. A) Actin was detected with mouse anti-actin primary and goat anti-mouse secondary antibody (700 nm; red). Transferrin was detected with rabbit anti-transferrin and goat anti-rabbit IRDye 800CW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

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



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

G. Secondary Antibody Dilution

The amount of secondary antibody used for NIR Western blot detection can vary widely. When using LI-COR IRDye® 800CW and IRDye 680RD conjugated secondary antibodies, the recommended dilution range is 1:5,000 to 1:25,000. When using LI-COR IRDye 680LT secondary antibodies, the recommended dilution range is 1:20,000 to 1:50,000. The dilution should be optimized for the primary antibody being used and the preferred appearance of the Western blot. Image Studio™ or Odyssey® imaging software can be used to optimize the appearance of the image, for a variety of secondary antibody dilutions (Figure 8). Higher dilutions (1:20,000 – 1:100,000) provide lower membrane background and fewer background bands than 1:1,000 or 1:5,000 dilutions (Figure 8).

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

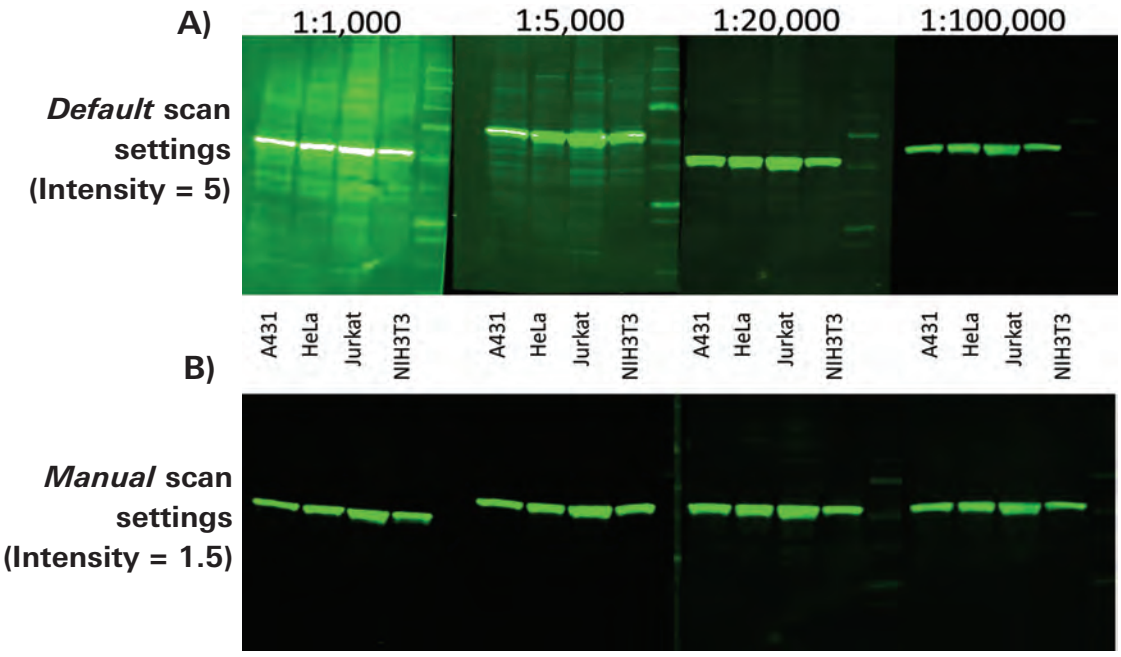
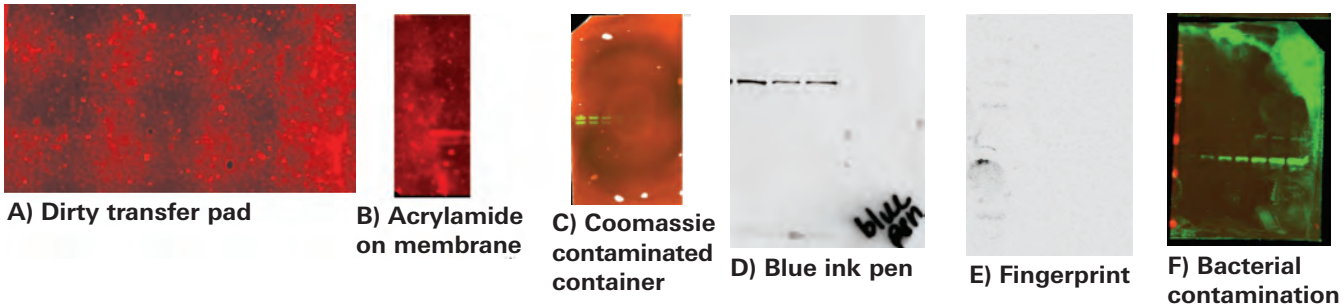


Figure 8. Effect of secondary antibody dilution on image data. IRDye 800CW secondary antibody (LI-COR) was used at the dilutions shown, to detect the target protein in various cell lysates. A) Blots were imaged with the Odyssey Classic system with the default scan settings (intensity = 5). The 1:1,000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

H. Miscellaneous Contamination

Many factors can cause contamination and increase the background levels of a near-infrared Western blot. Contamination can appear as a global increase in background, large smears of signal, or speckled blots. Common sources of contamination are listed in Table 1. Some example images are shown in Figure 9.



A) Dirty transfer pad

B) Acrylamide on membrane

C) Coomassie contaminated container

D) Blue ink pen

E) Fingerprint

F) Bacterial contamination in primary antibody

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

Table 1.

Contamination Source	Appearance	Solution
Blue loading buffer used during gel electrophoresis	Smeared signal in the 700 nm channel	Use LI-COR 4X Protein Sample Loading Buffer (P/N 928-40004).
Dirty transfer pads	Blotches can be seen on the blots that align with the transfer cassette holes	Replace transfer pads.
Acrylamide residue on membrane after transfer	Speckles and blotches can be seen in 700/800 nm channels	Carefully rinse off membrane in 1X PBS before it dries.
Blue pen used on membrane	Smeared signal in the 700 nm channels	Use pencil to mark blots.
Dirty processing containers: 1. Coomassie Stain/gel stain/ anything blue 2. Bacterial Growth 3. Acrylamide Residue	1. In the 700 nm channel, entire membrane dark, smeared signal, or speckles, depending on the amount of stain residue in container. 2. Speckles and blotches can be seen in 700/800 nm channels. 3. Speckles and blotches can be seen in 700/800 nm channels.	1. Use different containers for gel staining and Western blot detection. 2. Wash containers with detergent, rinse thoroughly with distilled water, and a final rinse with methanol. 3. Wash containers as indicated above.
Fingerprints	Blotches can be seen in 700/800 nm channels where gloved/ungloved hands have touched the membrane.	Handle Western membrane with clean forceps only.
Dirty Forceps	Blotches can be seen in 700/800 nm channels where forceps have touched the membrane.	Do not use rusty forceps. Forceps can be washed with detergent, rinsed with water, and a final rinse with methanol.
Bacterial growth in Antibodies (primary or secondary)	Speckles and blotches can be seen in 700/800 nm channels.	Replace antibodies.

III. Imaging Factors That Can Affect Western Blot Results

A. Handling of the blot

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

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

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

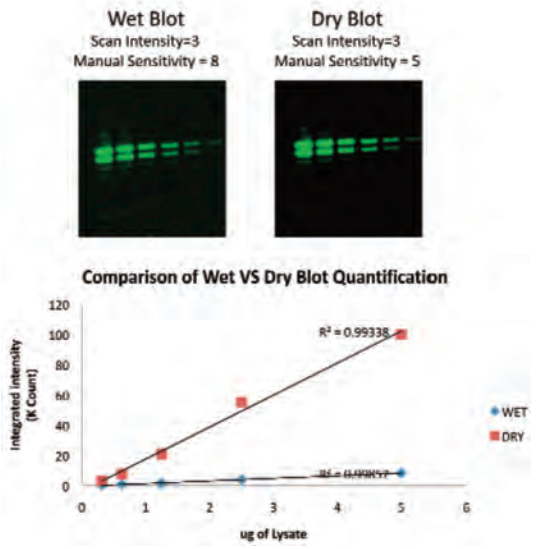


Figure 11. **Scanning of wet and dry Western blots**. The same Western blot was scanned wet and dry with an Odyssey Classic Imager. Optimal image display settings are shown. Quantification (graph below images) demonstrates higher signal intensity on the dry blot.

B. Imaging Parameters

Odyssey® Classic, Odyssey CLx, and Odyssey Sa

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

Scan Intensity – Improper optimization of the **Scan Intensity** can result in saturation of signal and reduced linear dynamic range. Figure 12 demonstrates how changes in the Scan Intensity setting affect the resulting signals on the Odyssey Classic Imager.

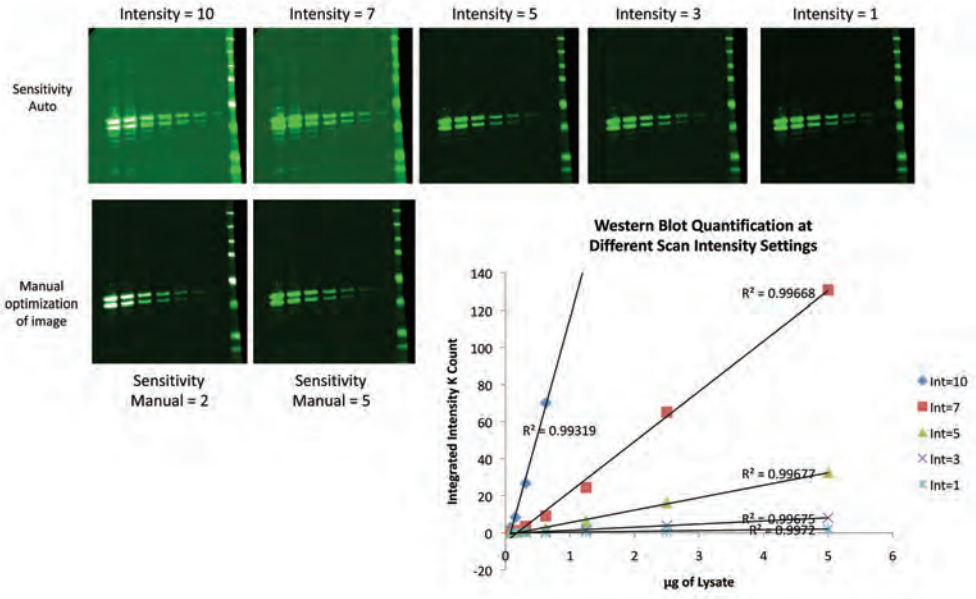


Figure 12. Effect of Scan Intensity settings on Western blot signals. The same Western blot was scanned at 5 different intensity settings with the Odyssey Classic Imager. A) Images shown with Auto Sensitivity settings for image display. Scan intensity setting is indicated above each imager. B) The same images (Intensity=10 and Intensity=7) are shown with optimized image display settings. C) Quantification of signals from the blots in A. Note that saturated signals at Intensity=10 (white pixels) cannot be quantified.

AutoScan function – The **AutoScan** imaging feature of the Odyssey CLx system is demonstrated in Figure 13. When Scan Intensity settings are manually adjusted, multiple scans may be required to generate an image without signal saturation. The Auto Intensity function automatically optimizes scan intensity to generate an image with no saturation (Figure 13A). For details, see the *Help System* of Image Studio™ Software.

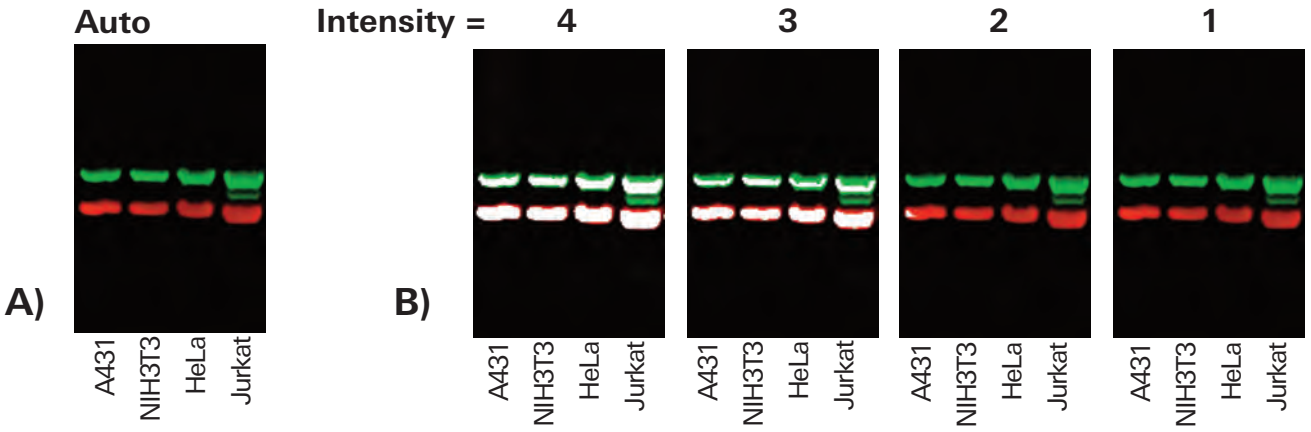


Figure 13. **The AutoScan function prevents signal saturation.** A) A two-color Western blot was imaged with the Odyssey® CLx system, using the AutoScan feature. No saturated signals were observed. B) The same blot was imaged repeatedly with manual Scan Intensity settings, beginning with Intensity=4. Pixel saturation is shown in white. Four separate scans were required to generate the desired image. In these lysates, tubulin (green bands) was detected with rabbit anti-Tubulin and IRDye® 800CW Goat anti-Rabbit IgG (LI-COR P/N 926-32211); actin (red bands) was detected with mouse anti-actin and IRDye 680LT Goat anti-Mouse IgG (LI-COR P/N 926-68051).

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

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

IV. Software Adjustments for Image Optimization

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

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

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

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

No Fluorescence – Blots that unexpectedly exhibit no fluorescence can be enhanced by changing the sensitivity setting of the image from **Linear Auto** to **Linear Manual**. These settings can be changed from the **View** menu, then **Alter Image Display** menu. To enhance the image, simply click the **Linear Manual** radio button and adjust the slider. By manually adjusting the sensitivity settings, the most desirable image can be chosen. Image display adjustments affect appearance only, and do not alter the raw data. For details, see the *User Guide*.

Dim Bands – Improving the appearance of dim bands is as simple as adjusting the **Brightness** and **Contrast** of the image. The default software setting is 5. Adjust **Brightness** and **Contrast** sliders until the image is optimal. Each fluorescence channel can be adjusted independently. Image adjustments can also be made in grayscale, and very faint bands are visualized more easily when bands are displayed in black against a white background. For details, see the *User Guide*.

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

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

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

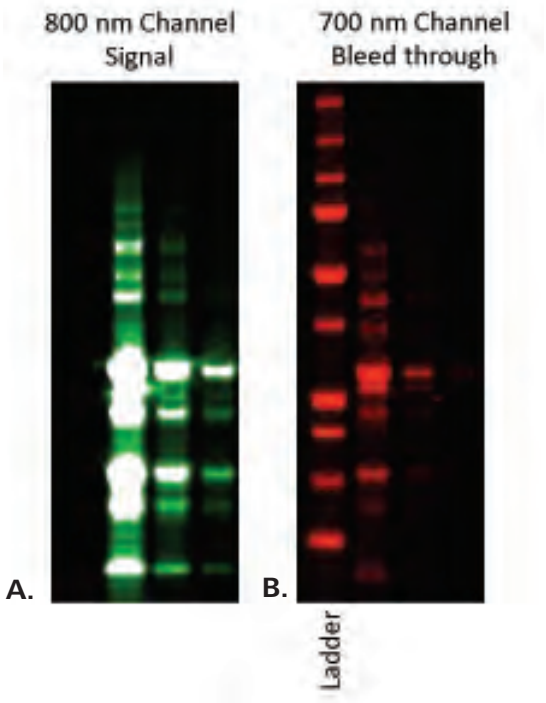


Figure 14. Saturated signal in the 800 nm channel (A) of the Odyssey Classic Infrared Imaging System can be visualized in the 700 nm channel (B). The only signal that should be seen in the 700 nm channel is the ladder on the far left of the image. The bands in the adjoining lanes are due to saturation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

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

Image Studio (ver. 1.x and higher)

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

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

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

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

For accurate Western blot quantification, the **Background** setting must be applied appropriately. This tool measures the intensity of the pixels selected as the background region. It is important to choose the most suitable Background calculation for your blot. There are several methods for background subtraction, and each method is unique to a specific need.

i. **No Background** selection uses zero for the background calculations. This is the best choice for assays with their own background calculation methods, such as concentration standards used with In-Cell Western™ Assays. The No Background method is rarely used for Western blot experiments.
ii. **Average Background** takes the average value of pixels on all four sides of the feature. A subset of sides (**All, Top/Bottom**, or **Right/Left**) can be selected to further optimize quantification. This customized background subtraction approach is used to address issues such as smears in lanes, or bands that are very close together. Choose the number of pixels to include in the calculation by changing the **Border Width**.
iii. **Median** function sets the background level to the median value of the pixels outside the feature.
iv. **User-Defined** background selection averages the intensity of pixels enclosed by a selected feature. To implement this method: display both image channels, draw a feature over an area of typical background (be sure not to include any saturated pixels), select the feature, choose the **Background** icon from the toolbar, and change the background method to **User Defined**. Click **Save**, and then **OK**. Notice that the feature is now designated as a Background feature. Multiple features can be selected for **User Defined Background**. This method is not preferred over Average or Median, due to possible inconsistencies in noise across the image.

VII. Summary

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

VIII. References

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

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07/14

Western Blot Normalization:
Challenges and Considerations for Quantitative Analysis

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LI-COR

1. Introduction

Researchers rely on Western blotting (also called immunoblotting) to detect and compare target proteins in complex samples. This trusted technique has become a widely-used tool for quantitative analysis and comparison of relative protein levels.

Quantitative immunoblotting relies on internal loading controls to confirm that changes observed in target protein abundance represent actual differences between the protein samples. Internal loading controls verify that samples are uniformly loaded across the gel, confirm consistent transfer from gel to membrane, and enable comparison of relative protein levels among samples.

Sample-to-sample variability is inevitable, even when sample concentrations are adjusted for uniform loading. Internal loading controls can mathematically compensate and correct for this variation. This process is called normalization, and is used to improve the accuracy and reproducibility of quantitative Western blot analysis. Careful, appropriate normalization is critical for meaningful comparison of relative protein levels and responses, and is particularly crucial when small or subtle responses are reported.[1-3] Accurate normalization begins with careful experimental design, to reduce the amount of sample-to-sample correction required. Reducing or eliminating sources of error will improve sample reproducibility and minimize the contribution of normalization to data analysis.

Internal loading controls are an important part of immunoblotting studies, and are often required for publication. But what's the best way to use internal controls for normalization and quantitative analysis? Many researchers who use a "housekeeping protein" (such as actin or tubulin) as an internal loading control assume that this method is reliable, because it's widely published. But recent studies indicate that housekeeping proteins are sometimes less reliable than expected as loading controls.[4-9]

This raises a number of questions. What's the best normalization strategy? How do loading controls affect the statistical properties of the data?[10] Is it better to stick with what we know, or explore different ways of thinking? As journals change their reporting requirements, what will editors and reviewers expect?

These questions circle back to the role of internal loading controls: confirming that the changes you see on the blot reflect actual change in the composition and/or biology of your samples. To demonstrate statistically significant changes in the abundance of target protein, you need a reliable normalization strategy that fits the context and biology of your experiment. But normalization can be more complex than it appears. It's important to understand the limits of each normalization approach – because your current method may not be the best fit for your experimental conditions.

This paper describes important considerations, strengths, and limitations of commonly used loading controls and normalization methods, to assist you in choosing a reliable method for your experimental context. Alternative approaches to Western blot normalization are examined, and the effects of normalization strategy on resulting data are explored. Important considerations for using a single internal loading control protein are discussed, as well as emerging alternatives like total protein staining that use aggregate measurements for normalization of sample loading.

2. Understanding Western blot normalization

2.1 How does normalization work?

Quantitative immunoblotting is often used for ratiometric analysis, to compare relative protein levels across a group of samples. Relative comparison requires that ALL samples be uniformly loaded with equal amounts of sample protein, without variation; ratiometric analysis is based on this fundamental assumption. But complete uniformity is a flawed assumption. A variety of factors can introduce unavoidable sample variability and inconsistency across each blot.[11]

In quantitative immunoblot analysis, a target protein is measured in samples from various experimental conditions. The intensity of each target protein band is then divided by the intensity of the internal loading control for that sample. This adjusts target protein signals with respect to small, unavoidable variations in cell number and sample loading.[10] The ratio of target protein to loading control is then used to compare target protein abundance in different samples (Fig. 1).

The concept of normalization assumes that both measures (target protein and loading control) are dependent on sample concentration, and they will vary together to the same degree.[11] If this is true, calculating the ratio of target protein to internal control (i.e., dividing or "normalizing" by the loading control) will correct for variability. Accuracy of the normalization process is critical for quantitative analysis and meaningful comparison of protein samples.

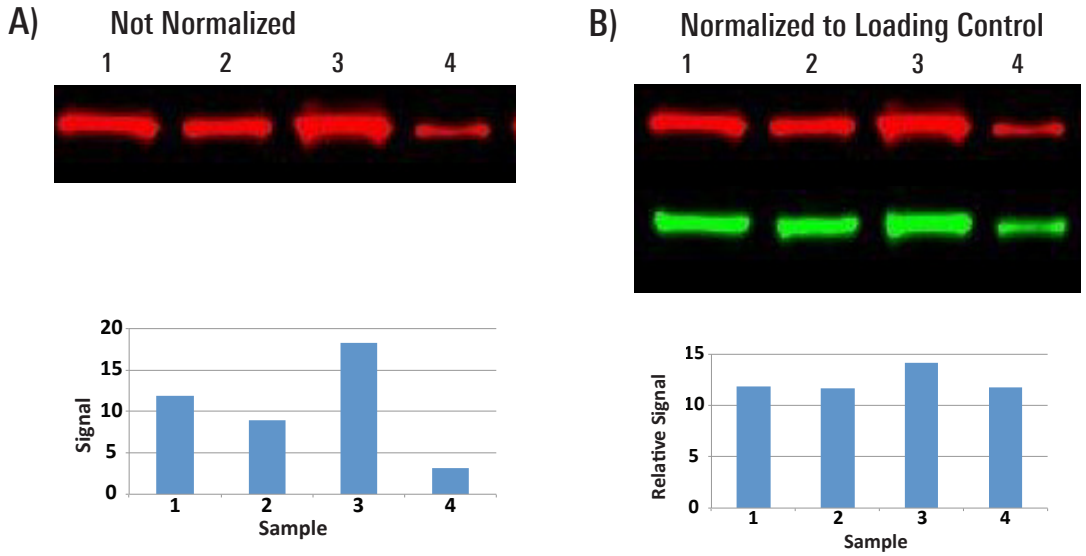

Figure 1. Normalization makes quantitative analysis more accurate. Internal loading controls and normalization ensure that observed changes in protein levels represent actual change in protein samples, not experimental artifacts. A) Raw data (not normalized). Target protein levels (red) are variable, with sample 4 showing the lowest protein level. B) Normalized data. The internal control protein (green) reveals that protein loading was inconsistent. The normalized data demonstrate that target protein levels are very similar in all samples.

Reducing or eliminating sources of variability throughout the experiment will improve sample reproducibility. This minimizes the amount of correction applied by normalization, enhancing the precision and reproducibility of sample-to-sample comparisons. Normalization is intended to compensate for small and unavoidable variation between samples, and cannot completely remove variability. If samples are inconsistent or large data corrections are applied, accuracy may be affected.

Precision and accuracy are even more crucial for interpretation of small changes in protein expression.[3] Reproducible measurement of a small response demands a much higher level of precision than a strong response, because the magnitude of the response must exceed the variability of the assay. If an immunoblot assay displays 20 - 30% variability (often cited as a typical coefficient of variation for Western blotting[1,11-12]), the measured response must exceed that variation by a statistically significant margin. If assay variability is low, it may be possible to reproducibly document a subtle response. Effective, carefully-planned normalization will more accurately reflect the amount of protein in each lane, and the actual composition of the samples.

2.2 Reporting normalization in published studies

Normalization is very relevant to the ongoing conversation about reproducibility in science. Although normalization is critical for quantitative immunoblotting, published studies frequently omit the experimental details needed for other researchers to evaluate the results.[13-18] Fortunately, scientific publishers are now increasing transparency – asking authors to report the "routine" details of their experimental methods, including data analysis methods such as immunoblot normalization.

In response to concerns about reproducibility, *Nature* and the *Nature* research journals introduced new editorial measures.[18] The updated guidelines ask authors to "describe methodological parameters that can introduce bias or influence robustness, and provide precise characterization of key reagents that may be subject to biological variability…[as well as] more precise descriptions of statistics". Space restrictions in the methods section were abolished to encourage detailed descriptions of experimental design and methods.[18] As more journals move in this direction, other publishers, and even funding agencies, may follow suit.

3. Common loading controls and normalization methods

A variety of biological factors can introduce error in Western blot analysis. The cell number may change in response to cell death or stimuli. Confluency of cultured cells drives considerable variability in protein expression.[19] Structural and metabolic proteins thought to have stable expression may be affected by growth conditions, stimuli, cell type, developmental stage, and other factors.[4-9] Biological context can and should influence the selection of appropriate internal loading controls and normalization strategies. It's not only important to have an internal control, but also to understand why that control is appropriate for the context and biology of the experiment.

An effective loading control meets two requirements: its expression is relatively constant across conditions and samples relevant to the experiment, and the resulting signal intensity is a linear representation of loading control abundance. If a single internal loading control cannot meet both requirements, more than one loading control should be used in that experiment.[1-3,11] As evidence emerges that single loading controls may not consistently meet both criteria, aggregate methods of normalization for quantitative immunoblot analysis are becoming popular alternatives.

A recent study reframed commonly-used normalization strategies and evaluated their statistical impact on data interpretation. Although this topic has been examined in depth for microarrays, the effects of normalization on the coefficient of variation (CV) and interpretation of Western blot data aren't well understood. For each method, the authors explored the effects of loading control variability on the normalized data.

Common immunoblot normalization strategies can be divided into two categories: normalization by a fixed point (using a single internal control protein) and normalization by sum (using total protein staining or multiple internal controls). The merits, liabilities, and statistical impact of each method are discussed below.

3.1 Normalization by a fixed point: Using an internal loading control protein

Normalization by a fixed point (using a single internal control protein) is the most commonly published method.[10] A second, unrelated protein that is common to all samples (often a "housekeeping protein" such as actin, tubulin, or GAPDH) is typically detected on the same blot as the target protein. Expression of the internal reference protein is assumed to be stable in all samples and unaffected by experimental conditions, such that reference protein abundance is entirely dependent on sample concentration.

This approach divides the data for each sample by measurement of a single reference point in that sample, the internal control protein. Biological variability of that fixed point can introduce uncertainty and increase the mean CV of the normalized data. The extent of influence depends on the choice of control for the experiment; internal controls with higher variability will more strongly affect data analysis. Normalization by a fixed point reduces false positives in the normalized data (samples with no statistically-significant difference in protein level that are mistakenly identified as different in the data analysis). But fixed-point analysis can also greatly increase the percentage of false negatives (samples that have small but statistically-significant changes in protein level that are not identified by data analysis). As a result, this method may sometimes fail to identify actual differences between samples.[10]

Using a single internal control protein alters the Western blot experiment. Rather than measuring the level of target protein relative to a sample characteristic such as total protein or cell number, you are measuring target protein levels relative to the protein selected as the internal control.[11] That protein may be very appropriate, if it is stably expressed and its abundance in all samples is dependent only on sample concentration.

Recent studies, however, indicate that stable expression of housekeeping proteins shouldn't always be taken for granted.[4-9] Although their expression is often thought to be relatively stable, steady-state levels of these proteins are known to vary in different tissues, or to be altered by some experimental conditions (discussed in section 7.1).[4-9] If a single internal reference protein will be used for normalization, stable expression should be validated with the appropriate sample types, experimental conditions, and antibodies.[1-9] This issue is explored in section 6, and several published examples are presented.

Band intensity and signal saturation also affect the accuracy of a single internal loading control. The intensity of internal control bands should fall in the middle of the linear range of detection whenever possible.[10] But many proteins used as internal controls, such as actin and tubulin, are highly expressed and generate strong bands – often falling outside the linear range of detection and becoming saturated.[1-3,11,13] As a result, signal intensity cannot increase in proportion to protein abundance. High-intensity normalization points increase the mean CV of the normalized data and should be avoided.[10]

Saturation artifacts are very deceptive – they can mask actual variation in protein levels by underestimating the amount of protein in the saturated bands. The similar intensities of saturated bands create the false impression that protein levels are nearly equivalent (Fig. 2).

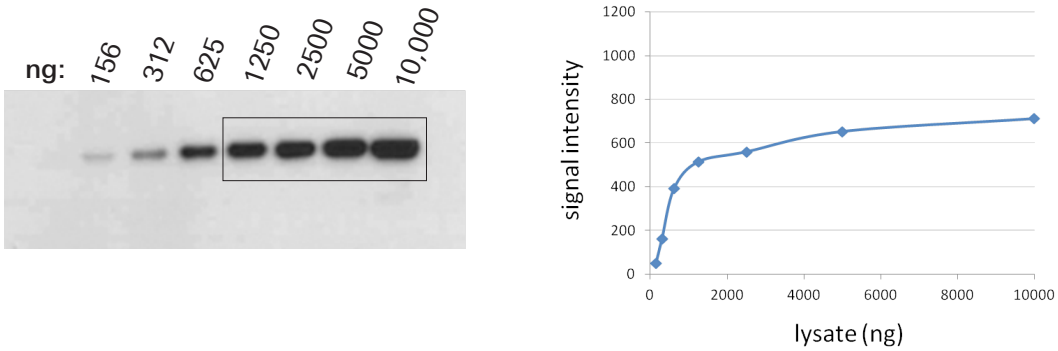

Figure 2. **Strong bands become saturated and underestimate protein abundance.** Target protein was detected in serial dilutions of NIH-3T3 cell lysate, using chemiluminescent substrate and 5-min film exposure. Strong signals (box) display saturation because they fall outside the linear range of detection. Band intensity can no longer increase proportionately to indicate protein abundance. As a result, the signal intensity of the saturated bands appears similar. High-intensity data points should not be used as controls for normalization.

3.2 Normalization by sum: Using total protein staining or multiple internal loading controls

Some researchers recommend the use of multiple internal control proteins on each blot.[1,11] This is a form of normalization by sum, an aggregate method for analysis of protein levels. Normalization by sum, using a multi-protein loading control strategy and aggregate measurements, is an emerging alternative to the fixed-point normalization strategy. In this approach, the target protein signal for each sample is divided by the sum of data obtained for that sample using total protein staining or multiple internal control proteins.

Unlike the fixed-point strategy, normalization by sum generally does not introduce uncertainty. It redistributes the uncertainty of the raw data in a mean-dependent manner, reducing the variability of high-intensity measurements and increasing the variability of low-intensity measurements.[10] This normalization strategy may increase false positives for high-intensity data points.

3.2.1 Total protein staining

Total protein staining is one type of normalization by sum. This approach uses a stain or chemical label to detect the total amount of sample protein and correct for variation. Total protein staining measures the aggregate protein signal (sum) in each lane, and eliminates the error that can be introduced by a single internal control protein.[3,5,9-11]

Total protein staining is emerging as a reliable and widely applicable strategy for quantitative immunoblotting.[2,11] It directly monitors and compares the aggregate amount of sample protein in each lane, rather than using an internal reference protein as a surrogate marker of sample concentration. This direct, straightforward approach to protein quantification may increase the accuracy of normalization. And it may also simplify your normalization workflow – because, unlike an internal control protein, total protein staining does not require validation for each experimental context and biological system.

An ideal total protein stain should meet several key requirements. It should produce linear signal output in response to sample concentration, across a wide range; correct for variation at all points in the Western blot process, including gel loading and transfer to membrane; and be compatible

with downstream immunodetection of target proteins.[1,11,20-22] The linear range of detection should be determined empirically to ensure that signal intensity is moderate, without saturation or low signal-to-noise ratios.[10]

Total protein staining is performed by gel staining or by staining of the membrane after transfer. Alternative methods for total protein detection, such as the Bio-Rad Stain-Free™ technology, replace conventional staining methods with chemical labeling of the sample proteins before or during electrophoresis. These methods are discussed in section 7.

3.2.2 Using multiple internal control proteins on a single blot

Multiple internal control proteins represent another approach to normalization in sum. In this strategy, several internal control proteins are detected on the same blot, to provide a more thorough assessment of sample loading. The band intensities of multiple loading controls are aggregated and used to calculate a mean estimate of sample concentration. This aggregate strategy is less sensitive to biological variability than a single loading control, but may require optimization.

A detailed 2015 study tested this approach, analyzing a single target protein in multiple cell lysate samples (biological replicates) using a fluorescent immunoblot method.[1] An antibody cocktail was also used to simultaneously detect five internal control proteins in each lane of the blot. After imaging, data analysis examined the effect of each loading control, as well as various combinations of loading controls, on reproducibility in these biological replicates. Without normalization, the coefficient of variation (CV) was 21%. Normalization with a single loading control produced variable CV results, depending on the protein used. GAPDH normalization decreased the CV to 9%, whereas normalization with tubulin did not improve the CV. The poor performance of tubulin in this experiment is likely due to saturation effects.

When additional loading controls were included in the analysis, the CV steadily improved to 7-8%. With more loading controls, reproducibility became less dependent on the specific combination of controls used for analysis. Because multiple controls are averaged, saturation or variability of one internal control has a negligible effect on the overall accuracy of normalization. This study concludes that using a single internal control protein for normalization can be risky, and recommends a multi-protein normalization strategy for quantitative immunoblot analysis. Three or more internal loading controls are suggested, if the sample concentration is unknown or cannot be directly measured.[1]

4. Important factors for effective normalization

4.1 Experimental design

Accurate normalization begins long before the gel is loaded, by carefully planning the experiment to make normalization as unnecessary as possible.[3,11] Although normalization is a powerful method, it cannot completely remove variation and should be used judiciously.[11]

Throughout the experiment, methodological choices and seemingly minor variations will affect the reproducibility and linearity of your immunoblot results. Without careful attention, immunoblotting may produce pseudo-quantitative results that are not proportional to the input.[1-3,20] A thoughtfully planned experimental strategy can control and reduce error, maximizing the consistency and reproducibility of the resulting samples. This, in turn, minimizes both the amount of correction required and the contribution of normalization to your data analysis.

A recent "Research Resource" article in *Science Signaling* proposed simple Western blot diagnostics to identify and optimize a variety of factors that can affect your results.[1,16] Several of these factors are briefly described here.

4.2 Sample preparation

Sample preparation can profoundly affect experimental outcome.[1-3] Even minor inconsistencies in plating, cell lysis, reagent volume, and other technical details can have a surprising impact.

Cell lysis conditions can affect sample composition, because they strongly influence protein extraction, solubilization, and modification status. Relevant proteins may be lost in the insoluble fraction; experimental treatments or stimuli may cause a shift between soluble and insoluble fractions.[1]

After sample preparation, a protein assay should be used to estimate the total protein concentration of each sample. Bradford, BCA, and Lowry assays are widely used; Nanodrop assays (A_{280}) are an option for cytosolic samples with no nucleic acid contamination.[23] The choice of protein assay should be influenced by the presence of detergents, buffers, or other components of your samples. Gel loading should be adjusted according to the estimated protein concentration, and samples loaded as accurately as possible to reduce the need for normalization.[1,10] Some researchers run a "pilot gel" and use Coomassie staining to confirm the results of the protein assay and fine-tune sample loading as needed.

4.3 Sample loading

Sample loading is a critical but underappreciated parameter. Many labs routinely load a specific, set amount of total protein per lane, generally 10-50 µg.[1-3,11] Sample loading is rarely optimized, yet overloaded gels are a significant problem.[24] If samples are overloaded, you may see very consistent band densities for an abundant internal loading control protein. However, this deceptive similarity may occur because those strong bands have exceeded the local capacity of the transfer membrane and/or exceeded the linear dynamic range of detection (signal saturation is discussed in detail in section 5). Running a standard curve with two-fold serial dilutions of cell lysate will identify the linear range and approximate point of saturation, which may be different for each target protein (Fig. 2).[3]

Janes examined this parameter using two-fold serial dilutions, from massive overloading (200 µg cell extract) to below the limit of detection (100 ng extract).[1] The linear range of sample loading was then determined for several target proteins. For p38 and actin, linear detection was demonstrated up to 50 µg of total protein. But for Hsp90 and tubulin, saturation was observed with less than 25 µg of total protein. Gomes et al[2] indicate that 20 µg/lane is excessive; Taylor and Posch report that sample loading in excess of 5-10 µg/lane are frequently overloaded and saturated.[3]

4.4 Detecting multiple proteins on the same blot

Quantitative immunoblotting is widely used to compare the relative levels of a target protein in a group of samples. Because this ratiometric analysis requires uniform loading of all samples, an internal loading control is detected and used for normalization.[11] For maximum accuracy, the target and internal control should be detected in the same context – at the same time, on the same blot, and in the same lanes.

4.4.1 Artifacts introduced by stripping and reprobing

When probing for multiple proteins using chemiluminescent detection, two options are available: stripping the blot and reprobing for a different protein, or simultaneously incubating the blot with all antibodies for multiplex detection. Although stripping and reprobing is very common, it's also time-

consuming and can introduce error. If stripping is incomplete, residual antibody will generate artifacts when the blot is reprobed; this is particularly troublesome if the two proteins migrate similarly on the gel. But complete removal of antibodies may be difficult to achieve. Stripping can damage or dissociate target proteins. It may also cause significant loss of sample proteins from the membrane – perhaps 25% or more of the target in a single stripping cycle.[2,25]

This issue was examined in a 2015 study, using immunoblots probed with phospho-ERK1/2 antibody and a GAPDH internal control.[1] The results showed that low-pH glycine stripping was very ineffective, with considerable residual antibody staining for p-ERK1/2 and GAPDH. Guanidinium stripping was more effective for removal of GADPH antibody, but a p-ERK1/2 artifact remained. An SDS stripping buffer with β-mercaptoethanol completely removed the GAPDH and p-ERK1/2 antibodies, but caused substantial loss of total protein from the membrane. This study describes stripping and reprobing of membranes as "a quantitative trade-off between antibody removal and total protein loss".[1] The potential effects of protein-specific factors (such as local overloading of the membrane, amino acid composition, and post-translational modification) on protein loss are an additional concern, although undocumented.

4.4.2 Multiplexing

Chemiluminescent detection is a single-color, single-channel method. If two proteins are detected simultaneously on a blot, chemiluminescent detection cannot identify or correct for antibody cross-reactivity artifacts. Proteins must be well separated on the blot, and controls should be used to check for antibody cross-reactivity. For multiplex detection, antibody incubations and detection are sometimes performed sequentially without stripping. However, this method is very time-consuming and may increase the membrane background.

Multiplex fluorescent immunoblotting makes normalization with an internal control simpler, more convenient, and more accurate. The blot is incubated with primary antibodies raised in different hosts. Secondary antibodies labeled with spectrally-distinct fluorescent dyes are then used to simultaneously detect the internal control on the same blot and in the same lanes as the target protein.[26-27] Blots are digitally imaged to detect the stable fluorescent signals (Fig. 3). Stripping is not required, co-migrating proteins can be used, and antibody cross-reactivity is easily identified. Stable expression of the internal control protein should be validated, as for any other fixed-point normalization strategy.

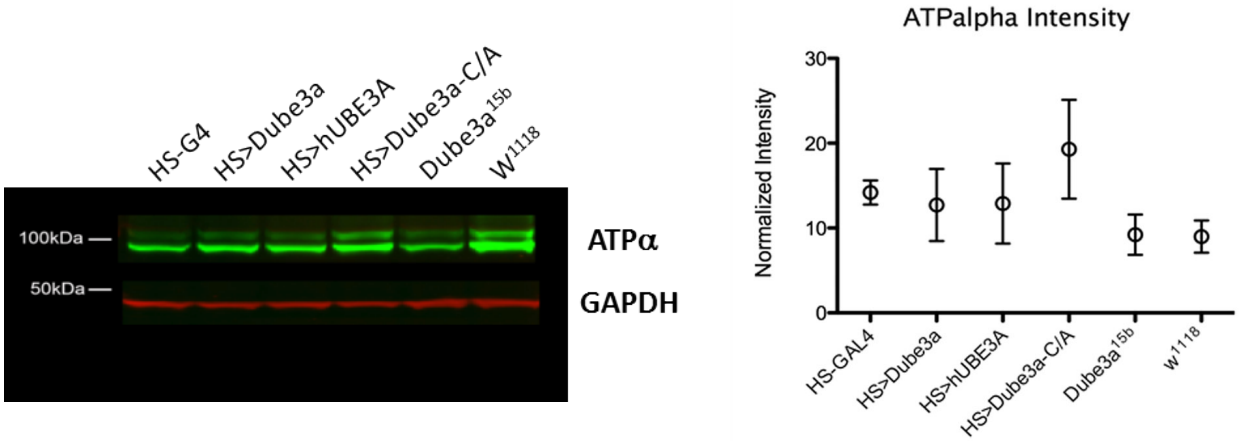

Figure 3. **Multiplex fluorescent Western blot detection with an internal control protein.** The stability and expression of ATPα (green) was examined in cell lines with differential expression of the ubiquitin ligase Dube3a. Normalization was performed with GAPDH as a loading control. NIR fluorescence imaging was used for detection. Normalized fluorescent intensities were graphed (n=3). Jensen et al. *PLoS ONE* 8(4): e61952.[28]

5. Saturation of highly abundant proteins

The impact of protein abundance and saturation on Western blot normalization is often overlooked. Saturation of strong internal control bands is especially problematic when a single internal loading control is used (normalization by fixed point). Many housekeeping proteins and structural proteins used as internal loading controls are highly abundant, but target proteins are often expressed at much lower levels.

Many factors contribute to saturation of strong bands, and saturation can occur at multiple points in the immunoblotting process. Saturation arises from limitations of protein transfer to the membrane, the detection chemistry used to generate signal, and the capacity of the imaging modality to record strong signals. When saturation occurs, strong signals will "plateau" and produce non-linear results that no longer reflect protein levels. These factors have a larger impact on data analysis when a single internal loading control is used for fixed-point normalization.

5.1 Membrane transfer

Overloading of samples is a common problem, but this parameter is not typically optimized. When an overloaded gel is transferred onto blotting membrane, highly abundant proteins can bind in layers on the membrane surface.[13,29] As a result, primary and secondary antibodies may only have access to the surface (top) layer of protein on the blot. This layering phenomenon contributes to underestimation of strong signals, and could interfere with detection of less abundant proteins. Even if other sources of saturation (such as detection chemistry and mode) are controlled, this layering effect is still able to cause saturation.

Membrane-related saturation is difficult to identify in Western blot images. A dilution series can quickly determine the upper limit, but the saturation point may be different for each target protein. Because it arises from the binding chemistry of proteins and blotting membranes, this type of saturation can occur with any detection chemistry or mode of imaging.

5.2 Detection chemistry

When internal control bands fall outside the linear range of detection, increases in protein level will not produce a proportional increase in signal intensity. Saturation artifacts can make strong bands appear to have similar intensity, hiding variations in protein levels and under-estimating the amount of protein present (see section 3.1, Fig. 2).

Large amounts of cell lysate are often loaded to facilitate detection of a target protein at endogenous levels, extremely overloading the abundant internal control protein (Fig. 4).[1-3,11,13] This pushes the internal control bands past the saturation point of the detection method. For accurate normalization, both the internal control protein and target protein must be detected within the linear range of the method used. When the abundance of the target and internal control are quite different, the linear range of detection and optimal sample loading will also be very different. This discrepancy makes quantitative analysis more complicated.

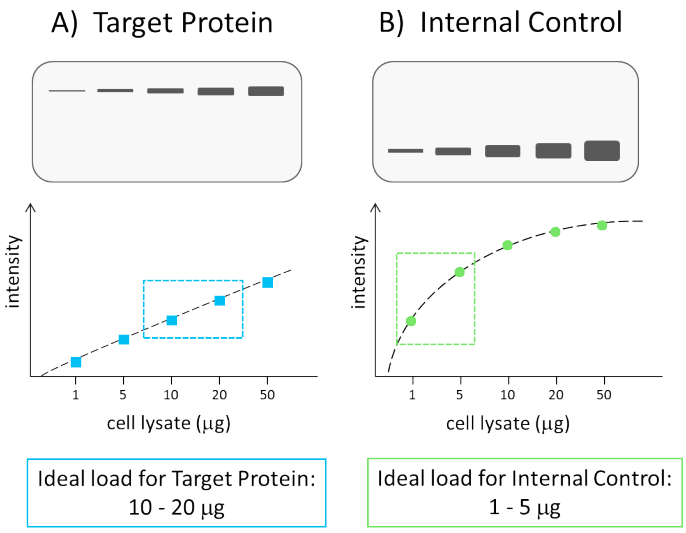

Figure 4. The linear range of detection may be different for the target protein and internal control protein. A) For the target protein depicted in this illlustration (blue), optimal sample loading would be ~10-20 µg lysate per lane. But for the internal control protein (B, green), that amount of lysate would be overloaded. Signals would be outside the linear range of detection. The amount of internal control protein would be underestimated, and could mask actual sample-to-sample variation in protein levels. Adapted from Taylor and Posch.[3]

The most commonly used detection chemistries for Western blotting are enhanced chemiluminescence (ECL) and NIR fluorescence. The reagents and detection chemistry used to visualize signals can be affected by saturation in several ways, and the enzymatic nature of chemiluminescent detection makes it particularly vulnerable to saturation artifacts.

5.2.1 Chemiluminescence

Chemiluminescent detection is an indirect, enzymatic method that typically uses secondary antibodies labeled with horseradish peroxidase (HRP) as an enzymatic reporter. This enzyme oxidizes a luminol-based substrate, transiently producing photons of light. Because signals are influenced by the kinetics of the enzymatic reaction, signal intensity does not always reflect the abundance of antigen.[1,24,30]

This detection chemistry provides high sensitivity, but may exaggerate or underestimate signal intensity. The enzymatic reaction is not necessarily proportional or linear, and a two-fold change in protein abundance may be measured as an exaggerated five-fold or greater increase in signal intensity.[1] But enzyme/substrate dynamics also cause underestimation of stronger signals, as the "runaway reaction" drives them to saturation.[1-3,11,13] These contradictory effects occur because the chemiluminescent reaction amplifies signal by turnover of substrate at the site of antibody binding. Signal amplification creates significant problems for quantitative analysis.

Local concentrations of enzyme and substrate can greatly impact the rate of the chemiluminescent reaction. The local concentration of substrate varies continuously throughout imaging, as the enzymatic reaction proceeds and consumes substrate.[25,31-32] Thus, substrate availability can vary widely across the membrane and is constantly in flux. When antibodies bind to a low-abundance protein on the blot, the local concentration of HRP enzyme is low and an excess of substrate is maintained. But an abundant protein such as a loading control will bind more antibody molecules, creating a high local concentration of HRP that consumes the reagent at a higher rate. Although excess substrate may initially be present, the kinetics and reaction rate will change as substrate is rapidly consumed.[31]

High local concentrations of enzyme-labeled antibody complex can also lead to loss of signal in several other ways. Side products of the peroxidase-catalyzed reaction may become oxidized and precipitate, causing the membrane to "brown out" and begin absorbing emitted light.[1] Additionally, the HRP enzyme itself can be inactivated by prolonged exposure to substrate, as free radicals produced by the reaction bind to the enzyme and reduce its ability to interact with substrate.[2]

The enzyme/substrate dynamics of chemiluminescent detection introduce variability and often generate non-linear responses. The limitations of this detection chemistry make quantitative analysis more challenging, and may affect the reproducibility of Western blot results – particularly when a single internal control is used for normalization by fixed point. These limitations apply to chemiluminescent Western blots in general, regardless of the method used to document the signals.

5.2.2 Fluorescence

Fluorescent detection is generally considered the most accurate method for quantitative immunoblotting.[26-27,33-34] Fluorescent immunoblotting is a direct, non-enzymatic method that uses secondary antibodies covalently labeled with fluorescent dyes (fluorophores), typically in the near-infrared (NIR) spectrum. Fluorophores are retained at the site of antibody binding, and generate signals across a wide linear range when exposed to excitation light of appropriate wavelength. When the appropriate fluorescent dyes are selected, fluorescent signals are very stable; blots can be stored for days or months and re-imaged later.[27]

Fluorescent detection is inherently more reproducible than enzymatic methods, because signal intensity is not affected by timing or enzyme/substrate dynamics.[1,26,35] In heavily loaded samples, tightly packed fluorophore-labeled antibodies do have the potential for self-quenching.

5.3 Imaging modalities

The detection technology that records your results may impose its own limitations. Signal saturation is extremely common with some detection modes; it truncates the linear and dynamic ranges and may greatly affect the accuracy of normalization. Wide linear range is an important consideration for quantitative immunoblotting – and is absolutely critical for comparison of samples when target protein abundance is variable.[1,33]

Chemiluminescent Western blots are documented by exposure to x-ray film or with a CCD imaging system. Both modalities are affected by saturation, but with very different mechanisms. Digital imaging is used to document fluorescent immunoblots using CCD, PMT, or APD-based imaging systems.

5.3.1 Film exposure of chemiluminescent blots

Although film is commonly considered to provide the highest quality Western blot image, this is a misconception.[13] In fact, film-based detection has two fundamental limitations that affect the analysis and reproducibility of immunoblotting data. It provides an extremely narrow linear range of detection, roughly 4-10 fold (Fig. 5A); and rapid saturation of strong signals makes it very difficult to accurately determine the limits of detection, particularly the upper limit.[13] Film can exaggerate small differences in abundance, while also masking sample-to-sample variability of strong bands.[1]

Plateau and saturation of strong signals are artifacts of the photographic emulsion that coats the film. Photons of light from the chemiluminescent reaction activate individual silver grains in the emulsion, which are then converted to black metallic silver to create a visible image. A strong signal

will quickly activate the majority of silver grains in that area of the film. Film responsiveness slows and eventually saturates, as new photons of light become less likely (and eventually unable) to activate additional silver grains in that local area. This effect, known as high-intensity reciprocity failure, causes the rapid plateau and saturation of strong signals commonly observed in film exposures.[36-38] As a result, the intensity of strong bands may be significantly underestimated and actual changes in relative protein level will not be detected.

The plateau and saturation of strong signals are particularly risky because they are largely undetectable to the user. Without a dilution series of controls on every blot, it's extremely difficult to determine the upper limit of detection. Film response becomes compromised before it reaches the point of saturation – but there's no reliable way to determine when this begins to affect data output. Because of these limitations, film should not be used for quantitative immunoblotting.[1,3,13,33]

5.3.2 CCD imaging of chemiluminescent blots

CCD imaging typically offers a wider linear range of detection than film (Fig. 5B).[3,10,13,26] Many CCD systems are able to detect some lower-intensity signals without extensive saturation of strong signals. However, saturation does occur with some imagers, and the resulting linear range may be less than 100-fold.[3,10,26] Sensitivity and linear range will vary, depending on the imaging hardware.

Saturation of CCD images is often represented by the imaging software as colored pixels. CCD imaging provides improved image quality and clarity, and eliminates the error and variability introduced by scanning of exposed films for densitometry.[39] Overall, digital imaging more accurately represents band intensity and protein abundance than film exposure (Fig. 5B). If immunoblot accuracy is optimized and results are carefully interpreted, this method can be useful for quantitative immunoblot analysis.[1-3] Even with a digital imaging modality, Western blot signals are still subject to the nonlinear enzymatic effects of chemiluminescent detection that may affect outcome.

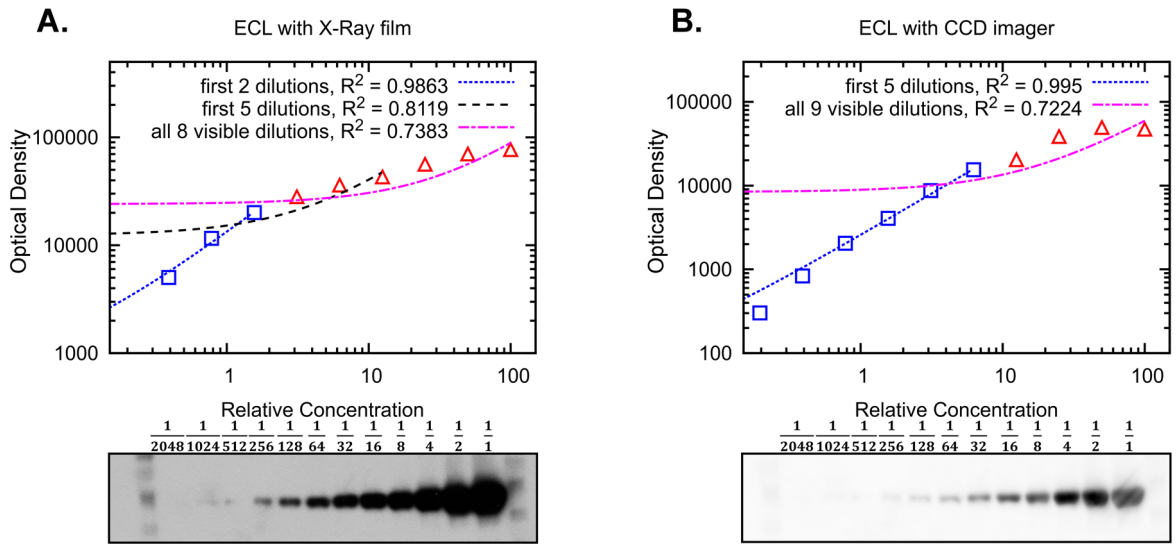

Figure 5. Film imaging provides a much narrower linear range than digital imaging with a CCD system. A) Two-fold serial dilutions were detected on replicate Western blots, using ECL substrate. A) Film exposure. Only the first 2-3 dilutions (blue squares) fell within the linear range, with $R^2 = 0.9863$. All other dilutions were subject to saturation (red triangles). B) CCD imager (longest exposure with no saturation observed). The linear range spanned 5 dilutions (blue; $R^2 = 0.995$). Image clarity was improved; stronger bands were more distinct and did not overlap. Membrane overloading and detection chemistry effects are likely both contributing to the saturation observed in both A and B. Degasperi et al. *PLoS ONE* (2014) doi:10.1371/journal.pone.0087293.[10]

5.3.3 Digital imaging of fluorescent blots

Fluorescent immunoblotting is best performed with near-infrared (NIR) fluorescent dyes and imaging systems.[1,5,12,26,30,33-35] Background autofluorescence of transfer membranes and biological samples is low in the NIR spectral region, enabling high sensitivity. In contrast, high background fluorescence in the visible spectrum causes poor signal-to-noise ratios, limiting sensitivity and performance. NIR fluorescent signals are typically documented by measuring fluorescence intensity with a laser-based APD scanner or CCD imager. The sensitivity and linear range of this method are also influenced by the imaging instrumentation used. The excitation light source and optical system affect sensitivity and background levels. The ability to detect low-intensity signals without saturation of strong signals, which is crucial for a wide linear dynamic range, is highly dependent on the imaging hardware and varies from 2.5 to > 6 orders of magnitude (Fig. 6).

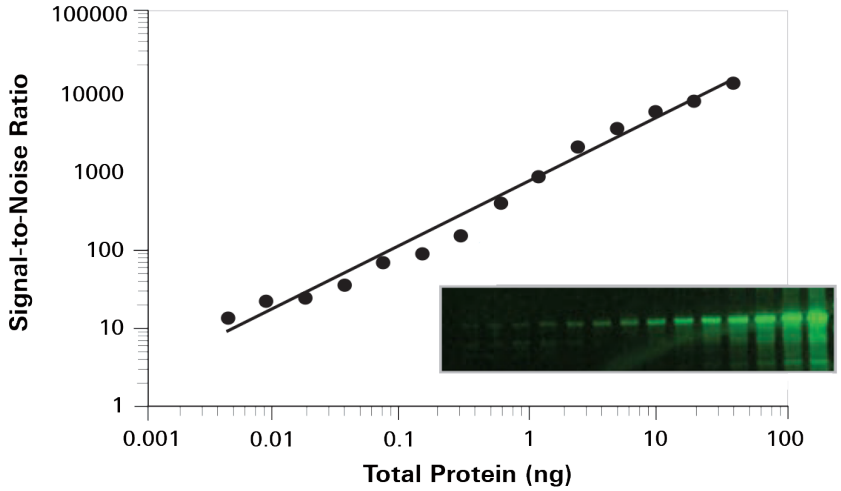

Figure 6. NIR fluorescence imaging provides a very wide linear dynamic range of detection. Two-fold serial dilutions of purified transferrin were detected by NIR fluorescent immunoblotting, using a laser APD scanner. All 14 dilutions, from 40 ng to 4.8 pg, fell within the linear range without signal saturation (an 8000-fold range).

6. Using internal loading control proteins for immunoblot normalization

The most commonly used internal loading control strategy is detection of a second, unrelated protein on the same blot (normalization by fixed point; described in section 3.1). An ideal internal control protein would be stably expressed in all cell types, with its expression level unaffected by changing experimental conditions. Housekeeping proteins such as actin, tubulin, and GAPDH are widely used and published as internal control proteins, but their expression may not be as stable as previously hypothesized.[4-9]

Multiplex immunoblot analysis of post-translational modification is a different type of internal control. The blot is incubated with a mixture of modification-specific and pan-specific primary antibodies against the target protein, and fluorescently-labeled secondary antibodies are used to detect and discriminate the two signals.[26] This ratiometric analysis approach uses the target protein as its own internal loading control (discussed in section 6.3).

6.1 Housekeeping proteins as internal loading controls

Use of housekeeping proteins as loading controls is based on the concept of "housekeeping genes," stable endogenous controls used for data normalization in gene expression assays that measure transcript abundance. A housekeeping gene is defined by its constitutive expression throughout all cells of an organism. Ideally, such a gene would not be subject to regulation and would be expressed at a constant level in every cell type.[40]

Published observations of relatively stable gene expression of housekeeping genes such as actin, tubulin, and GAPDH across cell types and tissues have led to widespread use of their protein products as internal loading controls for quantitative immunoblotting. But in a cellular context, expression of these proteins can be much more variable than expected.[4-9]

Validation of the internal reference protein is very important, to confirm stable expression across the relevant cell types, tissue types, disease states, and/or experimental treatments.[1-2,4-9,19] A housekeeping protein such as actin or tubulin may not be the most appropriate choice for every experiment, and fixed-point normalization with a single control protein warrants consideration of additional factors (as discussed in section 3.1).

Biological and methodological factors contribute to the variability of housekeeping proteins as internal controls for immunoblot analysis:

- Gene expression does not reliably predict the abundance of the corresponding proteins[41-42] (see section 6.1.1).
- Housekeeping protein expression is not always constant across cell types and tissues. It may be affected by a variety of biological factors, including tissue type, growth conditions, stage of development, and disease (see section 6.1.2).
- Housekeeping proteins are typically very abundant. The resulting strong bands frequently cause signal saturation, which reduces the accuracy of detection and under-estimates the actual amount of internal control protein in each lane (as discussed in section 5).

6.1.1 Gene expression levels do not reliably predict protein abundance

For many proteins, mRNA levels do not reliably predict protein abundance.[43-47] A 2014 mass spectrometry study built a draft of the human proteome.[47] Published mRNA abundance values were compared to quantitative protein-expression profiles for twelve human tissues. Correlation between mRNA and protein abundance was weak in all tissues, with an average RS value of 0.41 +/- 0.07 (Table I).

Tissue	R_s
Uterus	0.34
Kidney	0.56
Testis	0.33
Pancreas	0.38
Stomach	0.45
Prostate	0.41
Ovary	0.39
Thyroid	0.31
Adrenal gland	0.47
Salivary gland	0.46
Spleen	0.39
Esophagus	0.45
Average R_s	**0.41 +/- 0.07**

Table 1. Spearman correlation coefficients for mRNA versus protein abundance plots for proteins in 12 human tissues. mRNA values were drawn from an existing database, and were not measured in the same tissue samples used for protein analysis. Wilhelm et al.[47]

Because post-transcriptional regulatory mechanisms strongly influence protein abundance, gene expression observations should be validated at the protein level to confirm stable expression of any housekeeping protein used as an internal loading control.

6.1.2 Expression of housekeeping proteins may be influenced by biological factors

Several recent studies have raised concerns about the use of common reference proteins as loading controls, reporting differential expression of housekeeping proteins in certain circumstances.[2,4-9,11] One example is shown here. Examination of human adipose tissue samples demonstrates highly variable expression of beta-tubulin in the same tissue type from obese subjects and non-obese controls (Fig. 7).[7] Subject-to-subject variation was observed within each group, and also between the two metabolic states.

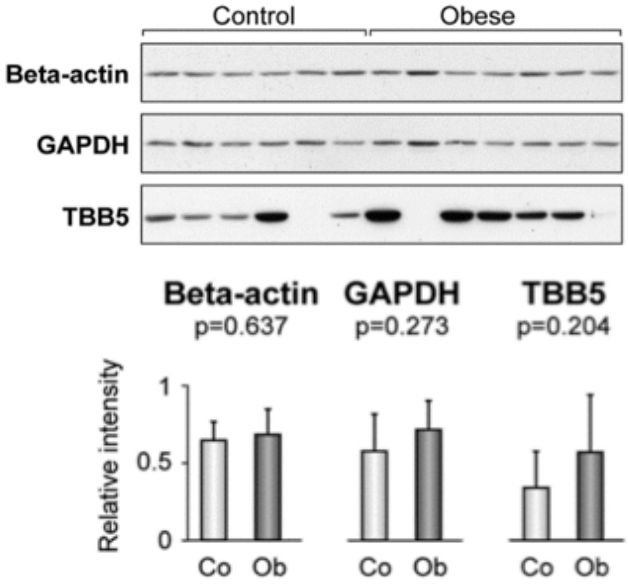

Figure 7. Variable expression of housekeeping proteins is observed in human adipose tissue from non-obese (control) and obese subjects. Western blot analysis of beta-actin, GAPDH, and tubulin beta chain (TBB5) expression in omental fat samples from 6 non-obese controls (Co) and 7 obese (Ob) subjects. Relative band intensity is expressed as mean ± SD. Pérez-Pérez et al. doi:10.1371/journal.pone.0030326.[7]

Developmental status is another important source of variable protein expression. To identify an appropriate housekeeping gene for use as an internal control in a retinal development study, expression profiles of three internal control candidates were examined across distinct developmental stages (Fig. 8).[6] From embryonic to post-natal development, each housekeeping protein displays a unique expression profile. Alpha-tubulin levels rise during early post-natal development, but then fall. Beta-actin is highly expressed in embryonic development, but expression drops off dramatically in post-natal stages. MAPK1 was also examined, and unlike the traditional housekeeping proteins, expression of this signaling protein was relatively constant throughout these stages of retinal development. The authors conclude that of the proteins tested, MAPK1 would be the most suitable internal control protein for retinal development.

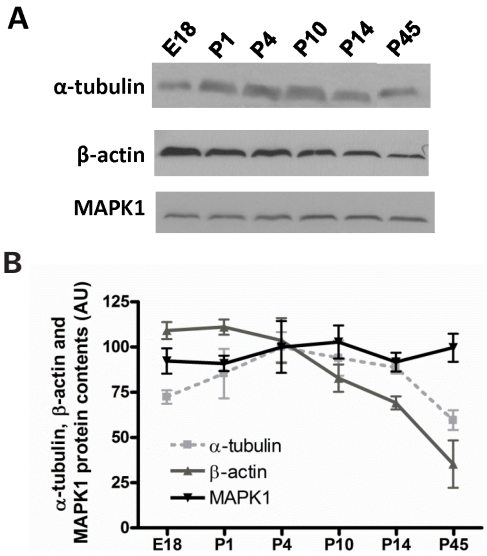

Figure 8. Variability of housekeeping protein levels throughout retinal development in rat. A) Western blot analysis of α-tubulin, β-actin and MAPK1 is shown for distinct retinal developmental stages, from embryonic to post-natal (E18, P1, P4, P10, P14, P45). B) Densitometric analysis of β-actin, α-tubulin and MAPK1. Values expressed as Mean ± SEM for 3 experiments. β-actin displayed the greatest variability during development, with CV = 36.6%. Rocha-Martins et al. *PLoS ONE* 7(8): e43028 (2012). doi:10.1371/journal.pone.0043028.[6]

Changes in protein expression also arise from environmental factors. Diurnal rhythm is one example. The neurexins are neuron-specific presynaptic proteins that participate in regulation of the excitatory/inhibitory balance in synapses. A 2012 study explored the hypothesis that neurexin expression might exhibit circadian patterns connected to the light:dark cycle.[48] Diurnal variations were demonstrated for expression of neurexins, exon splicing, and postsynaptic scaffolding proteins. Figure 9 shows one example.

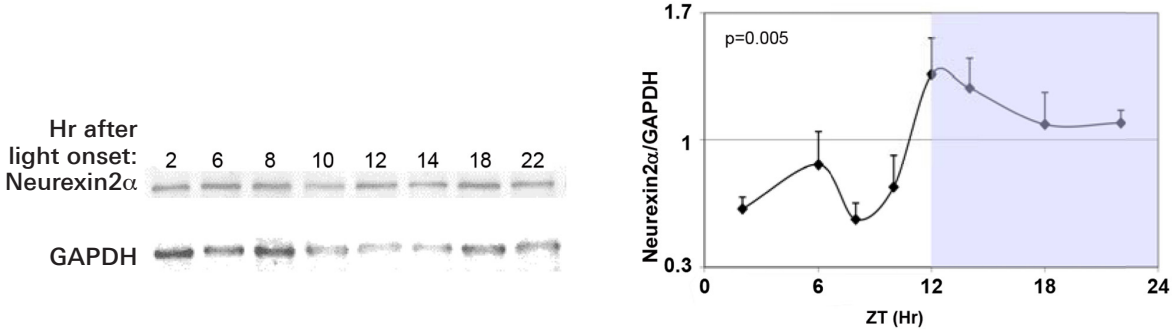

Figure 9. Diurnal rhythm influences neurexin 2α protein expression in mouse neurons C3H/J mice kept at 12:12 hours light:dark schedules were sacrificed at different times after light onset (0 hr). Expression of neurexin 2α (a synaptic receptor protein of the vertebrate nervous system) was examined by fluorescent immunoblotting. A) Representative blot shows levels at various times after lights-on. B) Values expressed as Mean +SEM relative to GAPDH (N = 3 per time point). Purple shading indicates the dark phase. Shapiro-Reznik eta l. *PLoS ONE* 7(5): e37894 (2012). doi:10.1371/journal.pone.0037894.[48]

Expression of reference proteins may even vary in different portions of a single tissue. The levels of reference proteins were examined in proximal and distal portions of sciatic nerve from the same mouse.[5] Although total protein concentration was constant, relative expression of actin and neurofilament-L (NF-L) was different in proximal and distal tissue samples. Abundance of actin was ~50% lower in the distal sample, whereas NF-L abundance was nearly 8-fold higher in the distal sample than the corresponding proximal sample. Although this study was performed with a structurally asymmetrical tissue, it emphasizes the importance of reference protein validation and implicates tissue sampling procedures as a possible source of variability in quantitative immunoblotting.

6.2 Saturation of strong bands

Housekeeping proteins are typically expressed at high levels. The resulting strong bands often cause saturation of signal, particularly when chemiluminescent detection is used.[2-3,11,13] Quantification of saturated bands generally underestimates the amount of internal reference protein in each sample. This effect may hide actual sample-to-sample variation in protein abundance,[1,11] as discussed in section 5.

6.3 Multiplex analysis of protein modifications: a different kind of internal control protein

Multiplex fluorescent detection is another option for immunoblot normalization. This approach is particularly useful for relative analysis of post-translational modifications such as phosphorylation. The method combines two primary antibodies raised in different hosts: a phospho-specific antibody (or other modification-specific antibody) and a pan-specific antibody that recognizes the target protein regardless of its modification state.[1,34,49-51] Fluorescently-labeled secondary antibodies are used to simultaneously detect and discriminate the two signals with digital imaging.[26] Phospho-signal is then normalized against the total level of target protein, using the target protein as its own internal control.

This method is widely used, and published examples are shown in Figures 10 and 11.[49-50] In validation experiments, Bakkenist et al. examined the possibility of binding interference from combined phospho-specific and pan antibodies, but detected little or no effect.[34] An additional, independent loading control may be used to verify sample loading, if desired.

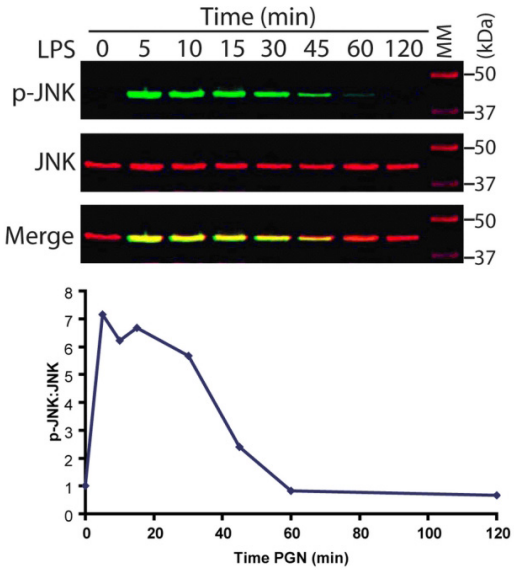

Figure 10. Time course of JNK phosphorylation in response to treatment with LPS. Multiplex fluorescence was used for ratiometric analysis of phosphorylation. Phospho-JNK is shown in green (800 nm) and total JNK in red (700 nm). Yellow indicates overlapping signals in the merged image. Reprinted with permission from Bond et al. *Biol Proced Online*. 10(1): 20-28 (2008).[49]

Two-color phospho-analysis has several advantages. Sample volume is reduced, and analysis is streamlined because all data are derived from a single blot without stripping and reprobing. Analytical confidence is increased by detection of unmodified and modified forms of the target protein on the same blot, in the same lane. No error is introduced by stripping and reprobing. Ratiometric analysis improves accuracy by correcting for loading and sampling error, and eliminates the uncertainty and variability that housekeeping proteins can introduce. Figure 11 illustrates the importance of detecting the internal control protein in the same lane of the same blot.[50] The Akt band in the last lane (far right) is irregular, likely because of a small bubble present during membrane transfer (Fig. 11, arrowhead). With multiplex detection, the same bubble is observed in the p-Akt (green) and total Akt (red) images and this transfer artifact is easily corrected.

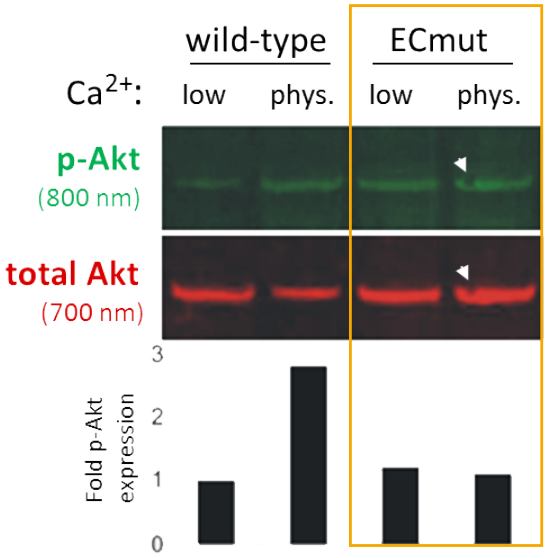

Figure 11. Loss of E-cadherin function affects PI3K/Akt signaling. Human urothelial cells with a dominant negative mutation in E-cadherin (ECmut) were grown in low or physiological Ca^{2+} conditions. Lysates were examined by multiplex Western blotting. Physiological Ca^{2+} increased phospho-Akt levels in wild-type cells but not ECmut cells (box). Arrowheads indicate a transfer artifact. Georgopoulos et al. *PLoS ONE* 5(10): e13621 (2010). doi:10.1371/journal.pone.0013621.[50]

Although ratiometric analysis is used to monitor and compare the relative phosphorylation response in a group of samples, relative comparisons do not indicate phosphorylation stoichiometry. Calculation of stoichiometry requires electrophoretic separation of phosphorylated and total protein forms; it cannot be determined using antibodies with different affinity characteristics.[1]

Analysis of other post-translational modifications, including ubiquitination, glycosylation, and acetylation, can also be performed in this manner.[28,52-53]

Chemiluminescent detection can make modification-specific immunoblotting much more difficult. Blots are sometimes stripped and reprobed with a pan-specific antibody, but it's essential to confirm that the original modification-specific antibody is completely removed. Any residual antibody will create artifacts when the blot is reprobed. Complete removal of antibodies can be difficult, and may require harsh stripping conditions that cause substantial loss of transferred proteins from the membrane.[1-2,25] Methods such as two-color fluorescence or detection of replicate blots are alternatives to stripping and reprobing.[1]

6.4 Internal loading control proteins: strengths, limitations, and considerations

For accurate normalization by fixed point, an internal control protein should meet several requirements:

Its abundance must be relatively constant for the conditions and samples relevant to the experiment (cell and tissue type, drug treatments, growth conditions, disease state, etc). A housekeeping protein may meet this need, or an alternative internal reference protein may be selected. Validation is essential to confirm stable protein expression in the samples, in your lab, and with your antibodies.[2] If the internal control is highly abundant, sample loading should be optimized to ensure that the protein is detected within the linear range and signals are not saturated. Target protein and internal control protein signals must both fall within the linear range of detection.

Signal intensity for the reference protein must display a linear relationship with the abundance of the reference protein. The upper and lower limits of the linear range can be determined empirically, and will be different for each protein detected.[1-3] At the upper end of the range, signals will become saturated and variations in protein abundance will not be detected. At the lower end of the linear range, low-intensity data points are affected by random noise and are generally not appropriate for normalization. Optimization of sample loading can help you identify and maximize the linear range of detection.

If a single internal reference protein does not meet these requirements, a multi-protein strategy (normalization by sum; discussed in section 3.2) may be needed.[1,11] Stripping and reprobing of blots can introduce detection artifacts and cause loss of blotted proteins from the membrane; it should be used with caution.[1-2,25]

7. Using total protein stains for immunoblot normalization

Concerns about the reliability of internal loading control proteins have prompted interest in alternative approaches for normalization. Total protein normalization, using a protein stain to detect the total protein in each lane of the gel or blot, is an increasingly popular option.[5,9,11,20-23,54-57] For each lane, the signal intensity of all proteins in the lane is measured in aggregate; that value is used in normalization calculations to represent the total protein content of the sample.

7.1 Total protein staining

This emerging method eliminates many of the challenges associated with internal control proteins. Total protein staining is a direct measure of the total amount of sample protein in each lane, and does not rely on an internal reference protein as an accurate indicator of sample concentration. Taking a more direct approach to assessment of sample proteins may increase the accuracy of normalization. And unlike an internal control protein, total protein staining does not require validation for each experimental context, biological system, and primary antibody. A total protein stain should produce a linear increase in signal intensity in response to increasing protein concentration. It should also correct for variation at all points in the Western blot process, including gel loading and transfer to membrane. And it must be compatible with subsequent immunodetection of the blot.[11,20-22]

Some protocols involve staining of a gel for comparison of total protein.[5] This is an excellent method for standardization of sample loading, but does not address potential inconsistencies in membrane transfer. Other protocols call for direct staining of the membrane after transfer.[20-23,55,57] This provides a more

detailed assessment of total lane density on the blot by accounting for variability in transfer efficiency, protein binding, and other factors. However, the membrane stain must be compatible with subsequent immunostaining. In some protocols, the membrane is stained after immunodetection is complete.[10] Alternative approaches have also been proposed; these "stain free" methods involve labeling of total sample proteins with a detection moiety.[13,23,56] Protein labeling occurs prior to or during gel electrophoresis (see section 7.4).

7.2 Coomassie staining of total protein for normalization

Although Coomassie staining has traditionally been quantified by densitometry, Coomassie Blue is also an excellent near-infrared (NIR) fluorophore. Gel imaging with an NIR fluorescence laser scanner generates strong 700 nm signals, with a wide linear response to protein concentration. Fluorescent signal is proportional to protein content across a range of 10 ng to 20 µg per band.[54] Because fluorescence is induced by binding to protein, background is very low and sensitivity is excellent.

Replicate gels or blots can be stained with Coomassie to standardize sample loading. It can also be used to stain the Western blot membrane after immunodetection.

7.2.1 Coomassie staining of replicate gels

The replicate gel approach was validated in a 2014 study.[5] Wishart and colleagues explored Coomassie staining as an alternative to commonly-used internal control proteins. First, they evaluated the linear range and sensitivity of total protein staining (Fig. 12). Coomassie staining (detected by NIR fluorescence and pseudo-colored in red) produced a total protein signal that was linear across the entire range of protein concentrations (Fig. 12, red line). Coomassie results directly correlated with protein concentration data from BCA assays (Fig. 12, blue line). Total protein analysis by Coomassie staining provided a valid, linear readout of sample concentration.

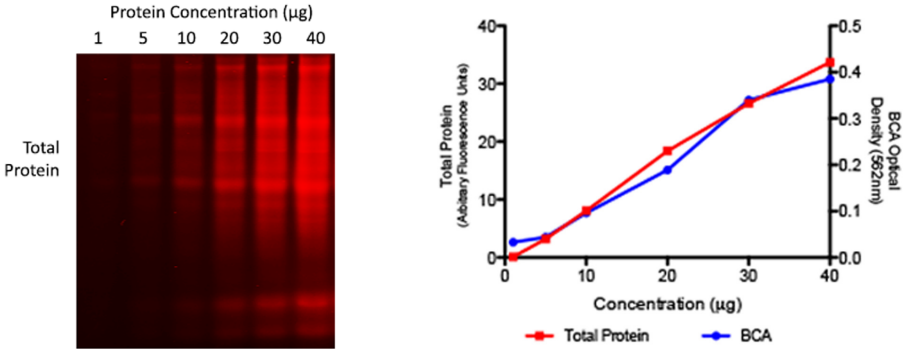

Figure 12. Coomassie staining of total protein is an accurate measure of protein load. Total protein was detected in whole brain homogenates with Coomassie stain and NIR fluorescence imaging. Coomassie signals (R^2=0.996) were directly correlated with BCA assay results (R^2=0.979) across a wide range of protein concentration. Eaton et al. doi:10.1371. journal.pone.0072457.[5]

Beta-actin expression was then examined in various tissues from wild-type mice, using total protein analysis for normalization.[5] Actin was detected by Western blotting in samples from six different tissues and was highly variable (Fig. 13A, green bands). In the quantitative analysis, Coomassie staining showed that total protein levels were relatively consistent but actin expression varied widely (Fig. 13B). Total protein is a more appropriate loading control than actin for cross-tissue comparison of these mouse samples.

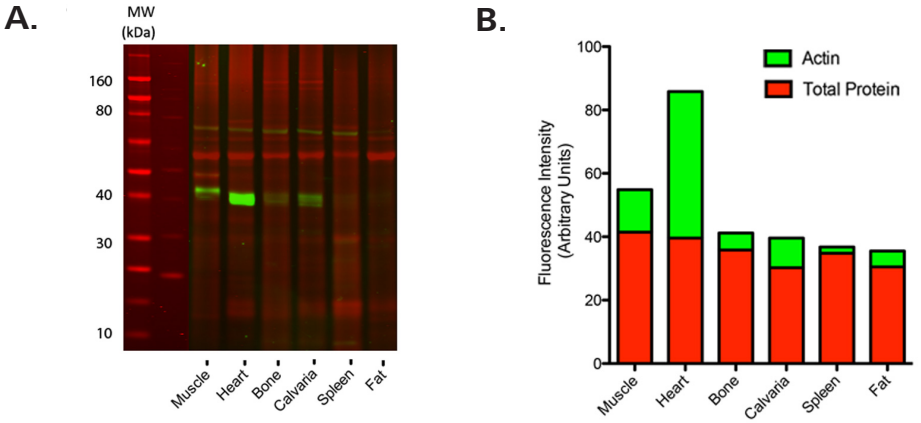

Figure 13. Expression of β-actin is highly variable in different mouse tissues. A) Actin expression was assessed by immunoblotting in tissue samples from muscle, heart, bone (femur), calvaria, spleen, and fat (gonadal). Multiplex immunoblot analysis with NIR fluorescence demonstrates variability of actin expression (green) in these tissues. A total protein gel image (red; overlaid on blot) was used to confirm the accuracy of protein loading across the samples. B) Stacked bar graph shows the comparative variability of β-actin (green bars) and total protein stain (red bars) for each tissue. Eaton et al. doi:10.1371/journal.pone.0072457.[5]

7.2.2 Coomassie staining of immunoblots

The replicate gel strategy is simple and useful, but does not account for variability in membrane transfer. Welinder and Ekblad demonstrated conventional Coomassie staining of PVDF blots after immunodetection as an internal control for protein loading.[55] The linearity of this method was superior to antibody-based detection of GAPDH, and more sensitive than the reversible total protein stain, Ponceau S. Although the blot had been blocked with nonfat dry milk, blocking proteins caused only negligible background staining and protein bands were clearly resolved. This post-staining approach corrects for variation in membrane transfer, and guarantees that total protein staining will not affect antibody binding and detection.

7.3 Other options for total protein staining of membranes

A variety of stains can be used to detect total protein on blotted membranes.[1-3,5,54-57] Some stains are reversible and can be removed from the blot prior to immunodetection. Depending on the binding chemistry, reversal of staining may require changes in pH or solvent hydrophobicity.[22] Commercially available total protein stains with signals of various colors and wavelengths are available. Depending on the stain, detection and quantification may be performed by densitometry or fluorescence imaging.

Several factors should be considered when you choose a total protein stain for normalization by sum. The sensitivity and linear range of each stain are different, and these parameters may affect your experiment. Although some blot stains are quite sensitive and able to detect low-nanogram amounts of protein, others are orders of magnitude less sensitive. Linear response range should be empirically tested to make sure it is appropriate for your sample concentrations.[2] Certain stains are compatible with only one type of membrane, nitrocellulose or PVDF. Some stains may cause increased membrane background,[1] and significant residual staining of protein bands is sometimes observed. The additional time required to stain and destain the blot is another consideration. It's also possible that use of a reversible stain could somehow affect certain epitopes. This is also a concern with staining methods that chemically modify sample proteins by covalent attachment of a detection molecule (section 7.4).[56] Coomassie staining performed after immunodetection would eliminate this possibility,[55] as discussed in section 7.2.2.

7.4 Alternative methods for total protein normalization

7.4.1 Stain-Free™ technology

The "Stain-Free" system from Bio-Rad takes a different approach to total protein detection – chemical labeling of the sample proteins.[3,13,56] The labeling moiety, a trihalo compound, is incorporated into the proprietary pre-cast gel. When this gel is exposed to UV light, the trihalo label covalently binds tryptophan residues in the sample proteins and forms a cross-linked fluorescent product. Fluorescently-modified proteins are then detected with a CCD imager and UV illumination.

Total protein can be assessed in the gel with good sensitivity across a linear range of 1-35 µg of cell lysate (Fig. 14A).[13] However, sensitivity is greatly reduced and the linear range is much narrower for detection of proteins on the transferred membrane. Loss of sensitivity is caused by high membrane background, due to autofluorescence of the PVDF membrane at UV wavelengths.[13] Membrane imaging of Stain-Free labeled proteins offers a linear range of only 10-70 µg (Fig. 14B), similar to Ponceau S.

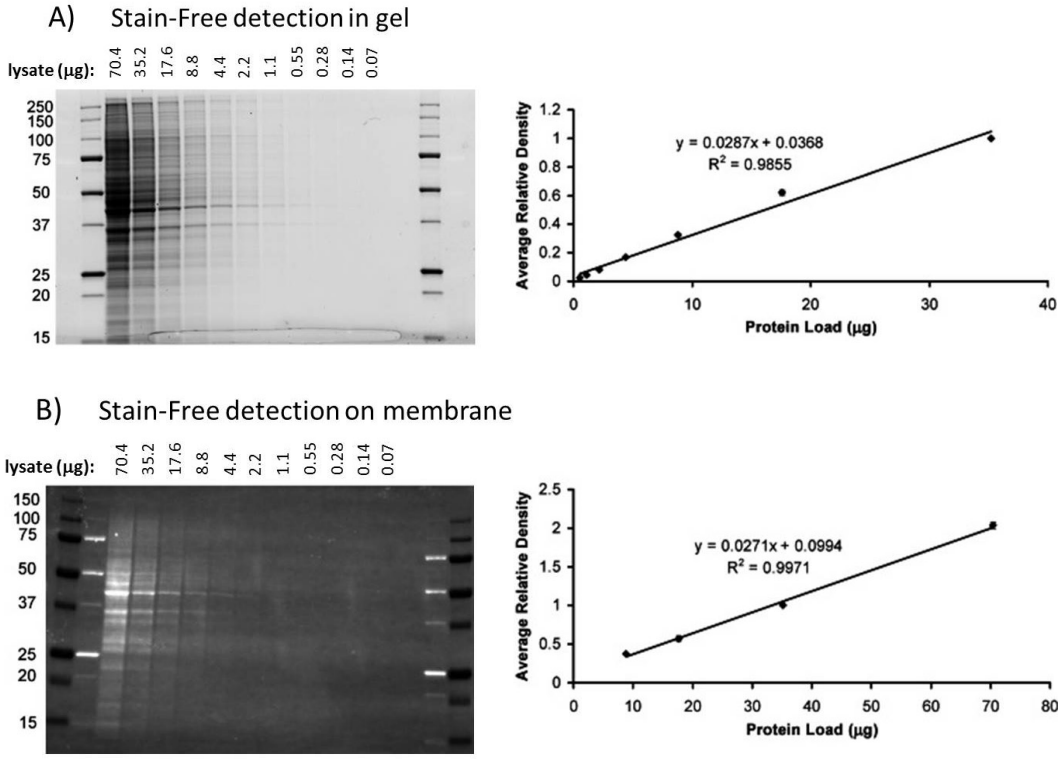

Figure 14. Stain-Free imaging of transferred membranes has a much narrower linear range than imaging of gels. Two-fold serial dilutions of HeLa cell lysate were loaded. A) Detection of Stain-Free labeled proteins in the gel. Graph displays the average relative lane density of the total protein load for three gels. MW markers were run in the first and last two lanes. B) Detection of Stain-Free labeled proteins after membrane transfer. Membrane autofluorescence is high at UV wavelengths, limiting the detection sensitivity and linear range. Taylor SC et al. 2013. *Mol Biotechnol.* 55:217-26.[13]

7.4.2 Limitations of the Stain-Free™ technology

The Stain-Free technique is convenient but has notable limitations. Unlike conventional stains that can be washed away, the Stain-Free chemistry is covalently, irreversibly bound to your protein samples. Chemical modification of tryptophan residues may interfere with immunodetection of some epitopes or have other unintended downstream effects.[3,13,56]

Although Stain-Free chemistry performs well for gel imaging, optimal normalization also requires correction of variability introduced by membrane transfer. Inconsistent transfer efficiency can result in two- to four-fold changes in signal across the blot.[10,13] If uncorrected, these effects add uncertainty to the data and may increase the coefficient of variation (CV).

The limited sensitivity of Stain-Free chemistry for total protein detection on membranes may also limit its usefulness. The published limit of detection is ~10 µg/well (Fig. 14B).[13] Given that sample loading of 10-25 µg/well is common,[1-2,13] total protein levels may routinely fall in the lower end of the Stain-Free linear range, near the lower limit of detection. These low-intensity data points may be unsuitable for normalization. Low-intensity normalization signals have larger CVs and may introduce uncertainty, increasing the mean CV of the normalized data and producing false negatives (undetectable differences between protein samples).[10-11] This may be a concern in the context of normalization by sum, because the redistribution of uncertainty may further increase the CV of low-intensity data points.

Increasing the amount of sample protein loaded on the gel will not overcome this sensitivity limitation. As discussed previously, overloaded samples may produce strong Western blot signals that become saturated. High-intensity data points outside the linear range will also affect the accuracy of normalized data.[10] It may be possible to boost the Stain-Free signal intensity by extending the UV crosslinking step, but this increases the extent of covalent modification and the risk of interference with antibody binding.[56]

This method also offers less flexibility than conventional stains. Proprietary gels and specific equipment for transfer and imaging are needed, requiring you to change established lab protocols.[21] The predetermined workflow makes it difficult to adjust the staining protocol to fit the sensitivity and linear range needs of different experiments or target proteins.

7.4.3 Total labeling of protein samples with reactive dye

Pre-labeling of protein samples with a lysine-reactive fluorescent dye is another option for normalization. In this method, amine-reactive fluorescent cyanine dye is mixed with the total protein sample prior to electrophoresis.[22] Reactive dye covalently binds to lysine residues in the sample proteins, forming a stable conjugate. The extent of protein labeling is controlled by the reaction stoichiometry, using a small amount of amine-reactive dye to label approximately 5% of total protein.

As with the Stain-Free method, covalent modification of sample proteins could affect downstream analysis. Conjugation of the cyanine dye to sample proteins may alter epitopes and affect antibody binding. Partial labeling of a complex mixture with reactive dye is not straightforward, and the extent of protein labeling in this method is inherently dependent on amino acid composition.

7.5 Total protein normalization: strengths, limitations, and considerations

An ideal total protein stain for normalization by sum has several key characteristics:

Linear signal output in response to sample concentration. Many protein stains can meet this need, but linear range and sensitivity can vary widely and may be specific to the type of membrane used. These parameters should be considered when choosing a stain for total protein normalization.

Corrects for variation at all points in the Western blot process. Gel-staining approaches are useful and inexpensive, but do not correct for membrane transfer variability. Staining of total protein on the blotted membrane may be the most accurate approach for Western blot normalization. Several good staining options are available, but the "best" choice for membrane staining is unclear. Sensitivity and linear range should not be sacrificed to enable detection on blotted membranes.

Compatible with immunodetection of target proteins. Membrane stains are a promising option, but staining of replicate gels or staining of blots after immunodetection may also meet researchers' needs. For a number of membrane stains, no downstream effect on immunodetection has been demonstrated. Methods that covalently modify target proteins for fluorescent detection have the potential for downstream interference.

Total protein staining after membrane transfer is emerging as possibly the most reliable and accurate method for normalization of Western blot data. This normalization strategy will require staining reagents that are affordable and flexible, offer a wide linear range of detection and excellent sensitivity on membranes, and add minimal handling time and effort to the quantitative immunoblot process.

8. Conclusions and future directions

Internal loading controls and normalization are critical for quantitative immunoblotting. An accurate loading control will display a linear relationship between signal intensity and sample concentration. When implemented, an effective normalization strategy should correct for variability in all stages of the immunoblotting process, including the transfer of sample proteins to membrane. It should also be compatible with immunodetection of target proteins and other types of downstream analysis.

As researchers detect and interpret subtle changes in protein samples, accurate normalization is becoming increasingly important. Although use of an internal control protein such as actin or tubulin is perhaps the most common strategy, recent studies are raising questions about this approach. The evidence indicates that an internal reference protein should be validated in the relevant experimental context, to confirm stable expression. But these proteins are still widely used for normalization, and routinely published without validation data. Many researchers may be unaware that an internal reference protein is not always sufficient for normalization, or that validation of the control protein is important. We see a constant stream of new publications that use this familiar, widely-accepted internal reference protein approach for quantitative Western blot analysis. But if this established method is failing us, how should we respond?[58]

As scientific publishers look more closely at experimental methods and data analysis,[18] we have a unique opportunity to re-evaluate our commonly-used normalization methods. These discussions may be uncomfortable, but we cannot simply ignore the issue. A deeper understanding of how our methods work, why they sometimes fail, and how they affect reproducibility is important for choosing the "best" normalization strategy – one that fits the context and biology of the experiment.[58]
Multi-protein normalization strategies such as total protein staining are now emerging as the new standard for immunoblot normalization.[5,20-23,54-57] Membrane staining of sample proteins may be an affordable, reliable

option that easily integrates into your existing laboratory workflow.

Several recent studies hint at the future of Western blot normalization and analysis. Some researchers are making existing internal loading controls more powerful by adding bioinformatics to the mix. Examples include the aggregate analysis strategies recommended by Janes[1] and Degasperi et al.[10] (as discussed in section 3.2). Andrews and Rutherford recently described an online tool for maximum likelihood calibration of immunoblotting data and other assays that measure samples in batches. This "1-step calibration method" computes calibration results iteratively from all measurements, and reduces the sensitivity of results to experimental noise. The data calibration software they describe is open source and in the public domain.[59]

Looking at normalization from new perspectives may help us more fully understand the limitations, and potential strengths, of our existing methods. The future "gold standard" for immunoblot normalization may combine familiar tools with new analytical paradigms that help us more effectively apply the techniques we already use.

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Technical Note

Protein Electrotransfer Methods and the Odyssey® Infrared Imaging Systems

Developed for:

Aerius
and Odyssey® Family of
Imaging Systems

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

LI-COR®

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Table of Contents

IMPORTANT: *The following methods describe suggested transfer conditions for use with the Odyssey Infrared Imagers, but are intended only as a supplement to the manufacturer's instructions. Before proceeding, familiarize yourself with the appropriate user manuals and troubleshooting guidelines. Due to the large number of factors that affect protein transfer efficiency and performance, the scope of this document is limited to the materials listed in the "Required Materials" section below. Alternate materials may be substituted, if desired; however, user optimization will be necessary.*

I. Introduction

Electrophoretic elution, also referred to as electrotransfer or electroblotting, is the fastest and most reliable blotting method for transferring proteins from a polyacrylamide gel to a membrane support. The two most commonly-used techniques for electrotransfer are wet tank transfer and semi-dry transfer. This document describes the specific application of these two transfer methods for detection on the Odyssey Infrared Imagers. An alternative high-speed electrotransfer method utilizing the iBlot Dry Blotting System (Invitrogen Corporation, Carlsbad, CA), is also described.

REMINDER: *Wear gloves at all times when handling membranes, gels, and other blotting materials. Gloves will not only prevent contamination, but also protect from exposure to potentially hazardous chemicals commonly used in blotting procedures. Avoid touching membranes directly and always use clean forceps when possible.*

II. Required Materials

- Processed polyacrylamide protein gel, from one of the following sources:
  - NuPage® Bis-Tris pre-cast gels (Invitrogen Corporation, Carlsbad, CA)
  - Novex® Tris-Glycine pre-cast gels (Invitrogen Corporation, Carlsbad, CA)
  - Ready Gel® Tris-HCl gels (Bio-Rad Laboratories, Hercules, CA)
- Odyssey Nitrocellulose Membrane (LI-COR, P/Ns 926-31090 and 926-31092); or, Immobilon®-FL PVDF Membrane (Millipore, P/N IPFL00010)
- 10X Tris-Glycine Transfer Buffer
- 10X PBS Buffer
- Reagent grade Methanol
- Power supply (not required for iBlot transfers)

Tank (wet) Transfer:

- Mini Trans-Blot® Electrophoretic Transfer Cell (Bio-Rad, P/N 170-3930)
- Blotting filter paper, thick (Bio-Rad, P/N 170-3932)

Semi-Dry Transfer:

- Trans-Blot SD Semi-Dry Electrophoretic Transfer Cell (Bio-Rad, P/N 170-3940)
- Blotting filter paper, extra-thick (Bio-Rad, P/N 170-3967)

iBlot® Transfer:

- iBlot Dry Blotting System (Invitrogen, P/N IB1001)
- For single mini-gel transfers: iBlot Gel Transfer Stack, Nitrocellulose, Mini (Invitrogen, P/N IB3010-02); or, iBlot Gel Transfer Stack, PVDF, Mini (Invitrogen, P/N IB4010-02)
- For single midi- or dual mini-gel transfers: iBlot Gel Transfer Stack, Nitrocellulose, Regular (Invitrogen, P/N IB3010-01); or, iBlot Gel Transfer Stack, PVDF, Regular (Invitrogen, P/N IB4010-01)

III. Comparison of Electrotransfer Techniques

Table 1. Advantages and disadvantages of electrotransfer techniques.

	Wet tank	Semi-dry	iBlot System
Advantages	Greatest flexibility for optimization	Short transfer time (15-30 minutes)	Very short transfer time (7-10 minutes)
	More complete elution of proteins	Small buffer volume requirement	Little or no buffer requirements
	Most favorable for a broader range of protein molecular weights, resulting in more consistent antibody recognition	Large surface area available for transferring large gels or several small gels	Self-contained system with very few additional components required (e.g. power supply)
	Many options available for alternate transfer equipment	Low equipment maintenance	Low equipment maintenance
Disadvantages	Longer transfer times required (1 - 16 hours)	Extended transfer times not possible (due to buffer depletion)	Relatively low flexibility for optimization
	Large buffer requirement (500 mL or more)	Low buffering capacity	Higher operating expense (disposable electrode stack must be purchased from Invitrogen)
	Cooling required for most systems	Variable transfer efficiencies for low and high molecular weight proteins	Transfer efficiency can vary between different proteins
	External power supply required	External power supply required	

Quantitation Comparison Examples

The following data are representative of PAGE gels transferred to a membrane support using either wet tank, semi-dry, or iBlot methods (Figure 1). The wet tank transfer technique generally provides more complete elution of proteins over a broad range of molecular weights. Semi-dry and iBlot transfer techniques typically give similar performance results, though semi-dry transfer allows for more optimization flexibility. See Table 1 for advantages and disadvantages of each technique.

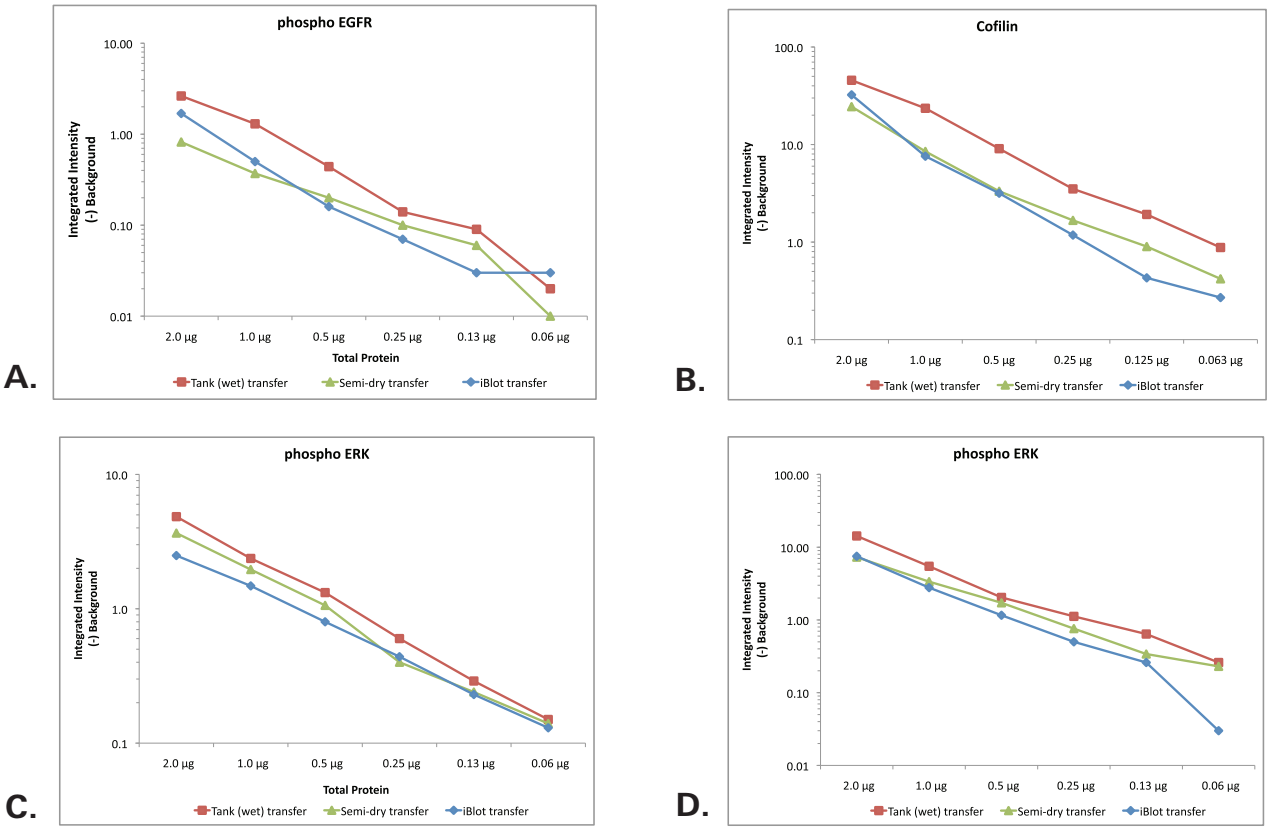

Figure 1. Western blot quantitation data comparing each of the three electrotransfer methods under varying conditions: (A) Phospho-EGFR data from NuPage 4-12% Bis-Tris gels transferred to Odyssey Nitrocellulose membranes. (B) Cofilin protein quantitation data from NuPage 4-12% Bis-Tris gels transferred to Immobilon®-FL PVDF membranes. (C) Phospho-ERK data from Novex 4-12% Tris-Glycine gels transferred to Immobilon-FL PVDF membranes. (D) Phospho-ERK data from gels prepared with NEXT GEL and transferred to Odyssey Nitrocellulose.

IV. WET TANK TRANSFER using the Mini Trans-Blot® System (Bio-Rad)

Description

Tank transfer is the traditional technique for electroelution of proteins to a support membrane. In this method, the gel/membrane stack is fully or partially immersed in a buffer reservoir and current is applied across the stack. Figure 2 shows a typical tank transfer setup.

Procedure

IMPORTANT: *Refer to "Optimization Considerations" following this section before proceeding.*

1. Preparation for transfer:
   a. Fill the Bio-Ice cooling unit with tap water. Place unit into a -20 °C freezer and allow complete freezing. After use, refill the unit and return to the freezer for future use.
   b. If necessary, cut the membrane and filter paper to the dimensions of the gel. For best results, the membrane and filter paper should be slightly larger than the gel.
   c. Prepare 1 liter of 1X transfer buffer. For improved heat dissipation during transfer, chill buffer to approximately 4 °C prior to transfer.

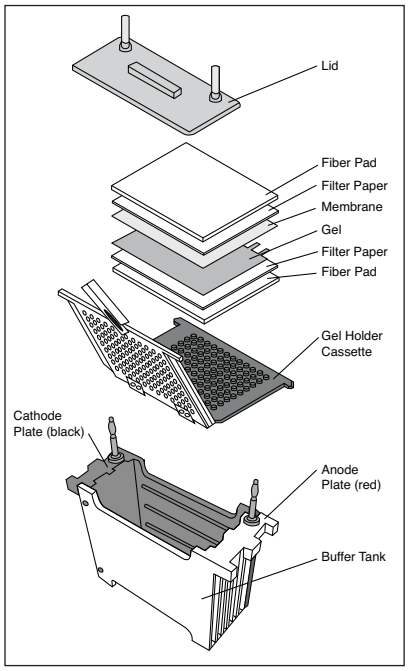

Figure 2. Diagram of tank transfer setup.

1X Transfer Buffer

10X Tris-Glycine Transfer Buffer	100 mL
Deionized water	700 mL
Methanol*	200 mL
Total	1000 mL

* Methanol should be added last to prevent precipitation

2. After gel electrophoresis is complete, trim the wells from the gel and place the gel into a suitable tray containing 1X transfer buffer. Allow the gel to equilibrate for 15-20 minutes.

3. Soak the membrane, two sheets of pre-cut blotting filter paper, and two fiber pads in 1X transfer buffer. *NOTE: Wet PVDF membranes in methanol first and rinse briefly in deionized water before soaking in transfer buffer.*

4. Prepare the gel sandwich:
   a. Place the cassette holder, gray side down, on a clean, flat surface.
   b. Place one of the pre-wetted fiber pads on the gray side of the cassette.
   c. Place one sheet of wetted filter paper onto the fiber pad.
   d. Carefully place the equilibrated gel onto the filter paper.*
   e. Place the pre-wetted membrane on the gel. Ensure that the membrane completely covers the gel, leaving no gel material protruding from the edges.*
   f. Place the second sheet of wetted filter paper on the membrane.*
   g. Place the second wetted fiber pad on top of the stack to complete the sandwich.
   h. Close and latch the cassette, being careful not to disturb the gel sandwich.
   i. Place the cassette into the electrode module, with the gray side of the cassette facing the black side of the module. This ensures that the gel is closest to the cathode (-) and the membrane is closest to the anode (+).

   * Roll out any bubbles which may have formed using a blotting roller, glass tube, or similar tool.

5. Place the electrode module, the frozen Bio-Ice cooling unit, and a magnetic stir bar into the buffer tank.

6. Fill the buffer tank with cold 1X transfer buffer and place the tank onto a magnetic stir plate. Set to stir rapidly to ensure even ion distribution throughout the transfer.

7. Attach the lid and plug the cables into the power supply. Set the power supply to run at a constant voltage of 100 V for 1 hour.

8. At the end of the transfer run, disassemble the gel sandwich. Discard the polyacrylamide gel and used transfer buffer according to your facility's waste disposal guidelines.

9. For greatest sensitivity, remove the membrane to a clean, dry container and allow to dry for 1 hour.

10. Wet the membrane in 1X PBS buffer and proceed with blocking and the remaining antibody incubation steps (Wet PVDF membranes in Methanol first, followed by brief rinsing in deionized water, before rinsing in 1X PBS buffer). Refer to LI-COR Protocol "Near-Infrared (NIR) Western Blot Detection" for details on performing Western Blot detection on the Odyssey Family of Imagers (available at www.licor.com/bio/support).

11. Clean the buffer tank, electrode module and other transfer equipment according to the manufacturer's instructions.

Optimization Considerations

Wet tank transfer allows for the greatest amount of optimization flexibility of the three electrotransfer methods described here. For this reason, the protocol described previously should be used only as a starting point for optimizing conditions for your particular sample. Detailed optimization guidelines are beyond the scope of this Technical Note; however, consider the following factors when beginning your optimization:

- *Buffer composition:* Typically, Towbin transfer buffer (25 mM Tris, 192 mM Glycine, 20% Methanol (v/v), pH 8.3) works well for most applications. This formulation provides a high buffering capacity, even for longer transfers, and promotes protein binding to the membrane. Alternative buffer components may be necessary for certain downstream applications such as protein sequencing, or for proteins that require a different pH for efficient transfer (due to unusually low or unusually high isoelectric points, for example).
- *Methanol:* Methanol in the transfer buffer helps to prevent gel swelling, which can produce uneven or fuzzy bands; also, methanol promotes protein binding to the membrane, particularly for nitrocellulose. Methanol can have negative effects as well, including gel pore size reduction, change in protein charge, and protein precipitation. Try reducing the methanol concentration to 10% or excluding methanol altogether to improve transfer efficiency.
- *Gel equilibration:* Generally, polyacrylamide protein gels should be soaked in transfer buffer prior to transfer. Lower percentage gels (i.e. < 12%) tend to shrink in methanol, so equilibration allows the gel dimensions to stabilize prior to transfer. Also, equilibration helps to reduce the amount of SDS and other buffer salts in the gel, which can interfere with protein adsorption to the membrane; however, in some cases where proteins are difficult to elute from the gel, the presence of SDS in the gel, and even the addition of SDS to a final concentration of 0.05-0.1% in the transfer buffer, can improve transfer efficiency. If you do not equilibrate the gel, or if you decide to equilibrate the gel in the presence of SDS, consider transferring to PVDF membrane. Adverse effects on protein adsorption caused by SDS will be reduced when using PVDF.
- *Power settings and transfer time:* The high-intensity power settings given in the above wet transfer procedure allow for a short transfer time. There are two main drawbacks to this high-intensity transfer: First, cooling is required to prevent the gel and the transfer buffer from overheating; high temperature not only poses a safety hazard, but also may damage the gel/membrane and result in poor transfer efficiency. Be certain to use chilled transfer buffer and the Bio-ice unit for high-intensity transfers. Second, high electric field strength may cause small proteins to be transferred too quickly or, conversely, incomplete transfer of large proteins. Performing the transfer overnight at low voltage (30 V), may result in a more quantitative transfer over a broader range of protein molecular weights.

Sample Wet Tank Transfer Data

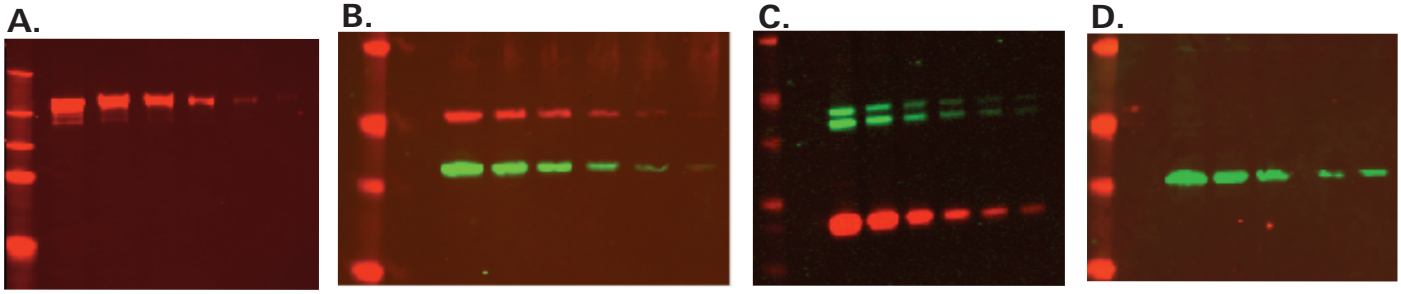

Figure 3. In each of the experiments shown above, serial dilutions of A431 cell lysate (protein concentration range 2.0 – 0.06 µg) were loaded onto polyacrylamide protein gels, electrophoresed, and transferred to membrane using the Mini Trans-Blot® (Bio-Rad) wet tank transfer system. Detection was performed using IRDye® labeled secondary antibodies and the Odyssey Infrared Imager. (A) Phospho-EGFR; Odyssey Nitrocellulose. (B) Tubulin (red, 700 channel) and ERK2 (green, 800 channel); Odyssey Nitrocellulose. (C) Cofilin (red, 700 channel) and phospho-ERK (green, 800 channel); Immobilon-FL PVDF. (D) GAPDH; Odyssey Nitrocellulose.

V. SEMI-DRY TRANSFER using the Trans-Blot® SD System (Bio-Rad)

Description

Semi-dry transfer utilizes two plate electrodes, which come in direct contact with the gel/membrane transfer stack, for electrical transfer of proteins to a membrane support. In this system, two pieces of buffer-soaked blotting filter paper replace the buffer tank and serve as the ion reservoir for current flow. Figure 4 shows a typical semi-dry transfer setup.

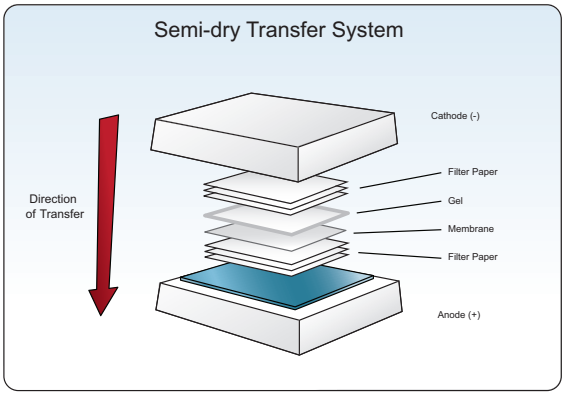

Figure 4. Diagram of semi-dry transfer setup.

Procedure

IMPORTANT: *Refer to "Optimization Considerations" following this section before proceeding.*

1. Preparation for transfer:
   a. Prepare 250 mL of 1X transfer buffer. For improved heat dissipation during transfer, chill to 4 °C prior to transfer.

1X Transfer Buffer

10X Tris-Glycine Transfer Buffer	25 mL
Deionized water	175 mL
Methanol*	50 mL
Total	250 mL

* Methanol should be added last to prevent precipitation

   b. Cut the membrane and two sheets of extra-thick blotting filter paper to the dimensions of the gel(s). For best results, the membrane and filter paper should be the same size or slightly larger than the size of the gel(s) to be transferred. Filter paper that is cut too large will result in inefficient current flow through the gel.

2. After gel electrophoresis is complete, trim the wells from the gel and place the gel into a suitable tray containing 1X transfer buffer. Allow the gel to equilibrate for 15-20 minutes.

3. Soak the membrane and two sheets of extra-thick blotting filter paper in 1X transfer buffer. *NOTE: Wet PVDF membranes in methanol first and rinse briefly in deionized water before soaking in transfer buffer.*

4. Remove the safety cover and cathode assembly and prepare the gel sandwich:
   a. Place a sheet of pre-soaked extra thick filter paper onto the platinum anode surface.*
   b. Place the pre-soaked membrane on top of the filter paper.*
   c. Carefully place the equilibrated gel on top of the membrane. Ensure that the gel is centered on the membrane, so that no part of the gel extends past the edges of the membrane.*
   d. Place the other sheet of pre-soaked filter paper onto the gel.*
   e. Carefully place the cathode onto the transfer stack. Press down on the cathode assembly to engage the latches, making certain not to disturb the stack.
   f. Replace the safety cover and plug the unit into a power supply.

** Roll out any bubbles which may have formed, using a blotting roller, glass tube, or similar tool.*

5. Set the power supply to run at a constant voltage of 20 V for 20 minutes (single mini gel), or 20 V for 30 minutes (two or more mini gels).

6. At the end of the transfer run, carefully remove the safety cover and cathode assembly. Disassemble the gel sandwich and discard the polyacrylamide gel and filter paper sheets according to your facility's waste disposal guidelines.

7. For greatest sensitivity, remove the membrane to a clean, dry container and allow drying for 1 hour.

8. Wet the membrane in 1X PBS buffer and proceed with blocking and the remaining antibody incubation steps (wet PVDF membranes in Methanol first, followed by brief rinsing in deionized water, before rinsing in 1X PBS buffer). Refer to LI-COR Bioscience's Protocol "Near-Infrared (NIR) Western Blot Detection" for details on performing Western Blot detection on the Odyssey Family of Imagers (available at www.licor.com/bio/support).

9. Clean the cathode assembly and the base/anode assembly according to the manufacturer's recommendations.

Optimization Considerations

Direct contact of the plate electrodes with the gel/membrane sandwich in the semi-dry transfer system results in relatively faster transfers, but allows for less flexibility for optimizing transfer conditions. Consider the following factors for optimizing semi-dry transfer:

- *Transfer Buffer:* The amount of transfer buffer required is greatly reduced in the semi-dry transfer system. Also, since the electrodes are in direct contact with the transfer stack and the distance between the electrodes is relatively small, high electric field strengths can be achieved. However, the buffering capacity of the system is low and, since there are no means for external cooling, transfer time is limited. Although Towbin transfer buffer (25 mM Tris, 192 mM Glycine, 20% Methanol (v/v), pH 8.3) is suitable in most cases, alternate transfer buffers should be considered for optimizing transfer efficiency. For example:
  - Bjerrum and Schafer-Nielsen transfer buffer (48 mM Tris, 39 mM glycine, 20% Methanol (v/v), pH 9.2)
  - Discontinuous buffer system: Semi-dry transfer confers the unique ability to use different buffers for each set of filter papers in the transfer stack. One good example of this system involves using a Tris-CAPS-methanol buffer on the anode side and a Tris-CAPS-SDS buffer on the cathode side (60 mM Tris, 40 mM CAPS, pH 9.6, plus either 15% methanol or 0.1% SDS).
- *Gel equilibration:* Some electrophoresis components, particularly SDS, increase the conductivity of the transfer buffer and thereby increase the amount of heat generated during transfer. Hence, gels should generally be equilibrated in transfer buffer prior to transfer to remove these residual components.
- *Electrode contact:* Efficient transfer depends significantly on complete contact of the two electrodes with the gel/membrane transfer stack. When preparing the stack, ensure that the membrane and filter paper sheets are trimmed to the dimensions of the gel (as described in the above procedure), and that bubbles are completely removed while assembling each piece of the stack. Do not use more than the recommended amount of filter paper in the stack. A stack that is too thick will result in excessive cathode pressure on the stack. The total thickness of filter paper used in the stack should be approximately 5.0 – 7.5 mm.
- *Power settings and transfer time:* The low buffering capacity and high amount of heat generated in semi-dry transfers necessitates a short (15 – 30 min.) transfer time. Because of the high electric field strength that can be generated, however, very efficient protein elution can be achieved. A number of factors dictate the optimal power settings and transfer time, including buffer composition and pH, gel composition, gel percentage, gel thickness, number of gels,

and protein molecular weight. Power conditions may require optimization when any of these factors changes significantly. If overheating is a problem, consider running the semi-dry transfer under constant current for a longer time (30 – 60 min.), rather than constant voltage for a short time. Under constant voltage, the current will drop off and cause the power and heat generation to increase. Maintaining constant current will decrease the amount of heat generated, although proteins will transfer slower. Refer to the manufacturer's instructions for recommended power settings.

Sample Semi-Dry Transfer Data

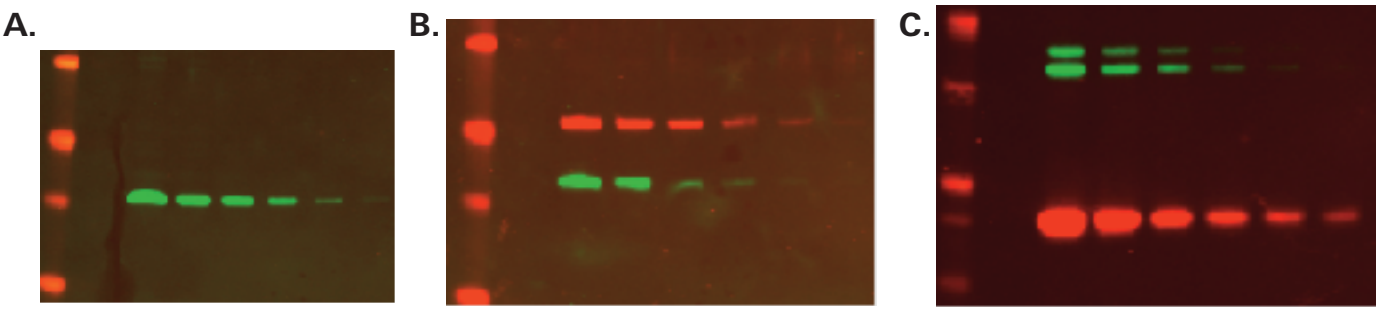

Figure 5. In each of the experiments shown above, serial dilutions of A431 cell lysate (protein concentration range 2.0 – 0.06 µg) were loaded onto polyacrylamide protein gels, electrophoresed, and transferred to membrane using the Trans-Blot® SD (Bio-Rad) semi-dry transfer system. Detection was performed using IRDye® labeled secondary antibodies and the Odyssey Infrared Imager. (A) GAPDH; Odyssey Nitrocellulose. (B) β Tubulin (red, 700 channel) and ERK2 (green, 800 channel); Odyssey Nitrocellulose. (C) Cofilin (red, 700 channel) and phospho-ERK (green, 800 channel); Immobilon-FL PVDF.

VI. iBLOT® DRY BLOTTING TRANSFER SYSTEM (Invitrogen®)

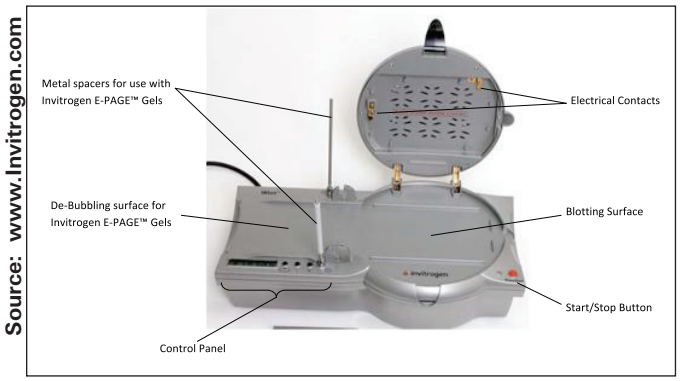

Figure 6. iBlot Gel Transfer Device

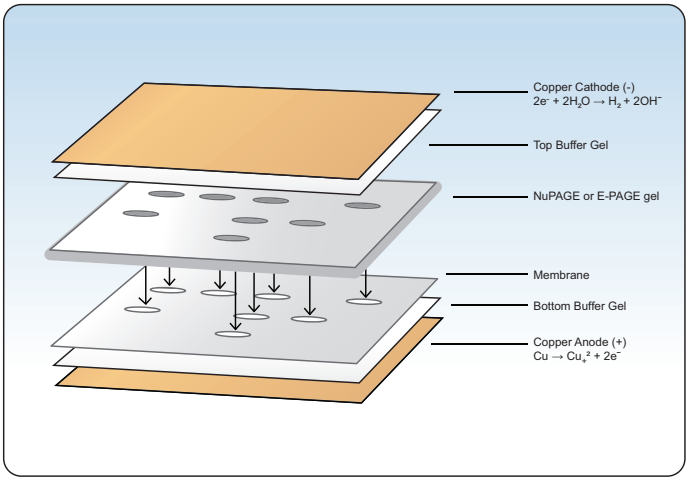

Figure 7. Gel/membrane sandwich with anode and cathode stacks

Description

The iBlot Dry Blotting System combines a patented gel matrix technology with a self-contained blotting unit to provide very fast, dry blotting of proteins to a support membrane. The concept of the iBlot system is similar to that of semi-dry electrotransfer, except that anode and cathode buffers contained in a solid gel matrix are used instead of buffer-soaked filter sheets. The iBlot device is shown in Figure 6. Figure 7 shows the transfer stack in a standard iBlot setup.

Procedure

IMPORTANT: *Refer to "Optimization Considerations" following this section before proceeding.*

1. Place the device on a stable flat surface, plug in the power cord, and turn on the power switch.
2. Soak the desired membrane in 1X PBS buffer.
   NOTE: Wet PVDF membranes in methanol and rinse briefly in deionized water before soaking in 1X PBS buffer.
3. Open the lid of the device.
4. Remove the sealing from the anode stack, and place the anode stack onto the blotting surface, with the tray tab facing toward the right.
5. Remove the membrane from the top of the anode stack using a pair of forceps. Replace with a piece of Odyssey Nitrocellulose or Immobilon®-FL PVDF membrane cut to the same dimensions.
6. Carefully place the pre-run gel on the transfer membrane, ensuring that the gel does not protrude over the edges of the membrane. One mini gel can be placed onto Anode stack (mini); or, two mini gels can be placed onto Anode stack (regular).
7. Wet a sheet of iBlot® filter paper in deionized water, and place on top of the pre-run gel(s). Roll out any bubbles which may have formed using the provided Blotting Roller.
8. Remove the sealing from the cathode stack and discard the red plastic tray.
9. Place the cathode stack, with the buffer gel facing down, onto the filter paper. Roll out bubbles using the Blotting Roller.
10. Remove the disposable sponge from its wrapper, and place it on the lid of the iBlot device. The metal contact should be in the upper right corner of the lid.
11. Close the lid of the device and secure the latch.
12. Select the appropriate program and run time:
    a. For NuPage® Bis-Tris gels, select program "P2" and set the run time to 10:00 minutes.
    b. For all other gels, select program "P3" and set the run time to 7:00 minutes.
13. Press the Start/Stop button. The red light changes to green, indicating the start of the run.
14. At the end of the transfer run, the device will automatically shut off and begin beeping. Press the Start/Stop button to complete the run.
15. Open the lid of the iBlot device, and disassemble the transfer stack. Discard the anode stack, gel, filter paper, and cathode stack according to your facility's waste disposal guidelines.
16. For greatest sensitivity, remove the membrane to a clean, dry container and allow to dry for 1 hour.
17. Wet the membrane in 1X PBS buffer and proceed with blocking and the remaining antibody incubation steps (Wet PVDF membranes in Methanol first, followed by brief rinsing in deionized water, before rinsing in 1X PBS buffer). Refer to LI-COR Bioscience's Protocol "Near-Infrared (NIR) Western Blot Detection" for details on performing Western Blot detection on the Odyssey Family of Imagers (available at www.licor.com/bio/support). A similar protocol is available for Aerius Imaging Systems.
18. Turn off the iBlot power switch. Clean the blotting surface and lid with a clean, damp cloth or paper tissue, and store the device according the manufacturer's recommendations.

Optimization Considerations

Invitrogen's iBlot® transfer technology uses a similar concept as semi-dry transfer, but replaces the user-formulated buffer system with a patented, high-performance buffer gel matrix to achieve very fast transfers. Limited flexibility for changing transfer conditions restricts the amount of user optimization necessary, but the following factors should be considered:

- *Power settings and transfer time:* Five transfer programs are available, which correspond to different voltage settings (10 – 25 V). Program selection, together with transfer time adjustment, has a considerable impact on transfer efficiency. The instrument settings given in the above procedure will typically provide good results in most cases; however, adjustments may be required, depending on the type and percentage of gel being used. When optimizing conditions, begin by adjusting transfer time up or down in increments of 30 seconds. As a general rule, decrease the voltage by one step for every 2-3 minutes of time added to the run. Refer to the manufacturer's instructions for detailed optimization guidelines.

- *Gel equilibration:* One of the advantages of the iBlot system is that transfer buffer is not generally necessary. However, in some cases, more efficient protein elution may be achieved with gel equilibration. Prepare 100 mL of transfer buffer in a shallow tray, and allow the prerun gel to soak for 5-15 minutes.

1X Transfer Buffer

10X Tris-Glycine Transfer Buffer	10 mL
Deionized water	70 mL
Methanol*	20 mL
Total	100 mL

* Methanol should be added last to prevent precipitation

- *Electrode contact:* Complete contact between the anode stack, membrane, gel, and cathode stack is essential to achieving optimal transfer efficiency. Ensure that all components of the transfer stack are aligned evenly, and that bubbles are completely removed during assembly. Also, be sure to remember to include the disposable sponge, and to secure the latch when closing the lid on the device. The disposable sponge and electrode stacks may be used only once.

- *Membrane choice:* Odyssey Nitrocellulose or Immobilon®-FL PVDF membranes give the best results for detection on the Odyssey Infrared Imaging Systems. The membrane provided with the anode stack is compatible with the Odyssey family of imagers, but higher background signal may result. When replacing the provided membrane, be sure to cut the new membrane to the appropriate dimensions and wet it in transfer buffer or deionized water prior to placing it onto the anode stack (wet PVDF membrane in methanol first, followed by soaking in transfer buffer or deionized water). No preparation is required if using the Invitrogen membrane provided with the anode stack.

Sample iBlot® Transfer Data

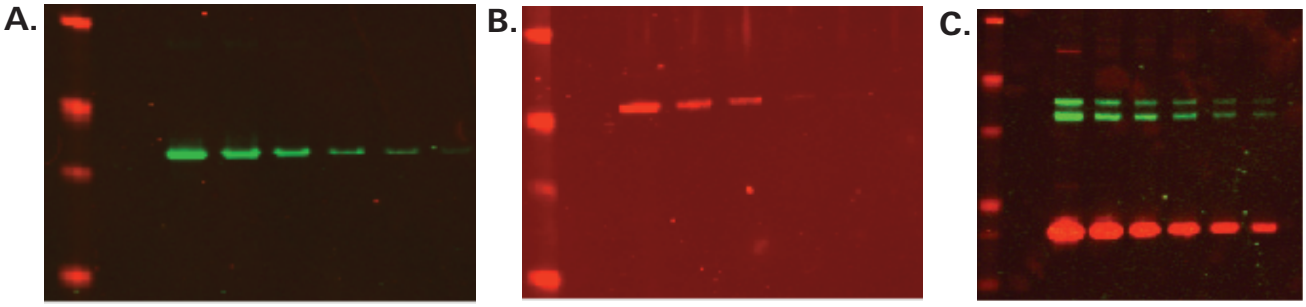

Figure 8. In each of the experiments shown above, serial dilutions of A431 cell lysate (protein concentration range 2.0 – 0.06 µg) were loaded onto polyacrylamide protein gels, electrophoresed, and transferred to membrane using the iBlot Dry Blotting System (Invitrogen). Detection was performed using IRDye® labeled secondary antibodies and the Odyssey Infrared Imager. (A) ERK2; Odyssey Nitrocellulose. (B) β Tubulin; Odyssey Nitrocellulose. (C) Cofilin (red, 700 channel) and phospho-ERK (green, 800 channel), Immobilon®-FL PVDF.

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Doc # 988-16191 05/16

Technical Note

One-Blot Western Optimization Using the MPX™ Blotting System

Developed for:

Aerius, and Odyssey®
Family of Imaging Systems

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

LI-COR®

Published May 2009. Revised May 2016.
The most recent version of this Technical Note is posted at
http://www.licor.com/bio/support

Table of Contents

I. Introduction

The independent channels of the LI-COR MPX (Multiplex) Blotter make it possible to optimize blocking buffer, primary antibody dilution, and secondary antibody dilution on a single Western blot. Western blotting procedures that generate a blot of 7.0 x 8.5 cm are easily adapted to the MPX format. The process fits into any laboratory's standard Western blot workflow. Both home-made and pre-cast gels can be used to generate blots.

Electrophoresis and transfer to nitrocellulose membrane are performed under standard conditions. Clamping the blot into the MPX Blotter creates up to 24 independent channels, allowing different conditions to be tested in each channel. The range of usable channels per sample is relative to comb size. For Western blot optimization, a single-well gel ("prep gel") is all that is needed. For this application, any detection method can be used, including near-infrared (NIR) fluorescence and chemiluminescence. This document presents general guidelines for use with the Odyssey® family of Infrared Imaging Systems.

II. Required Reagents

		LI-COR P/N
Sample Preparation	4X Protein Sample Loading Buffer	928-40004
Electrophoresis	Odyssey One-Color Protein Markers (Molecular Weight - 10 kDa to 250 kDa)	928-40000
	Chameleon® Duo Pre-stained Protein Ladder (8 kDa to 260 kDa)	928-60000
Blotting and Transfer	Tris Glycine	
	Odyssey Nitrocellulose (10 membranes; 7 x 8.5 cm)	926-31090
	or	
	Odyssey Nitrocellulose (1 roll; 30 cm x 3 m)	926-31092

		LI-COR P/N
MPX Detection	Blocking Buffer Optimization Kit	927-40040
	• Odyssey® Blocking Buffer (TBS)	927-50000; 927-50100
	• Odyssey Blocking Buffer (PBS)	927-40000; 927-40100
	• Casein Blocking Buffer	927-40200; 927-40300
	• Blocking buffer of your choice (BSA, Milk, etc.)	
	IRDye® Labeled Secondary Antibodies	LI-COR
	NewBlot™ IR Stripping Buffer, 5X	928-40028
	• 10X PBS • 10X TBS	
	Tween® 20	
	MPX Membrane Cushion	921-00120
Imaging	Odyssey Family Imager or Aerius Imager	

III. Gel Electrophoresis and Transfer

■ Gel Preparation

A wide variety of gel matrices are compatible with the MPX Blotter system.

If you are pouring your own gels, your gel casting system can be used with a single-well comb such as the LI-COR Single Marker/One Lane Comb (921-00200, 1 mm thickness).

Alternatively, pre-cast gels can be purchased and used. Table 1 lists several suggested types of pre-cast gels, and indicates the number of usable ports that each gel will provide for use with the MPX Blotter.

Table 1. Single-sample pre-cast gel options for use with the MPX Blotter.

Vendor	Well Designation	Sample #	MW Marker Well	Usable Ports
Invitrogen	2D	1	Yes	19
Bio-Rad	2D/Prep	1	Yes	21
C.B.S. Scientific	1 Well	1	No	23

■ Sample Preparation

When using a single-well gel, a larger volume of sample is required. Prepare your protein sample so that the sample volume and concentration is equivalent to running all the lanes on a standard 10-well gel. Example: 5 µg of lysate per lane = 50 µg in a total volume of 100 - 150 µL, including loading buffer.

The following procedure is suggested: Dilute the sample 1:4 in 4X Protein Sample Loading Buffer (LI-COR P/N 928-40004) with β-Mercaptoethanol. Heat the sample at 95 °C for 5 minutes.

■ Molecular Weight Marker

It is important to have a molecular weight marker that is visible to the eye because the marker is the primary tool used to align the blot in the MPX Blotter. Odyssey® One-Color Marker or Chameleon® Duo Protein Ladder is recommended.

■ Electrophoresis

IMPORTANT: The maximum length of the separating gel should not exceed 50 mm—the length of the channels on the MPX Blotter.

■ Transfer

- Always use clean forceps when handling membranes. Nitrocellulose membrane is recommended for this procedure.
- Once electrophoresis is complete, transfer proteins to Odyssey Nitrocellulose Membrane using standard transfer procedures.
- Mark the outside corners of the gel and sample wells with a pencil before separating the transferred gel from the membrane, as shown in Figure 1. The marks will help you correctly align the membrane when it is placed in the MPX Blotter.

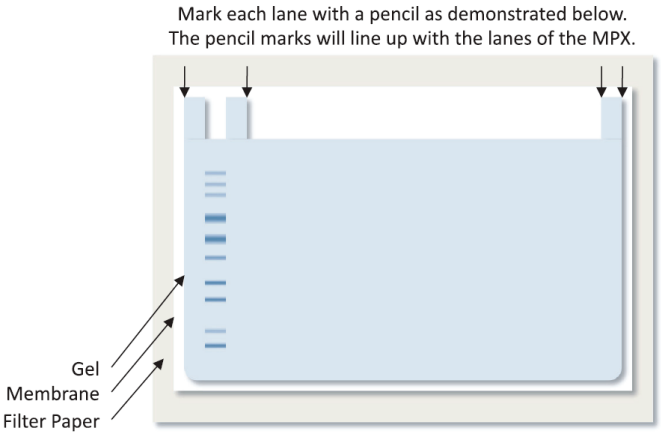

Figure 1. Mark the membrane with pencil, for later alignment into the MPX Blotter.

- Allow the membrane to dry for a minimum of one hour before proceeding with detection. *IMPORTANT! Use pencil to mark the blot. Ink from most pens will fluoresce on the Odyssey Imager and cause increased membrane background.*

IV. Membrane Blocking

■ Membrane Preparation

- Cut the membrane into four individual blots, as shown in Figure 2.
- Each individual blot will be processed with a different blocking buffer, and that blocking buffer will also be used for dilution of antibodies. TBS or PBS buffer systems may be used for blocking. **During washing steps, rinse and wash each blot with an appropriate wash buffer that matches the buffer system used for blocking.**

  Blot 1: Odyssey Blocking Buffer (TBS)
  Blot 2: Odyssey Blocking Buffer (PBS)
  Blot 3: Casein Blocking Buffer
  Blot 4: Blocking buffer of your choice (milk, BSA, etc. in TBS or PBS)

- Pre-wet each membrane with TBS or PBS buffer as appropriate (see above).

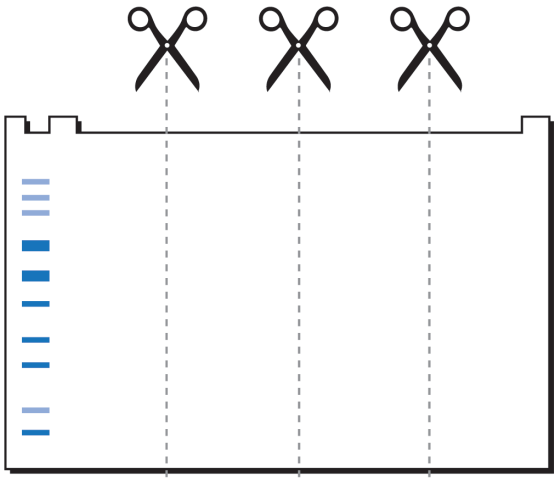

Figure 2. Cut the membrane into four individual blots for blocking buffer optimization.

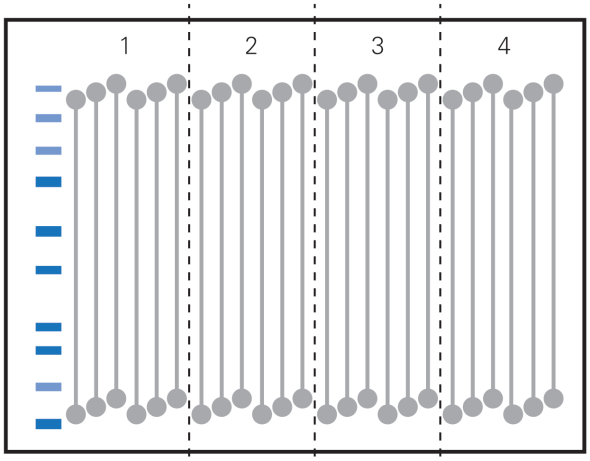

Figure 3. Place four individual blots into the MPX Blotter as shown.

- **Blocking**

  Place the membranes into 4 different incubation boxes. In each box, cover the entire membrane with blocking buffer (approximately 0.4 mL/cm^2), using a different blocking buffer for each membrane. Block the membrane for 1 hour at room temperature with gentle shaking.

  Blot 1: Odyssey® Blocking Buffer (TBS)
  Blot 2: Odyssey Blocking Buffer (PBS)
  Blot 3: Casein Blocking Buffer
  Blot 4: Blocking buffer of your choice (milk, BSA, etc.)

V. Alignment in MPX Blotter

Detailed instructions for use of the MPX Blotter are found in the MPX Blotter Multiplex Western Blotting Accessory User Guide at http://www.licor.com/mpxuserguide

Place the four blocked membranes into the MPX Blotter so that there are at least 4 channels available for use on each membrane. See Figure 3.

VI. Primary & Secondary Antibody Application

- **Primary Antibody Preparation**

  Two dilutions of primary antibody should be made for each blocking buffer that is tested. Suggested starting dilutions are 1:500 and 1:1,000. You may wish to modify these dilutions, based on vendor recommendations.*

  700 µL of each dilution will be needed. Dilute the primary antibody in the appropriate blocking buffer (see following table) with 0.2% Tween® 20.

**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.*

Blot	Blocker	Primary Antibody Dilutions	
1	Odyssey® Blocking Buffer (TBS)	1:500	1:1,000
2	Odyssey Blocking Buffer (PBS)	1:500	1:1,000
3	Casein Blocking Buffer	1:500	1:1,000
4	Blocking buffer of your choice (milk, BSA, etc.)	1:500	1:1,000

▪ Primary Antibody Application

- Load the primary antibody/blocker dilutions into the MPX Blotter in the indicated locations for each blocking buffer you are testing.
  – Apply 2 replicates of each primary antibody dilution, as shown in Figure 4.
- Fill the unused channels with the appropriate corresponding blocking buffer.
- Incubate for 1 - 4 hours at room temperature.

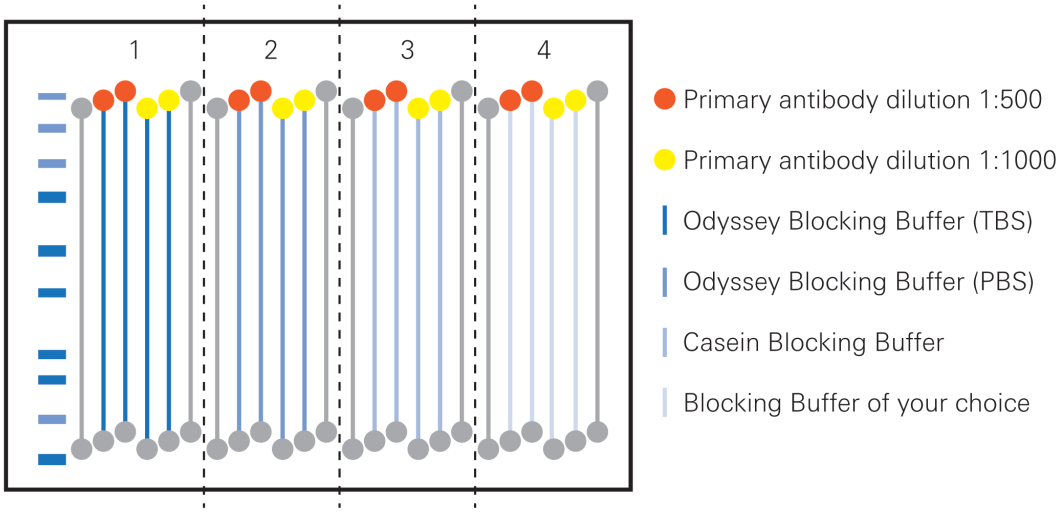

Figure 4. Placement of primary antibody dilutions in the channels of the MPX Blotter.

- Wash primary antibody from the channels thoroughly according to MPX Blotter manual instructions, using a buffer that matches the buffer system used for blocking. Wash buffers should contain 0.1% Tween® 20. ***Do not remove blot from MPX blotting manifold during washing.***

Blot 1:	TBS-T
Blot 2:	PBS-T
Blot 3:	PBS-T
Blot 4:	TBS-T or PBS-T, as appropriate

Secondary Antibody Preparation

Blot	Blocker	Secondary Antibody Dilutions	
1	Odyssey® Blocking Buffer (TBS)	1:5,000	1:10,000
2	Odyssey Blocking Buffer (PBS)	1:5,000	1:10,000
3	Casein Blocking Buffer	1:5,000	1:10,000
4	Blocking buffer of choice	1:5,000	1:10,000

Two dilutions of secondary antibody should be made for each blocking buffer that is tested. For IRDye® secondary antibodies, we recommend 1:5,000 and 1:10,000 as a starting point. Dilutions may be modified, based on vendor recommendations.

700 µL of each antibody will be needed. Dilute the secondary antibody in the appropriate blocking buffer with 0.2% Tween® 20.

Secondary Antibody Application

- Load the secondary antibody/blocker dilutions into the MPX Blotter in the indicated locations for each blocking buffer you are testing.
  - Add the secondary antibody dilutions to the channels previously stained with primary antibody, as shown in Figure 5.
- Fill the unused channels with the appropriate corresponding blocking buffer.
- Incubate 1 hour at room temperature. ***Protect from light during incubation.***

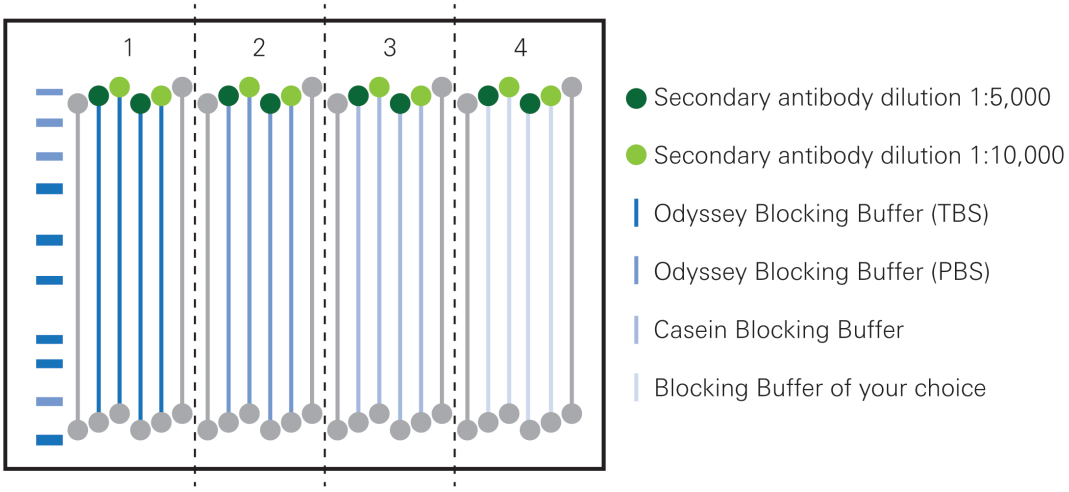

Figure 5. Placement of secondary antibody dilutions in the channels of the MPX Blotter.

VII. Imaging

Membranes can be imaged immediately.

- Image all four blots side-by-side, using standard Western blot imaging settings on any Odyssey® Family Imaging System.
- Visual inspection of images with Image Studio™ software or Odyssey application software can be used to determine which blocking buffer works best for the primary antibody you are testing.
  - View all blots together in a single image, with uniform image display settings, to compare membrane background levels and band intensity.
  - Individually adjust the image display settings for each blot to get the "best" image.
  - Evaluate non-specific banding in each blocking buffer condition.
- Look for blocking buffer conditions that provide strong signals for the expected band(s), low membrane background, and few non-specific background bands from the primary antibody.
  - Tradeoffs may be necessary. Blocking conditions that yield very strong bands might also have higher membrane background or non-specific banding.
- The "best" blocking conditions depend on the antigen-antibody pair you are using. Some primary antibodies are dramatically affected by blocking conditions. An inappropriate blocker can alter binding specificity, affecting the intensity of target bands and increasing non-specific banding. The pattern of non-specific bands may also be affected.
- Choose the blocking conditions that are most appropriate for the context and goals of your experiment.
- Quantitative analysis of specific bands on each blot will indicate if signal intensity (after background subtraction) is significantly different between blocker types.

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Doc # 988-16189 05/16

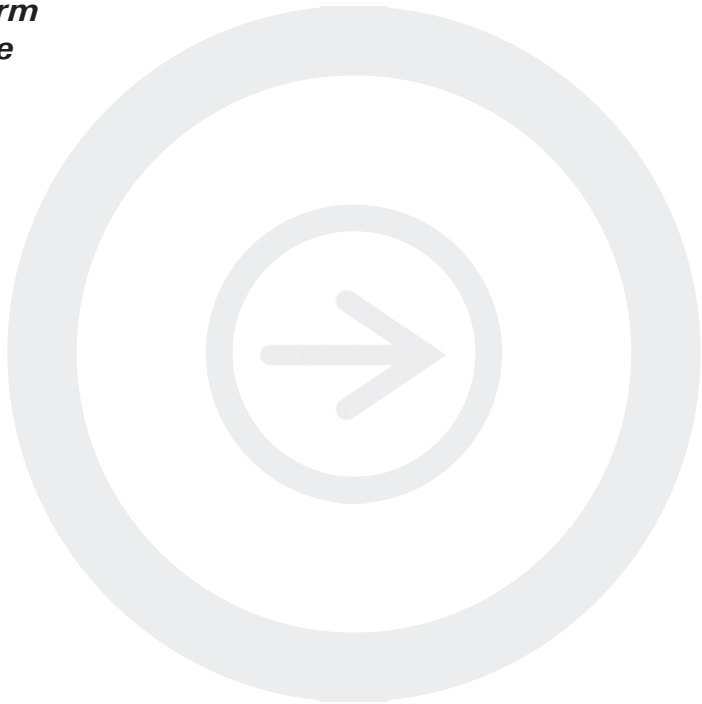
Odyssey Western Blot Blocker Optimization for Near-Infrared (NIR) Detection

Developed for:

Aerius and Odyssey® Family of Imagers

Please refer to your manual to confirm that this protocol and application are compatible with your Odyssey Imager model.

Part Numbers: 927-40040



LI-COR®

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The most recent version of this document is posted at http://biosupport.licor.com/support

Table of Contents

I. Introduction

This document describes a method to determine optimal blocking conditions for NIR Western blot detection with the Odyssey family of Imagers. The specific lysate and antibodies used in your system will be evaluated in four different blocking buffer systems. The buffering system will also be evaluated in this experiment. Odyssey Blocking Buffer is provided in either TBS (TRIS Buffered Saline) or PBS (Phosphate Buffered Saline) formulation. In general, the washing step should include the same buffering system as the blocking step (e.g., 1X TBS wash when using Odyssey Blocking Buffer (TBS)).

II. Required Reagents

	LI-COR P/N
■ Odyssey Protein Molecular Weight Marker	928-40000
■ IRDye® Secondary Antibodies (LI-COR)	
■ Blocking Buffer	
• Blocking Buffer Optimization Kit	927-40040
– Odyssey Blocking Buffer (TBS)	927-50000; 927-50100
– Odyssey Blocking Buffer (PBS)	927-40000; 927-40100
– Casein Blocking Buffer (PBS)	927-40200; 927-40300
• Blocking buffer of your choice (milk, BSA, etc.)	
■ Membrane	
• Odyssey Nitrocellulose (0.22 µm)	926-31090; 926-31092
• 4X Protein Sample Loading Buffer and PVDF Membrane Kit	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").

LI-COR P/N

- Tween® 20
- PBS Buffer (1X)
- TBS Buffer (1X)
- Methanol (when using Immobilon®-FL PVDF membrane)
- SDS (when using Immobilon-FL PVDF membrane)
- Western Blot Incubation Box 929-97201; 929-97205; 929-97210

  Aerius or Odyssey Family Imager

III. 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 two 15-well gels, load the following samples in the order indicated:

Lane	Sample	Amount
1	Primary Antibody (as positive control)	5 µL of a 1:1000* dilution in PBS
2	Sample Lysate	313 ng
3	Sample Lysate	625 ng
4	Sample Lysate	1.25 µg
5	Sample Lysate	2.5 µg
6	Sample Lysate	5 µg
7	Sample Lysate	10 µg
8	Protein Marker	1-3 µL
9	Primary Antibody (as positive control)	5 µL of a 1:1000* dilution in PBS
10	Sample Lysate	313 ng
11	Sample Lysate	625 ng
12	Sample Lysate	1.25 µg
13	Sample Lysate	2.5 µg
14	Sample Lysate	5 µg
15	Sample Lysate	10 µg

** Suggested starting point; may need to be altered, depending on concentration of primary antibody.*

IV. Western Blocker Optimization Method

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

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 membranes 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, because wetting in methanol will cause ink to run.

If using the gel configuration described in Section III (Gel Preparation for Blocker Optimization), cut each membrane along the protein marker in lane 8 as shown in Figure 1. Be careful not to touch the membranes with bare or gloved hands. Label appropriately with pencil.

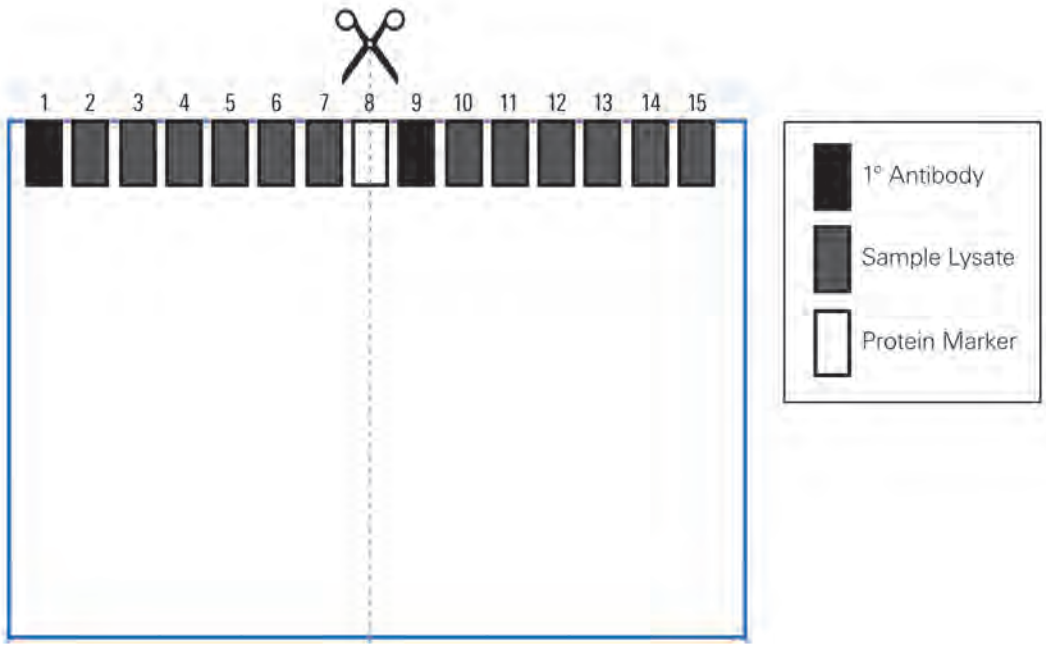

Figure 1. Cut both Western blot membranes along the Marker lanes to generate four individual optimization blots.

After cutting membranes, perform the following steps:

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

For Odyssey Nitrocellulose Membranes:

- Wet in one piece in 1X TBS for 2 minutes
  - Wet remaining pieces in 1X PBS for 2 minutes

2. Place cut membranes into 4 different Western Blot Incubation Boxes and block with 10 mL Blocking Buffer for 1 hour at room temperature while gently shaking.
   - Box 1 – Odyssey Blocking Buffer (TBS)
   - Box 2 – Odyssey Blocking Buffer (PBS)
   - Box 3 – Casein Blocking Buffer
   - Box 4 – Blocking buffer of your choice (milk, BSA, etc.)

3. Dilute primary antibody* in 10 mL of appropriate diluent listed below:
   - Box 1 – Odyssey Blocking Buffer (TBS)+ 0.2% Tween® 20 + Primary Antibody
   - Box 2 – Odyssey Blocking Buffer (PBS) + 0.2% Tween 20 + Primary Antibody
   - Box 3 – Casein Blocking Buffer + 0.2% Tween 20 + Primary Antibody
   - Box 4 – Blocking Buffer of your choice + 0.2% Tween 20 + 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 and wash each blot with a buffer that matches the buffer system used for blocking.**
     - Box 1: Rinse with 1X TBS-T (0.1% Tween 20).
       Cover blot with 1X TBS-T for washing.
     - Box 2: Rinse with 1X PBS-T (0.1% Tween 20).
       Cover blot with 1X PBS-T for washing.
     - Box 3: Rinse with 1X PBS-T (0.1% Tween 20).
       Cover blot with 1X PBS-T for washing.
     - Box 4: Use 1X TBS-T or PBS-T (0.1% Tween 20) as appropriate.

   - Wash blots by shaking 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 (TBS) + 0.2% Tween® 20 + 0.01% SDS + Secondary Antibody
   - Box 2 – Odyssey Blocking Buffer (PBS) + 0.2% Tween 20 + 0.01% SDS + Secondary Antibody
   - Box 3 – Casein Blocking Buffer + 0.2% Tween 20 + 0.01% SDS + Secondary Antibody
   - Box 4 – Blocking Buffer of your choice + 0.2% Tween 20 + 0.01% SDS + Secondary Antibody

   Secondary antibody diluent for Odyssey Nitrocellulose Membrane
   - Box 1 – Odyssey Blocking Buffer (TBS) + 0.2% Tween 20 + Secondary Antibody
   - Box 2 – Odyssey Blocking Buffer (PBS) + 0.2% Tween 20 + Secondary Antibody
   - Box 3 – Casein Blocking Buffer + 0.2% Tween 20 + Secondary Antibody
   - Box 4 – Blocking Buffer of your choice + 0.2% Tween 20 + Secondary Antibody

   ** *Dilution factors for secondary antibodies*
   - 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 blots in diluted secondary antibody for 60 minutes at room temperature with gentle shaking.
   Protect membranes from light during incubation.

8. ***Protect from light during washes.***
   Wash membranes:
   - Pour off secondary antibody solution.
   - **Rinse and wash each blot with a buffer that matches the buffer system used for blocking, as described in Step 5.**
   - Box 1: 1X TBS-T (0.1% Tween® 20)
   - Box 2: 1X PBS-T (0.1% Tween 20)
   - Box 3: 1X PBS-T (0.1% Tween 20)
   - Box 4: Use 1X TBS-T or PBS-T (0.1% Tween 20), as appropriate

   - Shake vigorously on platform shaker at room temperature for 5 minutes.
   - Pour off wash solution.
   - Repeat 3 additional times.

9. Rinse each membrane with 1X TBS or 1X PBS (as appropriate) to remove residual Tween® 20. Membranes can be imaged wet or dry.

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

11. Visual inspection of images with Image Studio™ software or Odyssey application software can be used to determine which blocking buffer works best for the primary antibody you are testing.

    - View all blots together in a single image, with uniform image display settings, to compare membrane background levels and band intensity (Fig. 2A).

    - Individually adjust the image display settings for each blot to get the "best" image (Fig. 2B).

    - Evaluate non-specific banding in each blocking buffer condition.

    - Look for blocking buffer conditions that provide strong signals for the expected band(s), low membrane background, and few non-specific background bands from the primary antibody.

    - Trade-offs may be necessary. Blocking conditions that yield very strong bands might also have higher membrane background or non-specific banding.

    - The "best" blocking conditions depend on the antigen-antibody pair you are using. Some primary antibodies are dramatically affected by blocking conditions. An inappropriate blocker can alter binding specificity, affecting the intensity of target bands and increasing non-specific banding. The pattern of non-specific bands may also be affected.

    - Choose the blocking conditions that are most appropriate for the context and goals of your experiment.

12. Quantitative analysis of specific bands on each blot (Section V; Fig. 2) can be used to determine if signal intensity (after background subtraction) is significantly different between blocker types.

V. Example: Analysis of Optimization Blots

1. In this optimization experiment, ERK1/2 was detected in serial dilutions of A431 cell lysates.

2. After imaging, blocking buffer performance was evaluated. In addition to visual comparison, quantitative analysis was used to examine signal intensity of the ERK1/2 doublet under the conditions tested.

Figure 2A. All blots were viewed together in a single image, with identical image display settings. Signal intensity values are shown in each lane. Higher membrane background was observed on the blot blocked with 5% Milk in TBS.

Figure 2B. After cropping, image display settings were adjusted individually to display the "best" image for each blot. Signal intensity values are shown in each lane.

Figure 2C. Quantification data from all four blots were plotted. Band intensity was highest in Odyssey Blocking Buffer (PBS). Intensity was lowest in 5% Milk and Odyssey Blocking Buffer (TBS).

Signal intensity values in Figures 2A and 2B were not affected by changes to the image display settings. *Signal intensity measurements are derived from the raw image data. Image display settings adjust the visual mapping of raw data to the pixels on the computer monitor, but do not affect the raw data.*

3. Analysis and interpretation of optimization data

   - Blocking with 5% Milk in TBS resulted in higher membrane background than other blocking conditions (Fig. 2A). For comparison of membrane background levels, blots were viewed together as a single image, with uniform image display settings.

   - The strongest signals were observed with Odyssey Blocking Buffer (PBS) (OBB-PBS, Fig. 2C). Signals observed with casein blocker were also good.

   - Weaker signals were observed with Odyssey Blocking Buffer (TBS) (OBB-TBS) and 5% Milk.

   - Non-specific background bands were seen with OBB (TBS), OBB (PBS), and Casein blocking buffers (Fig 2B, arrows). Non-specific banding was reduced with 5% Milk (TBS).

   - OBB (PBS) and Casein may be appropriate choices for this antibody-antigen pair.

   - If non-specific banding is a concern, 5% Milk (TBS) could be used with this primary antibody. Band intensity would be somewhat reduced, but strong bands would still be visible. However, increased membrane background may make it difficult to detect fainter bands. Optimization of other factors, such as antibody dilution, may also reduce non-specific banding.

   - **The "best" choice of blocking buffer depends on the antigen-antibody pair you are using. Choose the blocking conditions that are most appropriate for the context and goals of your experiment.**

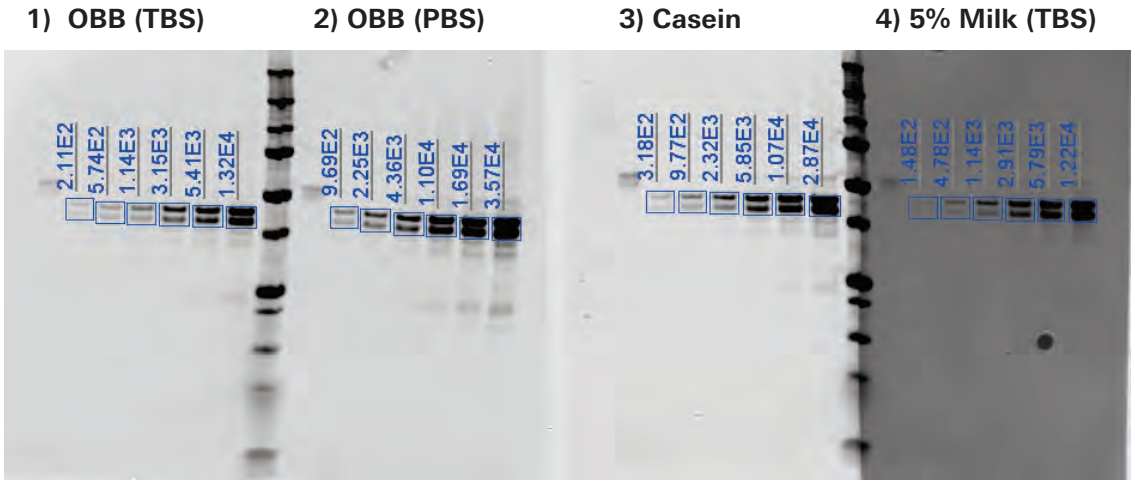

Figure 2A. Blots viewed as a single image, with uniform image display settings.

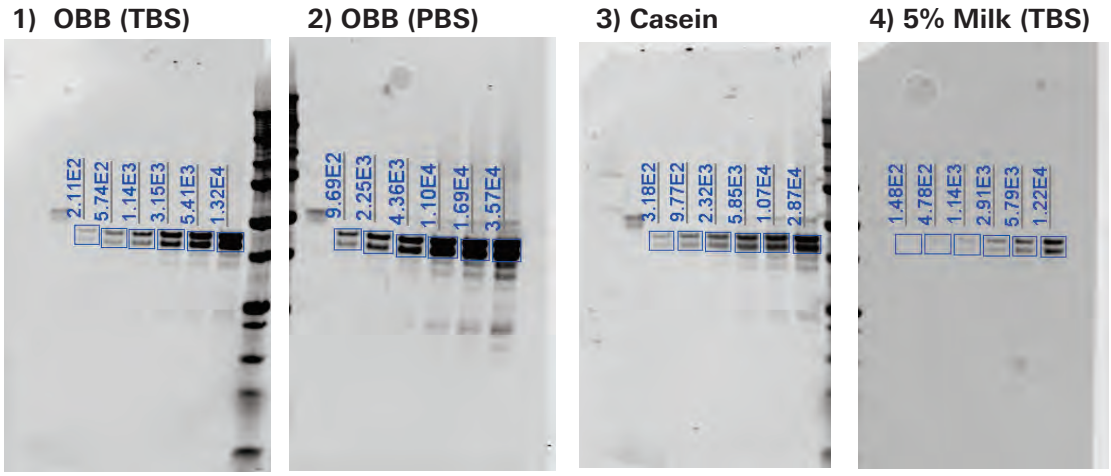

Figure 2B. Image display settings individually adjusted for each blot

Figure 2. Two-fold serial dilutions of A431 cell lysate were separated by electrophoresis (sample loads ranged from 313 ng to 10 µg, left to right). Primary antibody was loaded in the first lane of each gel as a positive control. Gels were transferred to nitrocellulose membrane and incubated in the designated buffer for blocking. Membranes were probed with rabbit anti-ERK1/2 (Santa Cruz Biotechnology; sc-94), and then IRDye® 680RD goat anti-rabbit IgG (LI-COR; P/N 926-68071) prior to imaging on Odyssey CLx.

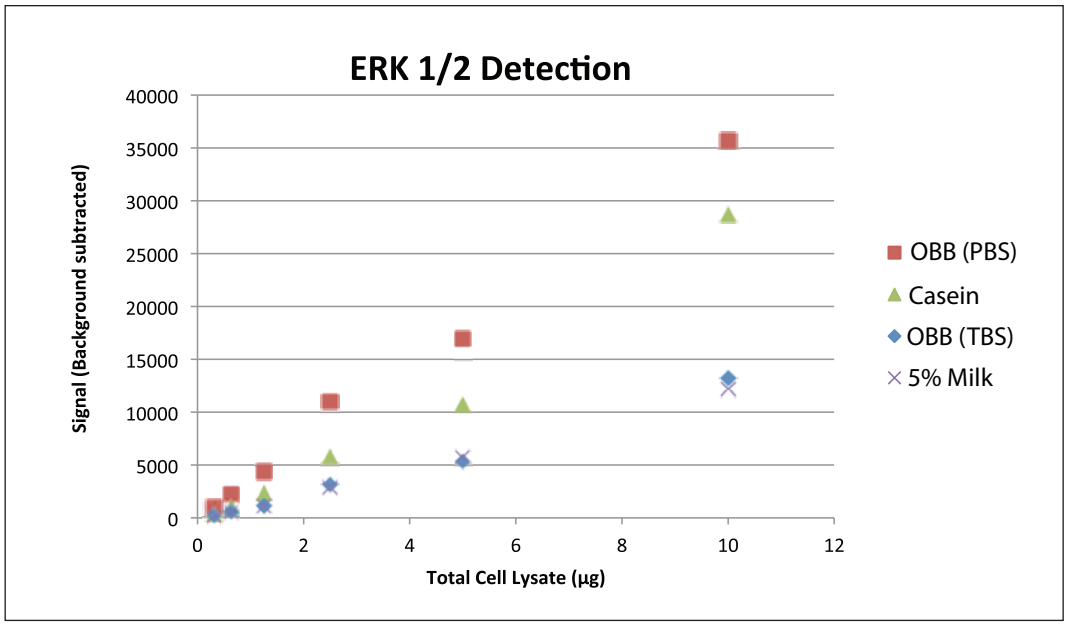

Figure 2C. Quantification data of images shown in Figures 2A and 2B.

Tips

- Follow the protocol carefully.
- For additional Odyssey Western detection tips, visit www.licor.com/WesternBlotTips

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Doc # 988-14403
05/14

CellTag™ 700 Stain

In-Cell Western™ Assay Kits I and II

Developed for:

Odyssey® Classic, Odyssey CLx, Odyssey Sa, and Aerius Imaging Systems

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

Part Numbers: 926-41091 and 926-41092

Storage:

IRDye® secondary antibody	4 °C
Odyssey Blocking Buffer	4 °C
CellTag 700 Stain	-20 °C

See Sections IV and V for complete storage recommendations

LI-COR® Experience Excellence

Published April 2013. The most recent version of this protocol is posted at http://biosupport.licor.com/support

Contents

I. Introduction

The In-Cell Western Kits provide detection reagents for cell-based In-Cell Western Assays. Each kit includes blocking buffer, IRDye® 800CW secondary antibody for detection of a specific protein target in the 800 nm channel, and CellTag 700 Stain to normalize well-to-well variations in cell number. This cost-effective normalization method makes quantification of the target protein more precise.

Detection of two proteins can be achieved using two different primary antibodies, followed by detection using two different secondary antibodies (one labeled with IRDye 800CW and the other with IRDye 680RD) in a multiplex assay. IRDye secondary antibodies for multiplex detection can be purchased at: www.licor.com/catalog. Dilution factors and blocking conditions should be optimized for target and primary antibody combinations.

II. Using CellTag 700 Stain for Cell Number Normalization

CellTag 700 Stain is a near-infrared fluorescent, non-specific cell stain that provides accurate normalization to cell number for In-Cell Western applications. The stain accumulates in both the nucleus and cytoplasm of permeabilized cells, and provides linear fluorescent signal across a wide range of cell types and cell numbers (see Figure 1). CellTag 700 Stain is detected in the 700 nm channel of Odyssey® CLx, Classic, and Sa Imaging Systems. CellTag 700 Stain is applied to the cells during incubation with IRDye 800CW secondary antibody, and enables accurate measurement of target protein levels (see Figure 2) with much higher throughput than Western blotting.

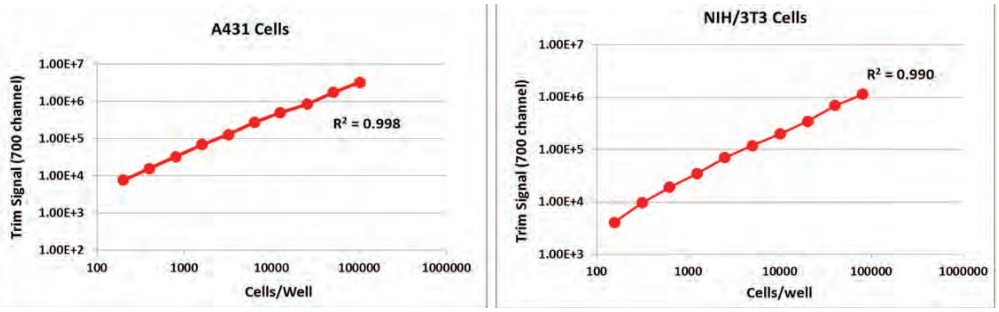

Figure 1. Linear Relationship between Fluorescence and Cell Number. Two-fold serial dilutions of A431 or NIH/3T3 cells were plated in 96-well plate. Cells were fixed, permeabilized, stained with CellTag 700 Stain (0.2 µM), and detected with Odyssey Classic Infrared Imaging System (Resolution: 169 µm; Quality: medium; Focus offset: 4.0 mm; Intensity: 5). The Trim Signals were used to generate the graphs. Linear fluorescent signal was obtained across a very wide range of cell numbers (~ 200 – 100,000 cells).

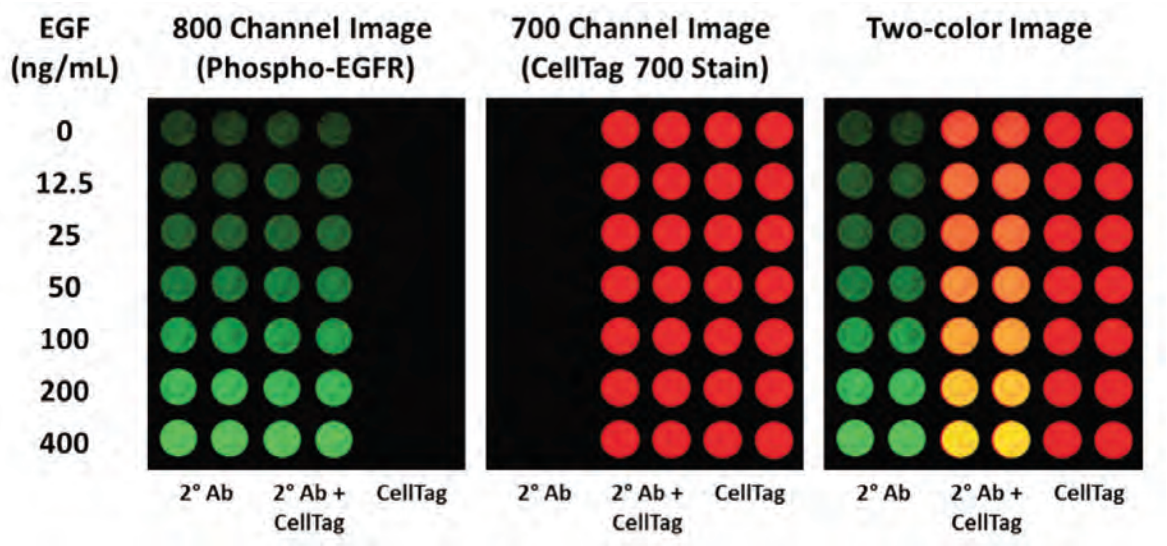

Figure 2. In-Cell Western Assay with CellTag 700 Stain in EGF-stimulated A431 Cells. EGF-stimulated A431cells were fixed and permeabilized. Phosphorylated EGFR was measured using rabbit anti-phospho-EGFR primary antibody followed by detection with IRDye® 800CW Goat anti-Rabbit IgG (LI-COR P/N 926-32211). CellTag 700 Stain (LI-COR, P/N 926-41090) was used for normalization to cell number. The data demonstrate that phosphorylated levels of EGFR increase with treatment of EGF. The plate was scanned on Odyssey® Classic Infrared Imaging System. (Resolution: 169 µm; Quality: medium; Focus offset: 4.0 mm; Intensity: 5 for both channels).

III. In-Cell Western Protocol

Kit Components (sufficient for 20 x 96-well plates)

- IRDye 800CW secondary antibody, 0.5 mg (lyophilized) (LI-COR, P/N 926-32210 or 926-32211)
- Odyssey Blocking Buffer, 1 x 500 mL (LI-COR, P/N 927-40000)
- CellTag 700 Stain, 2 x 10 nmole (LI-COR, P/N 926-41090)

Additional Reagents (required but not included)

- Primary antibody
- 1X PBS
- Tissue culture reagents (serum DMEM, trypsin, etc.)
- Clear or black 96-well microplate (see IX. Experimental Considerations)
- 37% formaldehyde
- 20% Tween® 20
- 10% Triton® X-100

IV. Reconstitution of Antibody

1. Protect from light. Store IRDye 800CW secondary antibody at 4 °C prior to reconstitution.

2. Reconstitute contents of antibody vial with 0.5 mL sterile distilled water. Mix gently by inverting, and allow to rehydrate for at least 30 minutes before use. Centrifuge product if solution is not completely transparent after standing at room temperature.

3. Dilute only immediately prior to use. Reconstituted antibody is stable for 3 months at 4 °C when stored undiluted as directed.

V. Reconstitution of CellTag 700 Stain

1. Protect from light. Store CellTag 700 Stain at -20 °C prior to reconstitution. Use the stain within 6 months.

2. Reconstitute contents of vial with 0.1 mL 1X PBS for a final concentration of 0.1 mM. Mix thoroughly by vortexing, and allow to rehydrate for at least 30 minutes before use. Store the reconstituted stain at 4 °C.

VI. Cell Preparation and Fixation

1. Treat cells as desired with drug, stimulant, etc. Detailed In-Cell Western (ICW) protocols for certain cell lines and target proteins may be downloaded at: http://biosupport.licor.com. See In-Cell Western Assay Cell Fixation/Permeabilization document at the ICW Assay Application page (http://biosupport.licor.com/docs/ICW_fix_and_perm.pdf)

2. Remove media manually or by aspiration. Immediately fix cells with Fixing Solution (3.7% formaldehyde in 1X PBS) for 20 minutes at room temperature (RT).

   a. Prepare fresh Fixing Solution as follows:

1X PBS	45 mL
37% Formaldehyde	5 mL
3.7% Formaldehyde	50 mL

   b. Using a multi-channel pipettor, add 150 µL of fresh, room temperature Fixing Solution to each well. Add carefully by pipetting down the sides of the wells to avoid detaching the cells from the well bottom.

   c. Allow incubation on the bench top for 20 minutes at RT with no shaking.
   NOTE: If optimal fixation conditions for immunofluorescent staining of your cell line and/or target protein are already known, these conditions may be more appropriate than the fixation protocol described here and would be an excellent starting point for In-Cell Western assay development. Most fixatives and fixation protocols for immunofluorescent staining may be adapted to the In-Cell Western format.

3. To permeabilize, wash five times with 1X PBS containing 0.1% Triton X-100 for 5 minutes per wash.

   a. Prepare Triton Washing Solution as follows:

1X PBS	495 mL
10% Triton X-100	5 mL
1X PBS + 0.1% Triton X-100	500 mL

   b. Remove Fixing Solution to an appropriate waste container (contains formaldehyde).

   c. Using a multi-channel pipettor, add 200 µL of room-temperature Triton Washing Solution to each well. Make sure to carefully add the solution down the sides of the wells to avoid detaching the cells.

   d. Allow wash to shake on a plate shaker for 5 minutes.

e. Repeat washing steps 4 more times, removing wash manually each time. Do not allow cells/wells to become dry during washing. Immediately add the next wash after each manual disposal.
NOTE: If an alternative permeabilization method (for example, ice-cold methanol) is known to work well for immunofluorescent staining of your protein target, you may prefer to use that permeabilization method rather than the Triton method described here.

VII. Cell Staining

1. Using a multi-channel pipettor, block cells by adding 150 µL of Odyssey® Blocking Buffer to each well. Add the solution carefully by pipetting down the sides of the wells to avoid detaching the cells.
NOTES: No single blocking reagent will be optimal for every antigen-antibody pair. Some primary antibodies may exhibit greatly reduced signal or non-specific binding in different blocking solutions. If you have difficulty detecting your target protein, changing the blocking solution may dramatically improve performance. If you have used the primary antibody successfully for immunofluorescent staining, consider trying the same blocking buffer for In-Cell Western detection.

Odyssey Blocking Buffer often yields higher and more consistent sensitivity and performance than other blockers. Nonfat dry milk or casein dissolved in PBS, or commercial blocking buffers, can also be used for blocking and antibody dilution. When using anti-goat antibodies, milk-based reagents may be contaminated with endogenous IgG, biotin, or phosphoepitopes that can interfere with detection.

2. Allow blocking for 1.5 hours at room temperature with moderate shaking on a plate shaker.

3. Dilute desired primary antibody in Odyssey Blocking Buffer or other appropriate blocker. As a general guideline, 1:50 to 1:200 dilutions are recommended, depending on the primary antibody. If the antibody supplier provides dilution guidelines for immunofluorescent staining, start with that recommended range.
NOTE: If using CellTag 700 Stain for normalization, only one primary antibody will be used. Alternatively, you may choose to normalize with a second primary antibody in your assay. The second primary antibody MUST be from a different host, and an appropriate IRDye® 680RD secondary antibody (not provided in the kit) will be required for detection.

   a. It is important to include control wells that DO NOT contain primary antibody. These wells will be treated with secondary antibody only, and should be used to correct for background staining in the data analysis.

   b. Remove blocking buffer from step 1.

   c. Add 50 µL of Odyssey Blocking Buffer to the control wells and 50 µL of the desired primary antibody in Odyssey Blocking Buffer to the rest of the wells.

4. Incubate with primary antibody for 2.5 hours at room temperature or overnight at 4 °C with gentle shaking.

5. Wash the plate five times with 1X PBS + 0.1% Tween 20 for 5 minutes at RT with gentle shaking, using a generous amount of buffer.

a. Prepare Tween® Washing Solution as follows:

1X PBS	995 mL
20% Tween 20	5 mL
1X PBS with 0.1% Tween 20	1000 mL

b. Using a multi-channel pipettor, add 200 µL of Tween Washing Solution. Make sure to carefully add the solution down the sides of the wells to avoid detaching the cells from the well bottom.

c. Allow wash to shake on a plate shaker for 5 minutes.

d. Repeat washing steps 4 more times.

6. Dilute the fluorescently-labeled secondary antibody in Odyssey® Blocking Buffer or other appropriate blocker. The recommended dilution range is 1:200 to 1:1,200, with a suggested starting dilution of 1:800. The optimal dilution for your assay should be determined empirically. To lower background, add Tween 20 at a final concentration of 0.2% to the diluted antibody. Avoid prolonged exposure of the antibody vials to light.

   a. Secondary antibody staining and normalization staining are carried out simultaneously. To stain for normalization, add CellTag 700 Stain to the diluted secondary antibody solution and apply this mixture to the cells. Suggested concentration for CellTag 700 Stain is 0.2 µM (1:500 dilution).

   b. For control wells (used to calculate background), do not add CellTag 700 Stain. Add only diluted secondary antibody to these wells.

7. Add 50 µL of secondary antibody solution without CellTag 700 Stain into each of the control wells and 50 µL of secondary antibody solution with CellTag 700 Stain into remaining wells. Incubate for 1 hour at room temperature with gentle shaking. Protect plate from light during incubation.

8. Wash the plate five times with 1X PBS + 0.1% Tween 20 for 5 minutes at room temperature with gentle shaking, using a generous amount of buffer.

   a. Using a multi-channel pipettor, add 200 µL of Tween Washing Solution. Make sure to carefully add the solution down the sides of the wells to avoid detaching the cells from the well bottom.

   b. Allow wash to shake on a plate shaker for 5 minutes.

   c. Repeat washing steps 4 more times. Protect plate from light during washing.

VIII. Imaging

1. After final wash, remove wash solution completely from wells. Turn the plate upside down and tap or blot gently on paper towels to remove traces of wash buffer. For best results, scan plate immediately; plates may also be stored at 4 °C for up to several weeks (protected from light).

2. Before plate scanning, clean the bottom plate surface and the scanning bed (if applicable) with moist lint-free paper.

3. Scan plate with detection in both 700 and 800 nm channels using an Odyssey® or Aerius System, as described below:
   NOTE: All settings may require adjustment for optimal data quality (see Section IX).

Instrument	Resolution*	Scan Quality*	Intensity Setting (700/800)	Scan Time Medium Quality
Odyssey Classic	169 µM	medium-lowest	5 / 5	7 min
Odyssey CLx	169 µM	medium-lowest	5 / 5	7 min
	169 µM	medium-lowest	AutoMode	16 min
Odyssey Sa	200 µM	medium-lowest	7 / 7	3 min
Aerius	200 µM	medium-lowest	7 / 7	3 min

**Higher resolution or scan quality may be used, but scan time will increase.*

IX. Experimental Considerations

Establish the specificity of the primary antibody by screening lysates of cells treated in the same manner as the In-Cell Western samples, using Western blotting and detection with an Odyssey or Aerius instrument. If significant non-specific binding is present, choose alternative primary antibodies to avoid non-specific signals that may affect In-Cell Western assay results.

Proper selection of microplates can significantly affect results, as each plate has its own characteristics, including well depth, plate autofluorescence, and well-to-well signal crossover. Use the following general considerations for microplate selection.

- In-Cell Western analyses use detection at the well surface with no liquid present. This results in minimal well-to-well signal spread, allowing the use of either clear plates or black-sided plates with clear well bottoms. Do not use plates with white walls, since autofluorescence from the white surface will create significant noise.
- Protect plates from light before imaging to ensure highest sensitivity. When storing plates after imaging, the plates should remain protected from light at 4 °C.
- In-Cell Western assays require sterile plates for tissue culture growth. The following plates are recommended by LI-COR Biosciences:

96-well format	Nunc® Plates (P/N 161093, 165305)
96-well format	Falcon™ Plates (P/N 353075, 353948)
384-well format	Nunc Plates (P/N 164688, 164730)
384-well format	Falcon Plates (P/N 353961, 353962)

- **Focus Offset Optimization** – If plates other than those recommended above are used, the focus offset can be determined empirically by scanning a plate containing experimental and control samples using the following focus offset settings.

Instrument	Focus Offset Determination (mm)
Odyssey Classic & Odyssey CLx	0.5, 1.0, 2.0, 3.0 & 4.0
Odyssey Sa & Aerius	1.7, 2.0, 3.0 & 4.0

Use the same intensity settings for each scan. After reviewing the scans, use the focus offset with the highest signal-to-noise for experiments. The actual minimum and maximum focus offset will vary with each instrument. Alternatively, consult the plate manufacturer for the recommended measured distance from the skirt to the bottom of the plate.

- All Odyssey® and Aerius imaging systems (excluding Odyssey Fc) require microplates that have a maximum 4.0 mm distance from the base of the microplate to the target detection area of the plate (actual maximum focus offset varies with each Odyssey Sa or Aerius instrument, and is found by choosing Settings > System Administration, then clicking Scanner Information). When using plates specified previously for In-Cell Western assays, the recommended focus offset is 3.0 mm.

- **Intensity Setting Optimization –**

Instrument	Initial Intensity Setting (700/800 nm)	Intensity Settings Weak Signal (700/800 nm)	Intensity Settings Saturated Signal (700/800 nm)
Odyssey Classic	5 / 5	7.5 / 7.5	2.5 / 2.5
Odyssey CLx	5 / 5	7.5 / 7.5	2.5 / 2.5
	AutoMode*	-	-
Odyssey Sa	7 / 7	8 / 8	4 / 4
Aerius	7 / 7	8 / 8	4 / 4

**The Odyssey CLx AutoMode function alleviates the need to scan the plate at multiple intensity settings.*

- Protect plates from light before imaging to ensure highest sensitivity. When storing plates after imaging, the plates should remain protected from light at 4 °C.

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Doc # 988-13623
0413

Electrophoretic Mobility Shift Assay (EMSA) Using IRDye® Oligonucleotides

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.

LI-COR®
Experience Excellence

Published May 2004. Revised October 2011.
The most recent version of this protocol is posted at http://biosupport.licor.com

Table of Contents

I. Introduction

Gel shift assays or electrophoretic mobility shift assays (EMSA) provide a simple method to study DNA-protein interactions. This assay is based on the principle that a DNA-protein complex will have different mobility during electrophoresis than non-bound DNA. These shifts can be visualized on a native acrylamide gel using labeled DNA to form the DNA-protein binding complex. To date, protocols require labeling DNA by (1) radioisotope, (2) digoxygenin, or (3) biotin. The Odyssey® Family of Imagers (LI-COR® Biosciences) offers a quick and easily-adapted alternative method to radioisotopic and chemiluminescent detection methods for EMSA analysis and visualization.

A DNA oligonucleotide end-labeled with LI-COR IRDye is a good substrate for protein binding. LI-COR offers pre-annealed oligonucleotides specific to eight unique binding proteins. DNA detection using IRDye reagents is linear within a 50-fold dilution range, from 9.1 fmol to 0.18 fmol. Additional benefits include no hazardous radioisotope, no gel transfer to membrane or gel drying, no chemiluminescent substrate reagents, and no film exposure. Following electrophoresis, the gel can be imaged while remaining in the glass plates. If necessary, the gel can be placed back in the electrophoresis unit and run longer.

Existing mobility shift assay protocols can be easily transformed into infrared assays by replacing the existing DNA oligonucleotides with oligonucleotides end-labeled with IRDye reagents. The binding conditions and electrophoresis conditions will remain the same as with any other EMSA detection method.

II. General Methodology

EMSA Oligonucleotides Labeled with IRDye 700

	Part Number
IRDye 700 p53 Consensus Oligonucleotide	829-07921
IRDye 700 STAT3 Consensus Oligonucleotide	829-07922
IRDye 700 CREB Consensus Oligonucleotide	829-07923
IRDye 700 NFκB Consensus Oligonucleotide	829-07924
IRDye 700 AP-1 Consensus Oligonucleotide	829-07925
IRDye 700 Sp-1 Consensus Oligonucleotide	829-07926
IRDye 700 HIF-1 Consensus Oligonucleotide	829-07929
IRDye 700 ARE (Androgen Receptor) Consensus Oligonucleotide	829-07933
EMSA Buffer Kit for the Odyssey	829-07910

Labeling DNA Fragments with IRDye Infrared Dyes

To obtain DNA fragments end-labeled with IRDye infrared dyes, oligos labeled with IRDye infrared dyes are used. It is critical that the DNA fragment is end-labeled rather than having dye incorporated into the DNA, which interferes with the formation of the DNA-protein complex.

Oligonucleotides are manufactured in single strand form; therefore, both forward and reverse DNA oligonucleotides must be purchased. Once oligonucleotides are obtained, they need to be annealed to form a double-stranded DNA fragment.

Oligonucleotides are annealed by placing the oligonucleotide set in a 100°C heat block for 5 minutes and then leaving the oligonucleotides in the heat block and turning it off to slowly cool to room temperature.

Important: *Both oligonucleotide sequences should be end-labeled with the same IRDye infrared dye. There is a significant decline (~70%) in signal intensity when using only one end-labeled oligonucleotide.*

III. Mobility Shift Sample Protocol (NFκB)

Each oligo labeled with IRDye 700 provided by LI-COR® Biosciences for EMSA reactions will have an optimized protocol to measure the protein-DNA interaction. See the specific EMSA oligo pack insert for more information. As an example, the NFκB protein-DNA interaction will be described in this document.

Gel Preparation: Native pre-cast polyacrylamide gels such as 5% TBE (BioRad) or 4-12% TBE (Invitrogen) are recommended. Alternatively, the recipe below can be used to prepare a 4% native gel.
NOTE: The protein shift detected on each gel type (i.e., 5% vs 4-12%) will be unique.

Prepare 4% native polyacrylamide gel containing 50 mM Tris, pH 7.5; 0.38 M glycine; and 2 mM EDTA:

For 40 mL mix:

5 mL 40% polyacrylamide stock (Polyacrylamide-BIS ratio = 29:1)
2 mL 1 M Tris, pH 7.5
7.6 mL 1 M Glycine
160 µL 0.5 M EDTA
26 mL H_2O
200 µL 10% APS
30 µL TEMED
Pour the gel between glass plates and wait about 1-2 hours to polymerize.

Oligo Preparation: EMSA oligonucleotides from LI-COR Biosciences are pre-annealed.

1. Dilute oligos in 1X TE for final concentration of 20 pmol/µL.
2. Place 5 µL of forward IRDye 700 oligo into a new tube and add 5 µL of reverse IRDye 700 oligo.
3. Anneal oligos by placing the oligo set in a 100°C heat block for 3 minutes. Leave the oligos in the heat block and turn it off to slowly cool to room temperature.
4. Dilute annealed oligos 1 µL in 199 µL water. This is your working DNA stock. Oligos can be stored at -20°C for up to a year if protected from light.

Binding Reaction: For NFκB IRDye 700 oligonucleotide, the following binding reaction is a good starting point.

Reaction	µL
10X Binding Buffer (100 mM Tris, 500 mM KCl, 10 mM DTT; pH 7.5)	2
Poly(dI•dC) 1 µg/µL in 10 mM Tris, 1 mM EDTA; pH 7.5	1
25 mM DTT/2.5% Tween® 20	2
Water	13
IRDye 700 NFκB	1
Raji nuclear extract (Positive control) (5 µg/µL)	1
TOTAL	**20**

After the addition of the DNA to the protein-buffer mix, reactions are incubated to allow protein binding to DNA. A typical incubation condition is 20-30 minutes at room temperature. Since IRDye 700 infrared dye is sensitive to light, it is best to keep binding reactions in the dark during incubation periods (e.g., put tubes into a drawer or cover the tube rack with aluminum foil).

Electrophoresis:

1. Add 1 µL of 10X Orange loading dye (LI-COR®, P/N 927-10100), mix, and load on a gel.
2. Run the gel at 10 V/cm for about 30 minutes in non-denaturing buffer (i.e., 1X TGE or TBE buffer).

NOTE: For best results, electrophoresis should be performed in the dark (simply put a cardboard box over the electrophoresis apparatus).

Imaging: Gels can be imaged either inside the glass plates or removed from the glass plate. When removing gel from the glass plates, take care not to deform or tear the gel. Scan the gel. Please refer to your manual for specific information on your model of imager.

Figure 1. IRDye 700 NFκB oligonucleotides were separated on a native polyacrylamide gel (4-12% TBE, Invitrogen EC62352BOX) and imaged on the Odyssey® Infrared Imaging System.
Lane 1) no nuclear extract;
Lanes 2 and 5) 10 µg Raji nuclear extract;
Lanes 3 and 6) 5 µg Raji nuclear extract;
Lanes 4 and 7) 2.5 µg Raji nuclear extract.

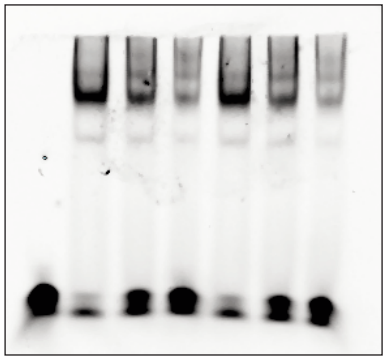
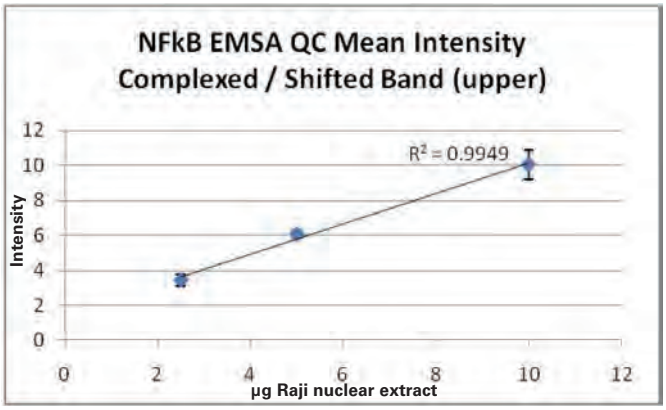


Figure 2. The uppermost shifted band in Lanes 2-7 of Figure 1 was analyzed to determine the level of NFkB binding to the IRDye 700 NFκB oligonucleotides.



One of the benefits of using the Odyssey® Infrared Imaging System for EMSA analysis is that it provides an easy method for quantification. However, there are issues to consider when using the Odyssey Imager to quantify EMSA results. The primary issue is that the free DNA fragment has much less signal than the DNA fragment when bound to a protein, making quantification of the unbound DNA inaccurate. The addition of DTT/Tween® 20 to the binding reaction stabilizes the dye and reduces this phenomenon. In addition, it is unrealistic to perform quantification analyses under the assumption that the free DNA band in the control, containing DNA only (no extract), should equal the sum of the signals of the free and bound DNA in the samples where the protein-DNA binding reaction occurs. Using end-labeled oligonucleotide duplexes as the DNA source and nuclear extract as a protein source renders this assumption impractical, due to the non-specific binding that occurs from using a nuclear extract. Oligonucleotides can also complicate quantification because the free oligonucleotides form a smear rather than a tight band. This makes it more difficult to assign an intensity value to bands.

Optimization

Binding Reaction

A universal binding condition that applies to every protein-DNA interaction cannot be recommended, since binding conditions are specific for each protein-DNA interaction. Thus, the user should establish binding reaction conditions for each protein-DNA pair. Binding buffer should be the same for this method as with any other mobility shift detection method used.

After the addition of DNA to the protein-buffer mix, reactions are incubated to allow protein to bind to DNA. Time required for binding is the same as when radioactively-labeled DNA fragments are used; a typical incubation condition is 20-30 minutes at room temperature. Since IRDye reagents are sensitive to light, it is best to keep binding reactions in darkness during incubation periods (e.g., put tubes into a drawer or simply cover the tube rack with aluminum foil). After the incubation period, native loading dye is added to the binding reaction.

NOTE: In some cases, it was observed that DNA control reactions (no protein) have lower signal than reactions containing protein. This may be due to lower stability of the dye in certain buffer conditions. The addition of 5 mM DTT and 0.5% Tween 20 to all reactions reduces this phenomenon.

IMPORTANT: It is critical not to use any blue loading dye (e.g., bromophenol blue), as this will be visible on the Odyssey® image. Use 10X Orange loading dye instead (LI-COR®, P/N 927-10100).

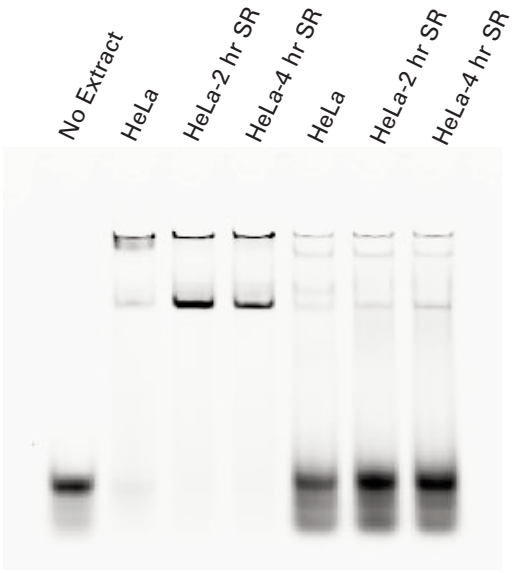


Figure 3. AP-1 EMSA using IRDye 700 end-labeled oligonucleotide duplex.
It is common to use unlabeled DNA duplex to determine binding specificity. Excess unlabeled DNA is added to the binding reaction; therefore, it competes with the labeled DNA for binding sites. If competition eliminates labeled DNA binding, no shift is observed (see last three lanes in gel), indicating that the binding reaction is specific.

Competition reactions contained 100-fold molar excess of wild-type oligonucleotide duplex. Nuclear extracts of HeLa, HeLa 2-hour serum response, and HeLa 4-hour serum response, were used to visualize an increase in AP-1 binding as a result of the serum response treatment to the HeLa cells.

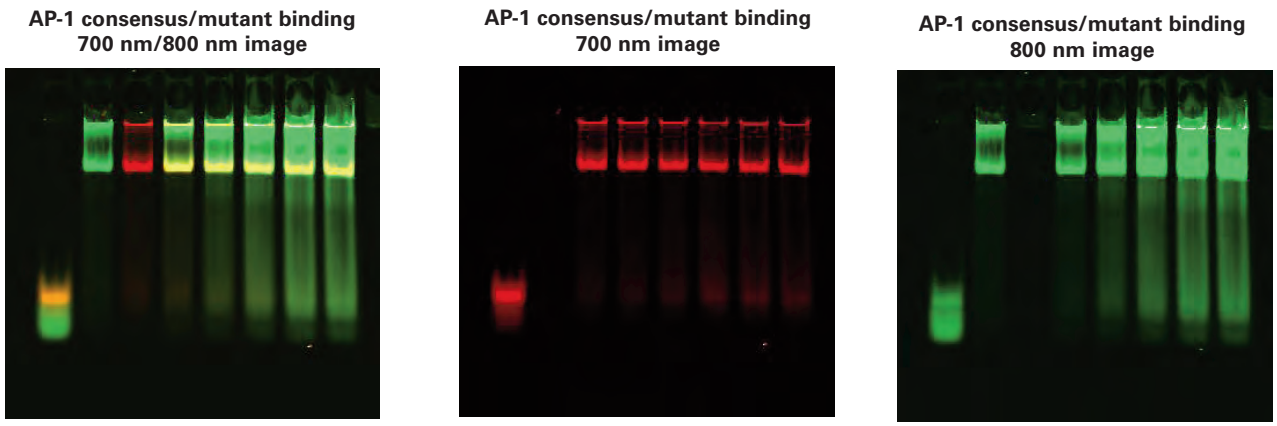


Figure 4. AP-1 EMSA using 2.5 µg HeLa 4-hour serum response nuclear extract to demonstrate binding specificity of AP-1 consensus DNA duplex. Binding specificity determination using Odyssey® two-color imaging.

Competition using mutant DNA duplexes is another common method to determine binding specificity. A mutant DNA sequence is used to compete with the wild-type binding sequence. Specific binding is observed when mutant DNA (unlabeled) does not reduce the binding of labeled wild-type DNA. Two-color analysis of mutant vs. wild-type binding is done using the Odyssey Infrared Imaging System. The wild-type oligos are labeled with IRDye 700 phosphoramidite and mutant oligos with IRDye 800 phosphoramidite. In the figure above, the mutant non-specific binding is very intense (800 nm image); however, there is no decrease in wild-type binding (700 nm image).

Lane 1 – Free IRDye 700 AP-1 consensus oligonucleotide and IRDye 800 AP-1 mutant oligonucleotide with no nuclear extract;
Lane 2 – Nuclear extract with 0:1 ratio of IRDye 700 AP-1 consensus oligonucleotide to IRDye 800 AP-1 mutant oligonucleotide;
Lane 3 – Nuclear extract with 1:0 ratio of IRDye 700 AP-1 consensus oligonucleotide to IRDye 800 AP-1 mutant oligonucleotide;
Lane 4 – Nuclear extract with 1:1 ratio of IRDye 700 AP-1 consensus oligonucleotide to IRDye 800 AP-1 mutant oligonucleotide;
Lane 5 – Nuclear extract with 1:2 ratio of IRDye 700 AP-1 consensus oligonucleotide to IRDye 800 AP-1 mutant oligonucleotide;
Lane 6 – Nuclear extract with 1:3 ratio of IRDye 700 AP-1 consensus oligonucleotide to IRDye 800 AP-1 mutant oligonucleotide;
Lane 7 – Nuclear extract with 1:4 ratio of IRDye 700 AP-1 consensus oligonucleotide to IRDye 800 AP-1 mutant oligonucleotide;
Lane 8 – Nuclear extract with 1:5 ratio of IRDye 700 AP-1 consensus oligonucleotide to IRDye 800 AP-1 mutant oligonucleotide;

References

1. Wolf, S.S., Hopley, J.G., and Schweizer, M. (1994) The Application of ^{33}P-Labeling in the Electrophoretic Mobility Shift Assay. *Biotechniques* **16**, 590-592.
2. Suske, G., Gross, B., and Beato, M. (1989) Non-radioactive method to visualize specific DNA-protein interactions in the band shift assay. *Nucleic Acids Research*, **17**, 4405.
3. Ludwig, L.B., Hughes, B.J., and Schwartz, S.A. (1995) Biotinylated probes in the electrophoretic mobility shift assay to examine specific dsDNA, ssDNA or RNA-protein interactions. *Nucleic Acids Research*, **23**, 3792-3793.

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Doc # 988-12448
1011

Syto® 60 Staining of Nucleic Acids in Gels

Developed for:

Aerius, and Odyssey® Family of Imagers

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

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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.

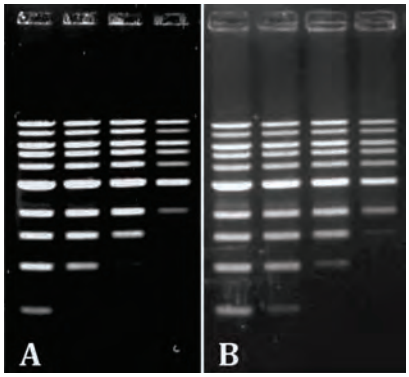

Figure 1. Two-fold dilutions of 1 kb ladder, from 1 µg to 0.125 µg, separated on a 1.2% agarose gel at 8V/cm in 1X TAE buffer for 1 hour. Panel A is the image of the gel obtained from the Odyssey Infrared Imaging System using an intensity of 5.0, gel face down. Panel B is the image of the gel acquired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

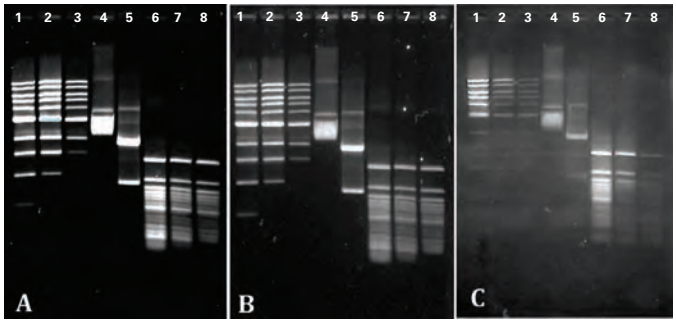

Figure 2. A 1.2% agarose gel was imaged using the Odyssey® Infrared Imaging System (panel A), Odyssey Fc Imaging System (panel B) or a UV transilluminator and the image captured using Polaroid 667 film (panel C). Lane 1) 1 µg 1 kb ladder; Lane 2) 0.5 µg 1 kb ladder; Lane 3) 0.25 µg 1 kb ladder; Lane 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.

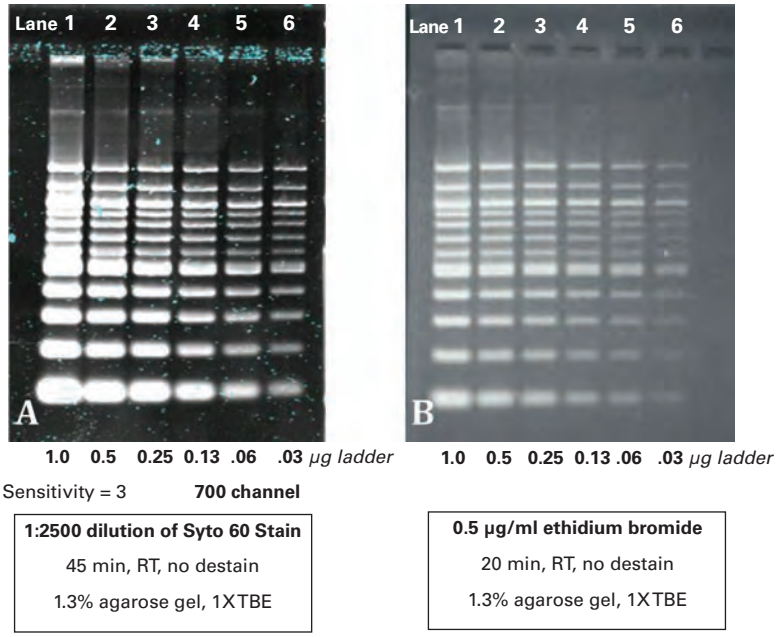

Figure 3. Panel A: Image of a Syto 60 stained gel using the Odyssey Infrared Imaging System. Panel B: Image of ethidium bromide-stained gel using a Polaroid camera.

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.

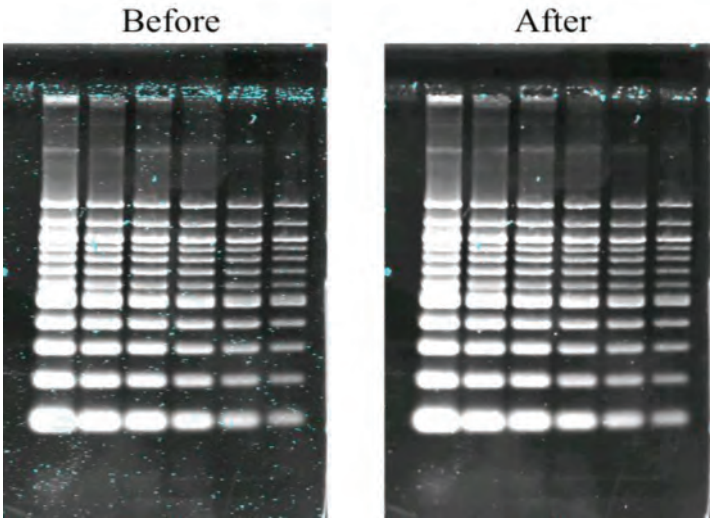

Figure 4. Image of agarose gel showing before and after using Odyssey Infrared Imaging System software's "FILTER" then "Noise Removal" function.

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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Doc# 988-12449

In Vivo Imaging

Scanning a Mouse on the Odyssey® System: *Hints and Tips*

Developed for:

Odyssey® Infrared Imaging System

Odyssey CLx (Manual mode only)

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Published September 2010. Revised October 2011. The most recent version of this technical note is posted at http://biosupport.licor.com/support

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1. DIET CONSIDERATIONS

Mouse chows generally have high fluorescent signal in the 700 and 800 nm channels due to plant-based ingredients that contain chlorophyll. If imaging in the abdominal region where intestinal fluorescence will be an issue, feed a purified diet containing no plant-based ingredients. Figure 1 illustrates imaging with regular mouse chow LM-485 (signal is saturated) compared to two purified diets provided by the same company.

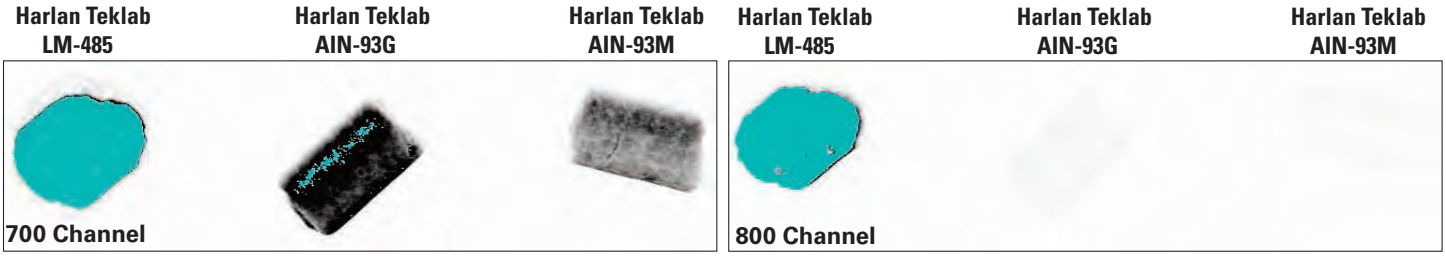


Figure 1. Three mouse chows from Harlan Teklab imaged on the Odyssey system.

An example of the level of interference that can be seen when imaging a mouse on the Odyssey system is shown in Figure 2. The circle on the 700 nm channel image indicates the intestinal signal due to the mouse diet, while the circle on the 800 nm channel image pinpoints the abdominal tumor.

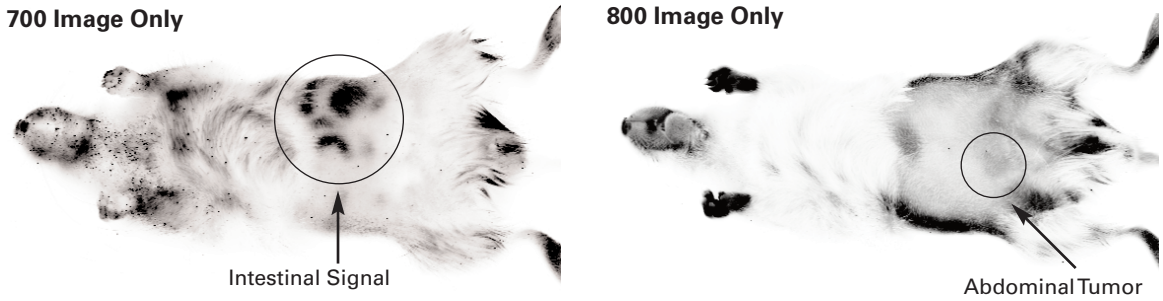


Figure 2. A typical mouse scan on the Odyssey Imager where an abdominal tumor was present. Scan parameters include resolution = 169 µm; quality = medium; focus offset = 2.0; intensity values = L1 for 700 channel and L3 for 800 channel.

II. PRE-SCAN

It is always beneficial to scan the mouse prior to probe injection, in order to document the amount of background/autofluorescence the mouse emits. Start with intensity setting of L1 and L3 for the 700 and 800 nm channels, respectively. When the level of expected signal is known, adjust intensity settings accordingly.

III. RESOLUTION

The first image should be at the lowest resolution (i.e., 337 µm), which gives a good preview of what to expect from the particular mouse model being evaluated. If the mouse is optimally positioned, this short preview scan will provide a more accurate estimate of the correct intensity settings. Generally, images scanned at 169 µm are a good compromise between resolution and scan time. Scanning quickly with live mice is important to minimize image anomalies caused by movement during a scan and to minimize stress to the animal.

IV. HAIR VS. SHAVED ANIMAL

If working with a haired mouse model (i.e., something other than nude mice), shave the animal in the region of interest prior to imaging. Up to 50% of the signal is blocked when imaging through hair. Hair removal can be accomplished by shaving (mustache shavers work well) or with the use of Nair®. To demonstrate, a mouse was implanted with a tube containing IRDye® 800CW in the thoracic cavity and imaged before and after shaving. Figure 3 shows the effect of hair, with a 53% difference between Panel A (before shaving) and Panel B (after).

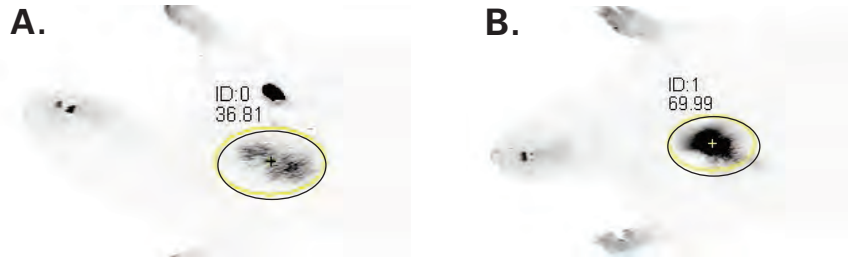
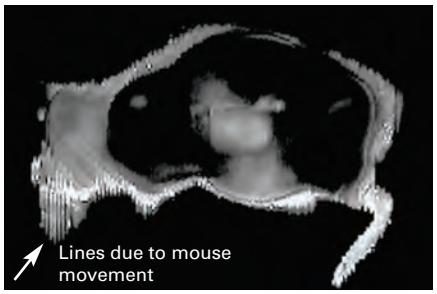


Figure 3. Signal from IRDye 800CW in the thoracic cavity when imaged on the Odyssey system before (Panel A) and after (Panel B) shaving.

V. MOUSE MOVEMENTS

When using injectable anesthesia, mouse movements due to breathing are relatively slow compared with the Odyssey scan speed, and images have a normal appearance like those shown earlier. With inhalation anesthesia, however, mice may breathe at a faster rate and cause lines to occur on the image because the breathing movement changes the position of the mouse during the scan.

There are several approaches to resolving this problem. One way is to keep mice under a constant supply of anesthesia so breathing is regular. Another approach is to maximize scan speed by adjusting the scan parameters. The Resolution parameter is a good place to start. When scanning at less than 169 µm resolution, you are much more likely to see lines, or missing lines, in images. If lines are present at 169 µm, try 337 µm; however, you may want to try decreasing the Quality parameter before changing to a lower resolution.



In the default scanning presets, image quality is set at Medium. Changing the Quality parameter to Low will increase the scan speed and reduce the occurrence of lines. Changing to Lowest quality, 337 µm resolution, is also an option.

If the lines cannot be eliminated, the image filters in the Odyssey software may reduce the problem. Start a new analysis and select the images with lines to use in the analysis. This will make copies of the images and leave the original images unchanged. In the New Analysis window, click the Filter button, select the Noise Removal filter option, and click OK. The noise removal filter calculates the median pixel value within

the 3 x 3 filter region and replaces the current pixel value with the median. This will generally improve the appearance of the image unless the frequency of the lines on the image is high (every other scan line, for example). This filter does change the quantification results.

VI. REFLECTIONS

Any areas that may cause a reflection will be an issue. Examples include: 1) when shaving, avoid nicking the skin, as the open nick will cause reflection and signal; 2) moisture; 3) sharp positioning angles with high offset; and 4) glossy or shiny connective tissue.

VII. FOCUS OFFSET FOR SURFACE AND ABDOMINAL TUMORS

A focus offset of 0 - 1.0 mm is a good starting point for a surface (subcutaneous) or intra-abdominal tumor.

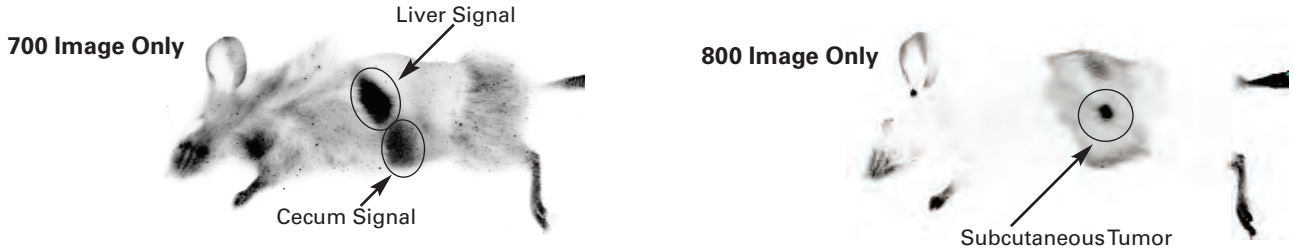
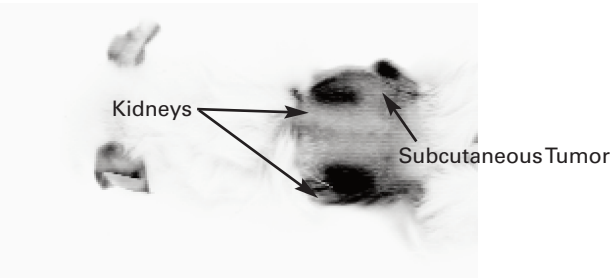


Figure 4. Liver and cecum signal present in the 700 nm channel and subcutaneous tumor (800 nm channel).

Figure 5. Intestinal signal in the 700 nm channel, and abdominal tumor in the 800 nm channel.

Figure 6. The 800 nm channel image illustrates clearance of the particular IRDye® 800CW-labeled probe from the kidneys. A subcutaneous side tumor is also visible.



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Doc # 988-12589
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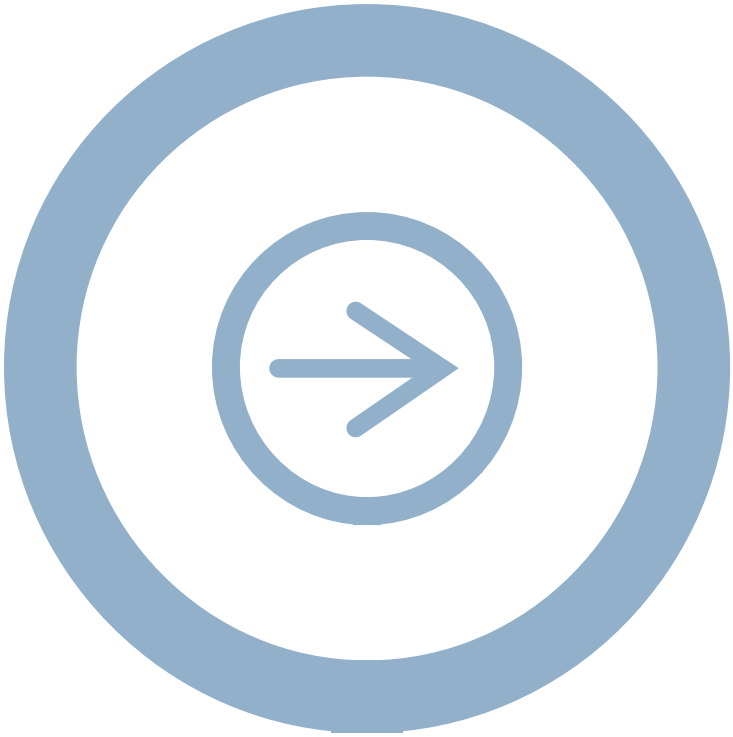
Technical Note

Image Studio™ Software Work Area Folder Best Practices

Developed for:

Image Studio Software 5.*x*

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



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I. Work Area Definition

Work Area: A folder used to store images, analysis data, software settings, and log files. The following are contents of a **Work Area**:

- **Database file**: Contains analysis data and software settings.
- **Log Folder**: Contains log files of Image Studio Software operations.
- **Images Folder**: Contains images from acquisitions taken in the current **Work Area** and images imported into the **Work Area**.

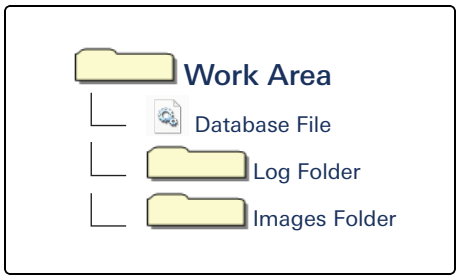


Figure 1. The **Work Area** folder contains a database file, log folder, and an images folder.

This Technical Note will explain how **Work Areas** can be used to facilitate data management, and it will address some common questions about **Work Areas**.

II. Avoid Renaming, Moving, or Adding Files to a Work Area

Under most circumstances, **Work Areas** should not be renamed, moved, or have files from outside Image Studio Software saved to them. If **Work Areas** are renamed or moved, Image Studio Software will not recognize them.

If Image Studio Software Does Not Recognize a Work Area That Was Renamed or Moved

If a **Work Area** has been renamed or moved, see "Adding Work Areas Back to the List" on page 9 for how to add an unrecognized **Work Area** back into Image Studio Software.

III. Creating a Work Area

Follow the procedures in this section, starting with "Before Launching Image Studio Software" below, to create **Work Areas** in a way that will facilitate data management.

Before Launching Image Studio Software

a. **MULTIPLE USERS:** If multiple people or multiple labs will be sharing the same computer account to run Image Studio Software, create an **Overall** folder on a local drive where each lab member using the system can create their own

Work Area or each lab can create a **Lab Folder**. Each group member will create an individual **Work Area** in the appropriate **Lab Folder** (see Figure 2 below).

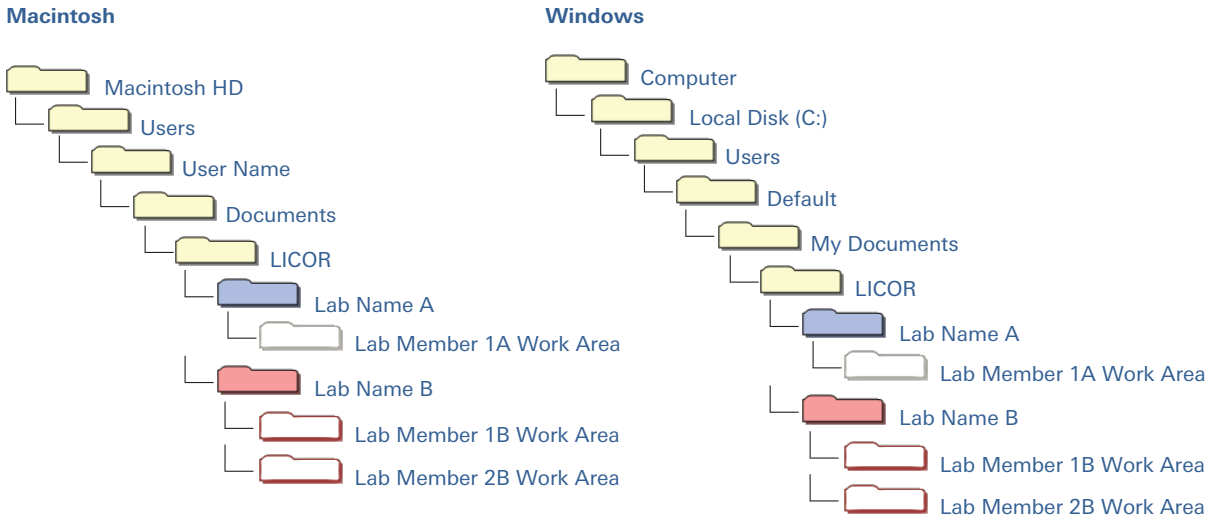


Figure 2. Efficient way to organize **Work Area** folders when multiple lab groups will be using the same computer account.

b. **SINGLE USER:** If each Image Studio Software user has an individual account on the computer, create an **Overall** folder on each account to save each individual's **Work Area**.

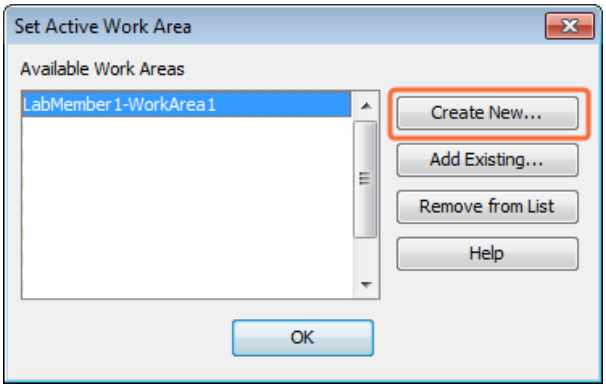
Avoid Creating a Work Area on a Network Drive

Avoid creating **Work Areas** on a network drive. Image Studio Software uses a **Work Area** to save data while scanning, so a scan may be aborted if the connection to the network drive is not fast enough.

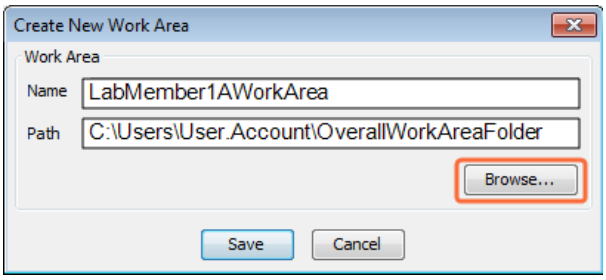
If data must be transferred to a network drive, flash drive, or cloud based system, use the Image Studio Software Export option to export zip files.

Procedure for Creating a Work Area

1. Double click the Image Studio Software icon.
2. The dialog box will open. Click **Create New…** to open the **Create New Work Area** dialog box.



3. In the **Create New Work Area** dialog box:



   a. Type a name for the **Work Area** in the **Name** field.
   b. Click **Browse** and navigate to the appropriate folder where the **Work Area** should be created.
   c. Click **Save** and Image Studio Software will open with the newly created **Work Area** as the active directory for saving new scans and analyses.

IV. One Work Area Per User

Maintaining a single user per **Work Area** provides an orderly system for file management and saves time by allowing Image Studio Software to automatically load the last used settings and analysis tools. Custom scan presets, views, and protein markers that are not standard in Image Studio Software settings will need to be entered and saved manually in each new **Work Area**.

File Management

Having too many **Work Areas** can make it difficult to remember which **Work Area** contains the files of interest. Multiple **Work Areas** are also inconvenient because the dialog box for selecting the active **Work Area** may contain many entries, and scrolling through the list can be difficult (see Figure 3).

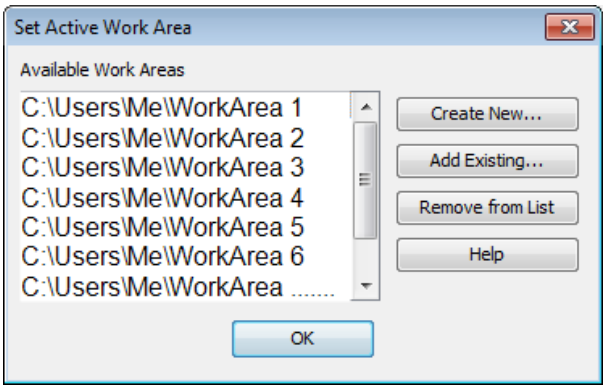


Figure 3. Too many **Work Areas** can make scrolling through the **Set Active Work Area** dialog box difficult.

Sort and View Advantages

In addition to cleaner file organization, leaving all of one's user data in a single **Work Area** takes advantage of the Image Studio Software tools for viewing and filtering files. Image Studio Software can display thumbnails of all images when the **Multiple Images** button is selected. Although a large number of images may be present in the **Images Table**, these images are easily managed using the Image Studio Software **Filter** function (see Figure 4 below).

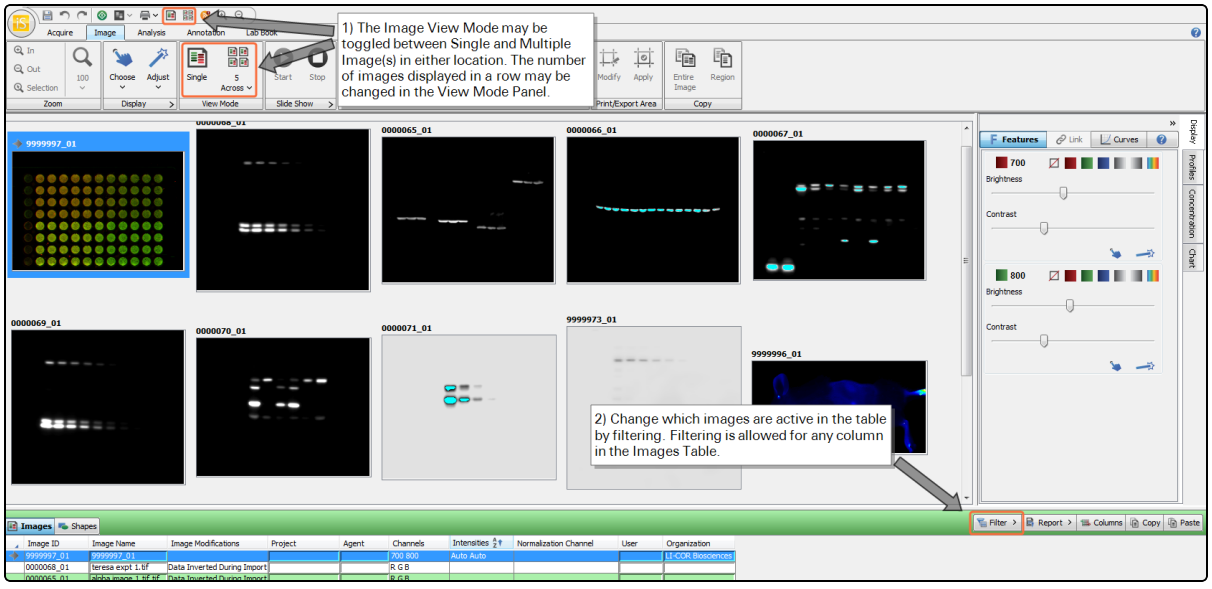


Figure 4. This picture shows 1) the **Image View Mode** in the upper left corner where the view may be changed from single to multiple images and 2) the **Filter** menu in the lower right hand corner that can be used to organize images.

V. Switching the Active Work Area

To switch the active **Work Area**:

1. Click the Image Studio Software **Application** menu, mouse over **Work Area**, and click **Switch** (see Figure 5).
2. In the **Set Active Work Area** dialog, click the desired **Work Area** in the list of Available **Work Areas**.
3. Click **OK** and the active **Work Area** will be switched.

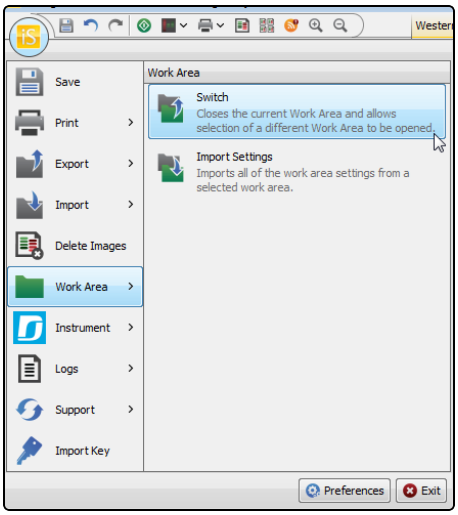


Figure 5. The Switch Work Area option in the Image Studio Software **Application** menu.

VI. Import Work Area Settings

Settings can be imported from a different **Work Area** into the current **Work Area**. Settings in the current **Work Area** will be overwritten.

To import **Work Area** settings:

1. Click the **Image Studio Application** button .
2. In the **Image Studio Application** menu, point to **Work Area** and click **Import Settings**.
3. In the **Import Work Area Settings** dialog, select the **Work Area** with the correct settings to be imported.
4. Click **Import**.

Most settings will be imported from the chosen **Work Area** and will overwrite settings in the current **Work Area**. For example, the imported settings include:

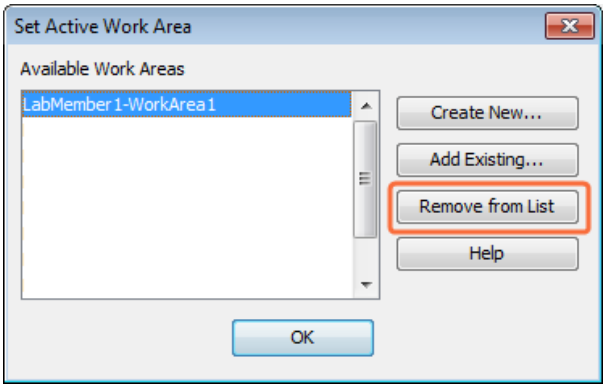
- Custom added protein markers (*i.e.* ones that have entered manually)
- **Work Area** preferences located in the **Preferences** dialog

- Show shape options in the **Show** group
- Image display options
- Table settings for all tables including which columns are displayed and how the table is sorted
- Instrument settings including scan presets (such as scan resolution, scan quality, and focus offset) and other options on the **Acquire** tab
- Custom grid, grid array, plate, and plate array templates
- Custom **Lab Book** templates
- Custom chart templates

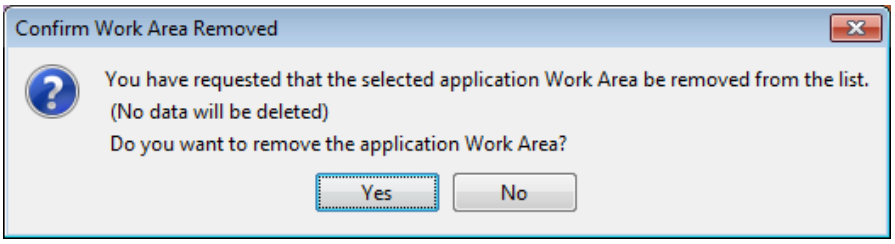
VII. Removing Unused Work Areas

Unused **Work Areas** can be removed from the list of **Work Areas** in the **Set Active Work Area** dialog box without deleting any data.

1. Click the Image Studio Software **Application** menu, mouse over **Work Area**, and click **Switch** (or just open Image Studio Software). The **Set Active Work Area** dialog box will open.
2. Scroll to find the **Work Area** to be removed, and click once to highlight it.
3. Click **Remove from List**.



4. The **Confirm Work Area Removed** dialog box will open. Click "Yes" to remove the **Work Area**.



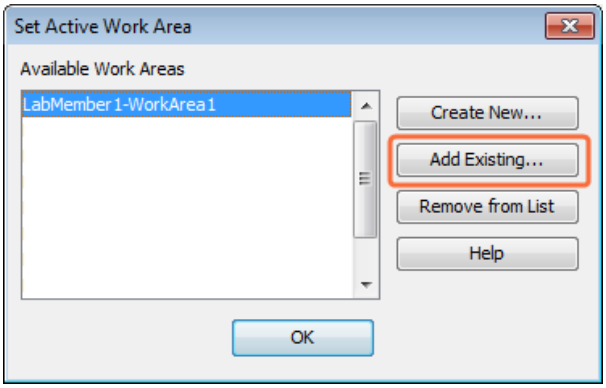
VIII. Adding Work Areas Back to the List

Use the following procedure to add a **Work Area** to the list of **Work Areas** in the **Set Active Work Area** dialog if:

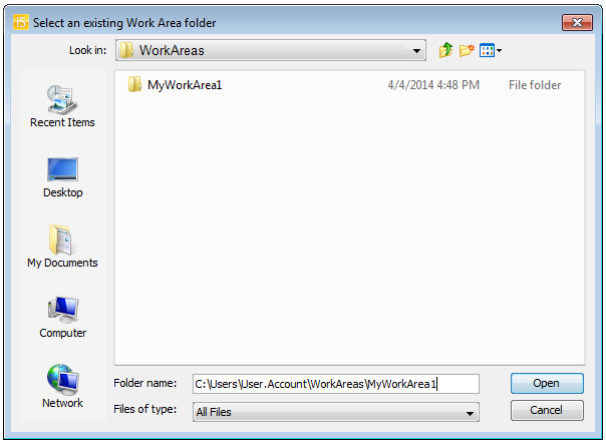
- A **Work Area** was removed using the **Remove from List** option and needs to be added back to the list.
- A **Work Area** was moved or renamed using the operating system's file manager. The **Work Area** in its new location or with its new name will need to be added using the **Add Existing...** option.

Procedure to Add Existing Work Areas

1. Click the Image Studio Software **Application** menu, mouse over **Work Area**, and click **Switch** (or just open Image Studio Software). The **Set Active Work Area** dialog box will open.
2. Click **Add Existing...**



3. The **Select an existing Work Area Folder** dialog box will open. Navigate to the **Work Area** that needs to be added.



4. Click once to highlight the **Work Area** to be added.
5. Click **Open** and the **Work Area** will be added to the list in the **Set Active Work Area** dialog box.
6. Click **OK** in the **Select Active Work Area** dialog box and Image Studio Software will open with the newly added **Work Area** as the active directory for saving new scans and analyses.

IX. When to Add Another Work Area

- A user may choose to create more than one **Work Area** to separate different projects, or if one **Work Area** is so full that it takes too long to open.
- When dealing with projects that require different settings and analysis types, keeping a **Work Area** for each project may be expedient, because Image Studio will recall the last used settings specific to each project when that project's **Work Area** is opened.

X. Other Considerations

IP Address Must Be Manually Entered For Each New Work Area

On some computer networks, IP addresses for the Odyssey® Classic and Odyssey Sa may need to be entered manually for each new **Work Area**. Consult the appropriate instrument manual for guidance, the Odyssey Sa Operator's Manual or the Odyssey Operator's Manual.

If New Work Areas Cannot Be Created

Nested Work Areas

The most common reason for this is trying to create a **Work Area** within another **Work Area** (nesting **Work Areas**). Starting with Image Studio Software version 3.1, nested **Work Areas** can no longer be created to prevent potential data loss.

Solving the Nested Work Area Problem

If nested **Work Areas** were created in previous versions of Image Studio Software, use the following procedure so **Work Areas** can be created again.

1. Using the operating system's file manager, create a higher level folder where the nested **Work Areas** can be stored at the same level in the folder structure (see Figure 6).
2. After the **Work Areas** have been moved to the higher level folder, they will need to be reassigned as **Work Areas** in Image Studio Software.
3. Reassign the **Work Areas** by clicking the **Add Existing...** option in the **Set Active Work Area** dialog box and choosing the **Work Area** in its new location. See "Adding Work Areas Back to the List" on page 9 for the complete procedure.
4. Remove unused **Work Areas** from the list. See "Removing Unused Work Areas" on page 8.

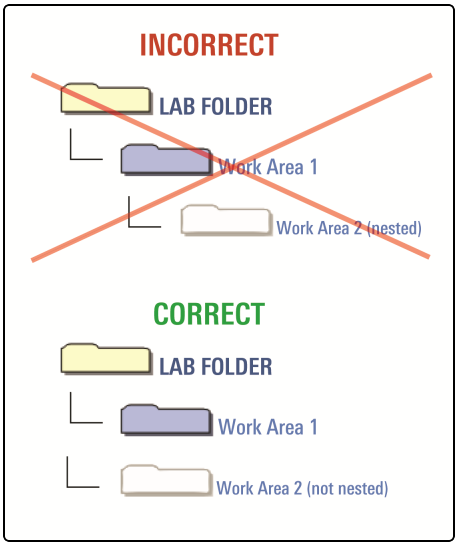
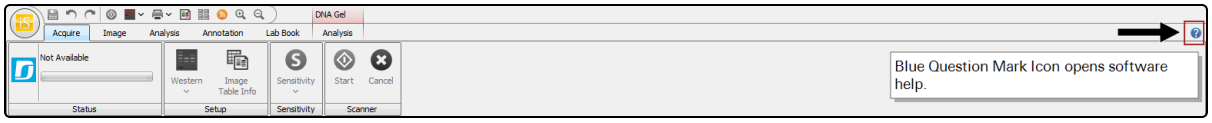


Figure 6. Use the operating system's file manager to move all **Work Areas** to be in the same folder at the same level (no **Work Area** within another).

XI. Further Questions

1. See the software help. Click the blue question mark in the upper right corner of the window to open the help. The upper right corner of the software help has a search box for quickly finding answers.



2. Contact LI-COR Technical Support (details in the footer of this document).

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