ODYSSEY® CLx

Application Protocols Manual

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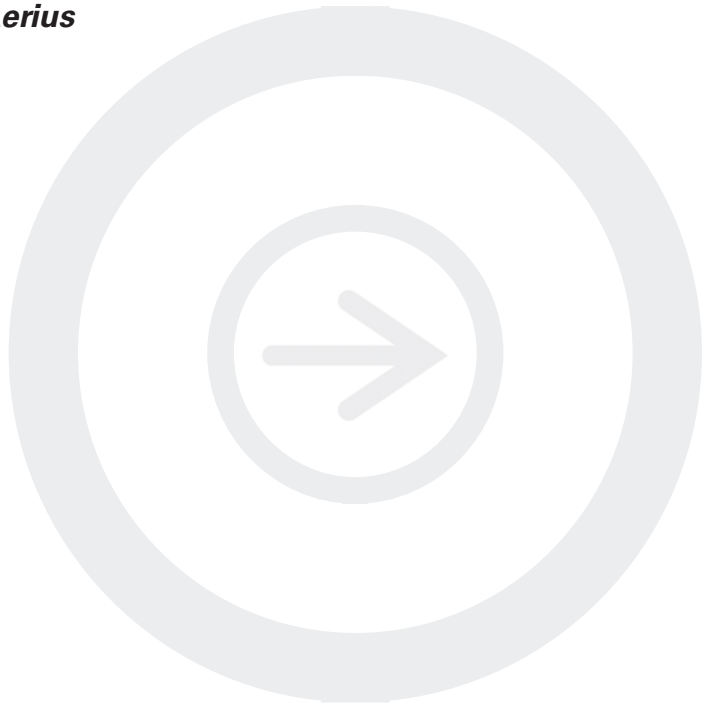
988-13858
0713

Western Blot Analysis

Developed for:

Aerius, and Odyssey® Family of Imagers

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



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I. Required Reagents

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

II. Western Detection Methods

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

After transfer, perform the following steps:

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

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

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

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

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

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

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

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

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

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

Molecular Weight Marker

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

Optimization Tips

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

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

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

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

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

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

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

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

III. Guidelines for Two-Color Detection

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

The following guidelines will help design two-color experiments:

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

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

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

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

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

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

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

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

IV. Stripping the Membrane

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

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

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

Primary Antibody Concentration

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

Secondary Antibody Concentration

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

Detergent Concentration

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

Tween® 20:

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

SDS:

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

VI. General Tips

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

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

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

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

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

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

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

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

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

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

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

VII. Imaging of Coomassie-Stained Protein

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

1. Wash gels with ultrapure water for 15 minutes.

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

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

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

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

VIII. Troubleshooting Guide

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

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

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

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

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Good Westerns Gone Bad:

Tips to Make Your NIR Western Blot Great

Developed for:

Odyssey® Family of Imagers

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

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Contents

I. Introduction to Western Blotting

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

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

II. Factors That Alter the Performance of a Western Blot

A. Membrane

A low-background membrane is essential for IR Western blot success. Background can be attributed to membrane autofluorescence or to detection of antibody non-specifically binding to the membrane. Polyvinylidene fluoride (PVDF) and nitrocellulose are typically used for Western blotting applications. There are many brands and vendors for both types of membrane. Before any Western blot is performed on an Odyssey System, the membrane of choice should be imaged "out of the box" on an Odyssey System to determine the level of autofluorescence. LI-COR has evaluated many different membranes for Western blotting; examples of membrane performance can be seen in Figure 1. There is typically more variability in PVDF performance than nitrocellulose.

NOTE: Not all sources of PVDF and nitrocellulose have been evaluated by LI-COR; therefore, it is important to evaluate the membrane before use. Membranes can be quickly evaluated by imaging them both wet and dry on any Odyssey System.

B. Blocking Reagent

There are many different sources and types of blocking reagents sold for Western blot applications. Antibody performance can sometimes be compromised by the blocking reagent chosen. Milk-based blockers may contain IgG that can cross-react with anti-goat antibodies. This can significantly increase background and reduce sensitivity. Milk-based blockers may also contain endogenous biotin or phospho-epitopes that can cause higher background.

If an antibody fails with one blocking condition, it may be advantageous to try another. Figure 2 is an example of the behavior of the anti-PKCα antibody in 5% BSA, 5% Milk, and Odyssey® Blocking Buffer on a nitrocellulose membrane. Figure 3 is a similar example using Odyssey Blocking Buffer, I-Block™, and 5% BSA for detection of anti-pAkt and β-tubulin in 293T Cells stimulated with TGF-β.

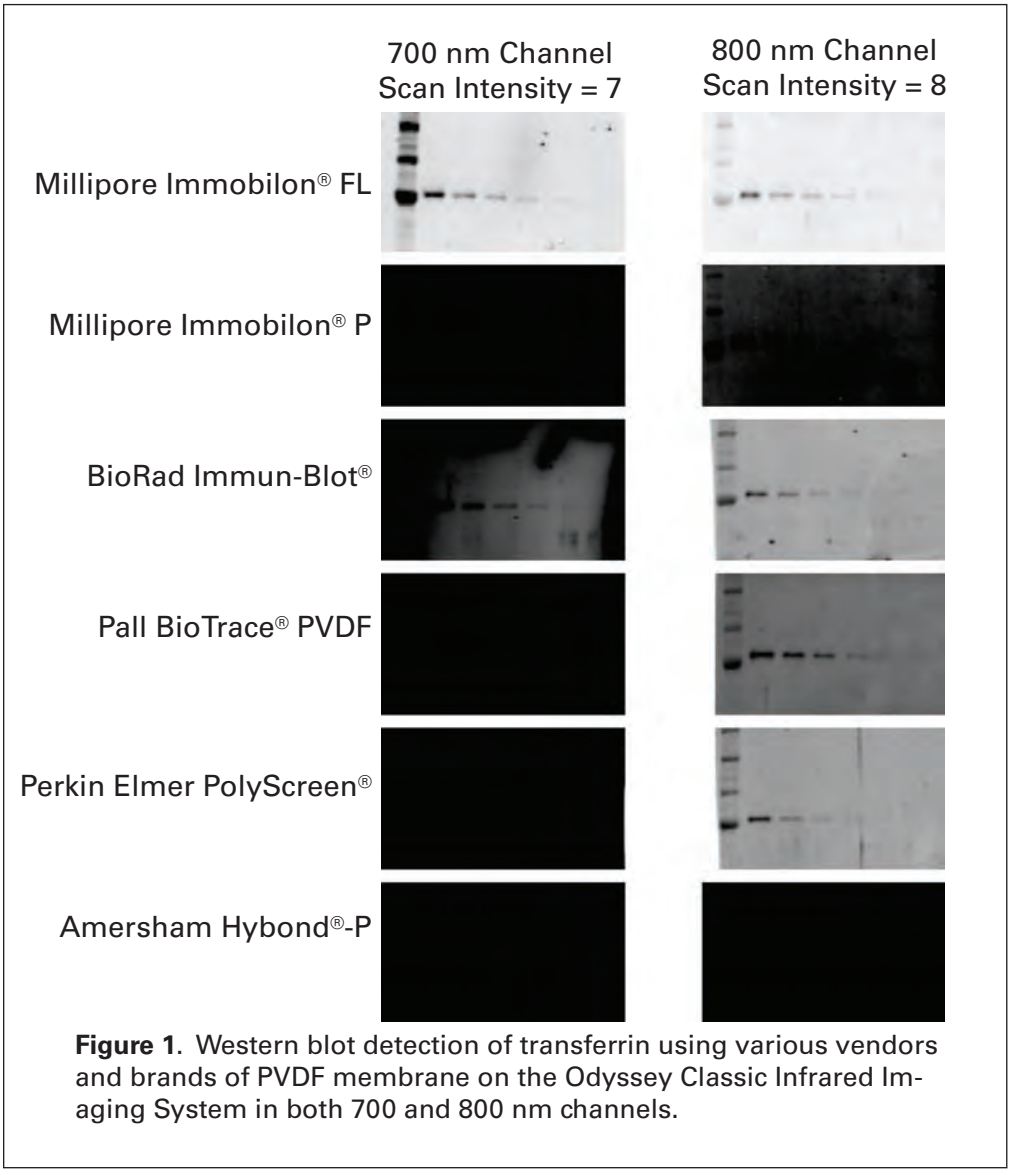


Figure 1. Western blot detection of transferrin using various vendors and brands of PVDF membrane on the Odyssey Classic Infrared Imaging System in both 700 and 800 nm channels.

We tested the PathScan® PDGFR Activity Assay: Phospho-PDGFR, Phospho-SHP2, Phospho-Akt, and Phospho-p44/42 MAPK (Erk1/2) Multiplex Western Detection Kit #7180, using five different blocking/diluent solutions. Figure 4 shows results from this experiment. The five phosphoproteins could be clearly visualized with each of the blocking solutions, with the exception of 5% Milk, which had very high background. The S6 Ribosomal protein (total protein loading control) was almost completely absent in blots where Odyssey® Blocking Buffer (P/N 927-40010, 927-40003, 927-40000, 927-40100) was used. This data clearly suggests that there is not a universal blocker that is best for all antibodies.

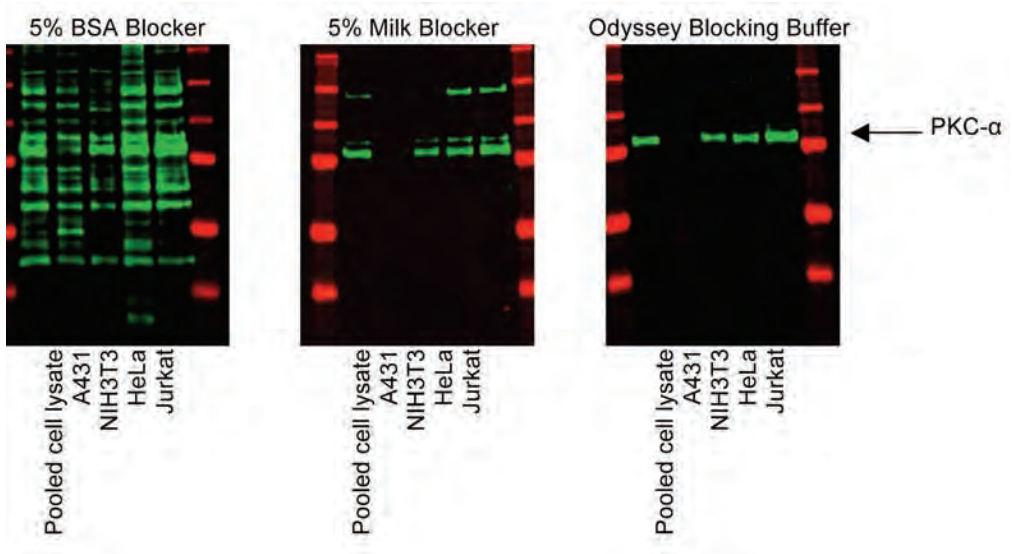


Figure 2. Western blots detected with anti-PKCα and IRDye® 800CW Goat anti-mouse. All blots were treated equally, with the exception of blocking reagent. All images were generated on the Odyssey Classic Infrared Imager with scan intensity setting of 5, sensitivity of 5.

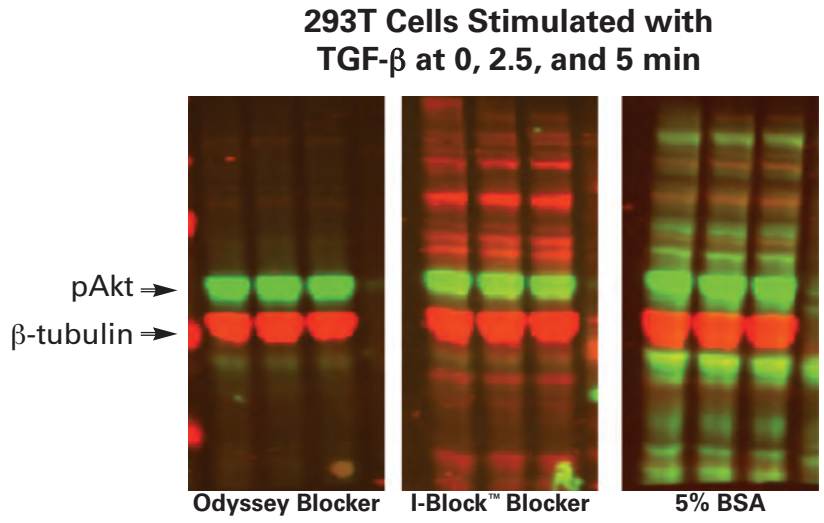


Figure 3. Western blots of 293T Cells stimulated with TGF-β (0, 2.5, and 5 minutes) detected with anti-pAkt and β-tubulin. All blots were treated equally, with the exception of blocking reagent. All images were generated on the Odyssey Classic Infrared Imager with scan intensity setting of 3.5/5 (700/800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

C. Detergents

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

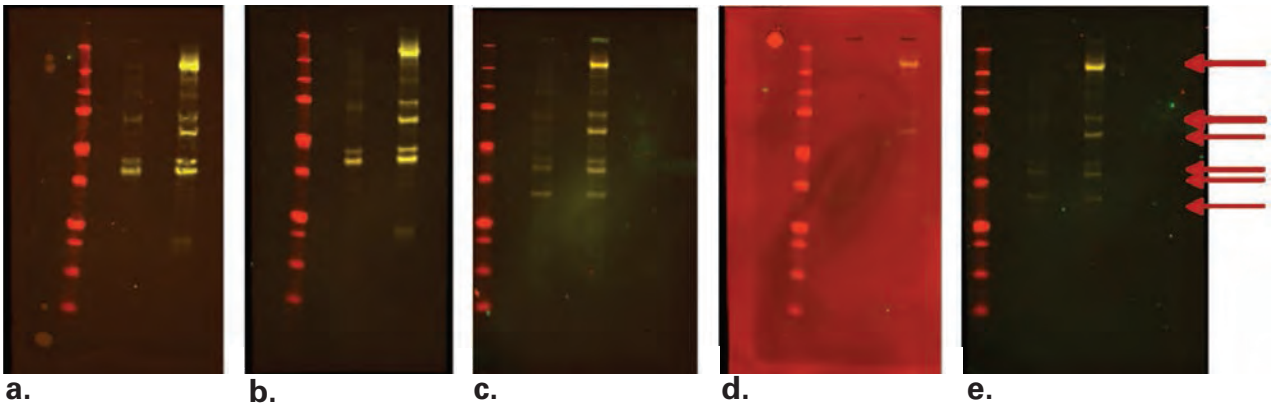
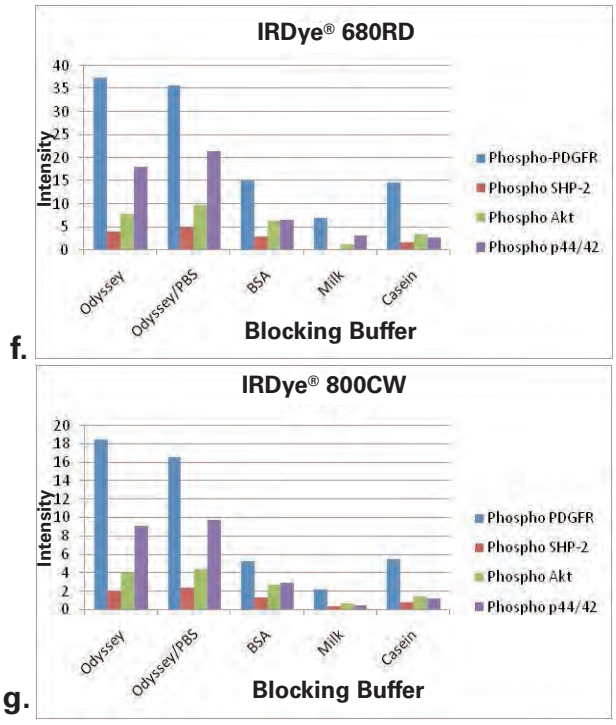


Figure 4. Above: Western blots utilizing PathScan® Multiplex primary antibody and both IRDye® 680 and IRDye 800CW goat anti-rabbit for detection. Five different solutions were used for blocking and antibody dilution (antibody dilutions included 0.2% Tween® 20):

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

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

f. Quantification of 700 nm signal in each blocking solution.
g. Quantification of 800 nm signal in each blocking solution.



1. **Tween® 20**
   a. Blocker – do not put Tween 20 into the blocking reagent during blocking.
   b. Primary and secondary antibody diluents should have a final concentration of 0.1 - 0.2% Tween 20 for nitrocellulose membranes, and a final concentration of 0.1% for PVDF membranes. A higher concentration of Tween 20 may increase background on PVDF.
   c. Wash solutions should contain 0.1% Tween 20.
2. **SDS**
   a. Blocker - do not put SDS into the blocking reagent during blocking.
   b. When using PVDF membrane, secondary antibody diluents should have a final concentration of 0.01 - 0.02% SDS. SDS can be added to the antibody diluents when using nitrocellulose to dramatically reduce overall membrane background and also reduce or eliminate non-specific binding. It is critical to use only a very small amount. SDS is an ionic detergent and can disrupt antigen-antibody interactions if too much is present at any time during the detection process. When working with **IRDye® 680LT** conjugates on **PVDF** membranes, SDS (final concentration of 0.01 - 0.02%) and Tween 20 (final concentration of 0.1. - 0.2%) **must** be added during the detection incubation step.
   c. Wash solutions should not contain SDS.

D. Primary Antibody

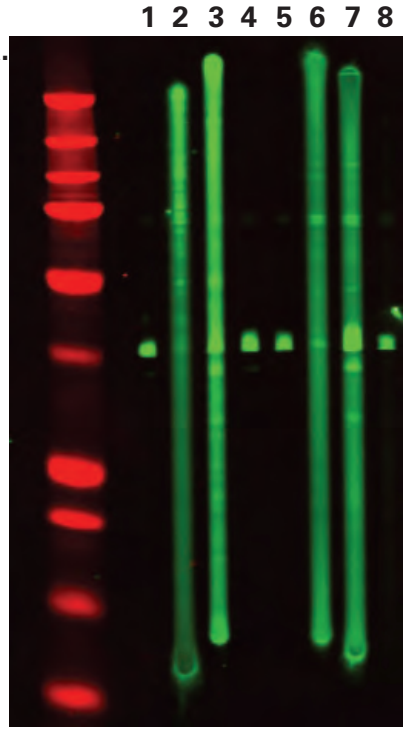
An antibody produced to detect a specific antigen is called the primary antibody, and it binds directly to the molecule of interest. Primary antibodies can be produced in a wide variety of species, such as mouse, rabbit, goat, chicken, rat, guinea pig, human, and many others. Primary antibodies for the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

E. Secondary Antibody Quality

One of the primary benefits of using an Odyssey® System for Western blot detection is the ability to detect two targets simultaneously. Two-color detection requires careful selection of primary and secondary antibodies. The two primary antibodies must be derived from different host species so they can be discriminated by secondary antibodies of different specificities (for example, primaries from rabbit and mouse will be discriminated by anti-rabbit and anti-mouse secondary antibodies). One secondary antibody must be labeled with IRDye® 680LT or IRDye 680RD, and the other with IRDye 800CW.

The exception to this is when using IRDye Subclass Specific Antibodies. IRDye Goat anti-Mouse IgG_1, Goat anti-Mouse IgG_{2a}, and Goat anti-Mouse IgG_{2b}, allow for two-color detection using primary antibodies derived from the same species (mouse). IRDye Subclass Specific antibodies react only with the heavy (gamma) chain only of the primary antibody. In mice, there are five unique subclasses of IgG: IgG_1, IgG_{2a}, IgG_{2b}, IgG_{2c}, and IgG_3. Each subclass is based on small differences in amino acid sequences in the constant region of the heavy chains, so antibodies directed against a particular subclass will not recognize antibodies directed against other subclasses. For example, IRDye goat anti-mouse IgG_1 recognizes mouse gamma 1, but will not recognize mouse gamma 2a, 2b, 2c or gamma 3. For details and a complete description, refer to *Western Blot and In-Cell Western™ Assay Detection Using IRDye Subclass Specific Antibodies*.

A.



B.

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

Figure 5. MPX™ screening of eight different GAPDH primary antibodies on a HeLa cell lysate sample. Primary antibodies were diluted in Odyssey® Blocking Buffer according to manufacturer's recommendations.

Always use highly cross-adsorbed secondary antibodies for two-color detection. Failure to use cross-adsorbed antibodies may result in increased cross-reactivity as shown in Figure 6. LI-COR® IRDye®-conjugated secondary antibodies are optimized for two-color Western blot detection. They are highly cross-adsorbed with a dye-to-protein ratio maximized for optimal signal-to-noise ratio in both Western blot and In-Cell Western™ assay detection. Figure 7 shows a comparison of LI-COR highly cross-adsorbed IRDye goat anti-mouse to a non-cross-adsorbed goat anti-mouse secondary antibody and their reactivity to the different mouse IgG sub-classes.

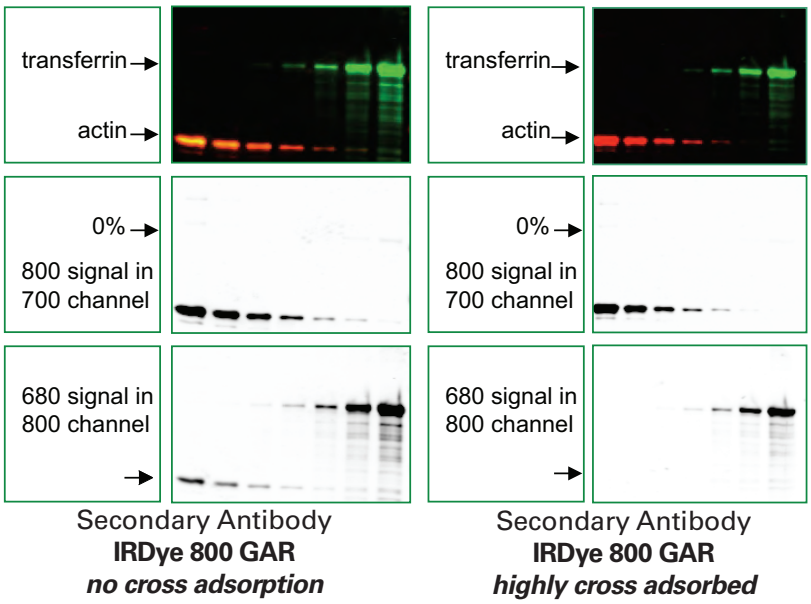
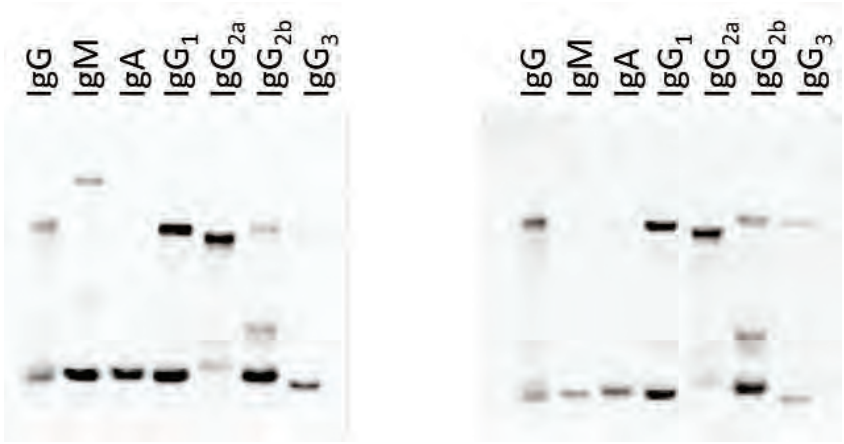


Figure 6. Example of a secondary antibody not cross adsorbed, cross-reacting with the second antibody pair in a two-color Western blot.

There are many choices in secondary antibodies for Western blot detection. LI-COR offers IRDye whole IgG (H + L) secondary antibodies and IRDye Subclass Specific secondary antibodies. Figure 8 demonstrates the performance of LI-COR IRDye goat anti-mouse compared to various other secondary antibody options for detection of a mouse IgG primary antibody. Figure 9 demonstrates the differences between IRDye Subclass Specific detection and IRDye whole anti-mouse IgG detection.



GAM IgG
LI-COR®
Highly Cross Adsorbed

GAM IgG
***Not* Cross Adsorbed**

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

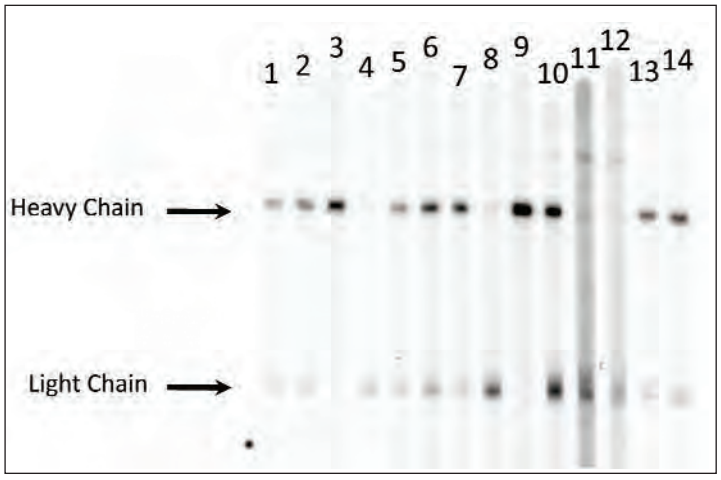
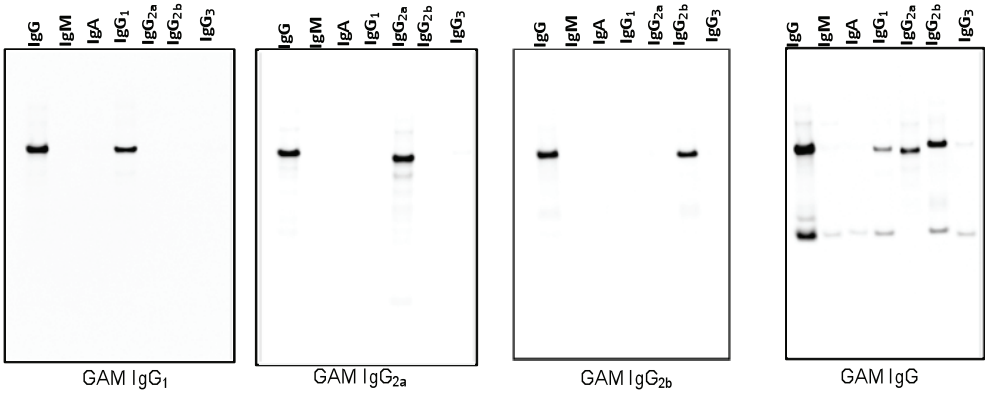


Figure 8. IRDye 800CW labeled anti-mouse antibodies against purified mouse IgG.

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

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

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



F. Secondary Antibody Dilution

The amount of secondary antibody that is used for IR Western blots can vary a great deal. When using LI-COR® IRDye® 800CW and IRDye 680RD conjugated secondary antibodies, the recommended dilution range is 1:5,000 to 1:25,000. When using LI-COR IRDye 680LT secondary antibodies, the recommended dilution range is 1:20,000 to 1:50,000. The dilution should be optimized for the primary antibody being used and the preferred appearance of the Western blot. The Odyssey® imaging software can be used to maximize the appearance of the image using a wide range of secondary antibody dilutions (Figure 10).

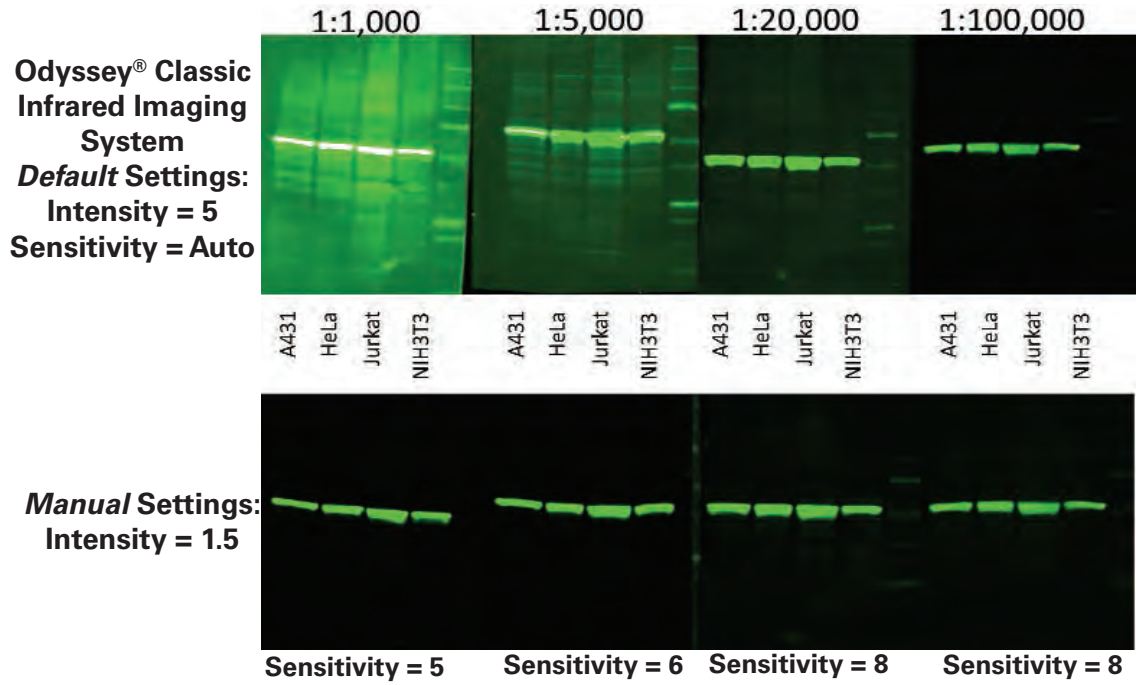


Figure 10. Secondary Antibody Concentration of IRDye 800CW goat anti-mouse with maximized Odyssey Classic imaging capabilities.

G. Miscellaneous Contamination

There are many things that can cause contamination of an infrared Western blot. Contamination can appear as a global increase in background, large smears of signal, or speckled blots. Common sources of contamination are listed in Table 1. Some example images are shown in Figure 11.

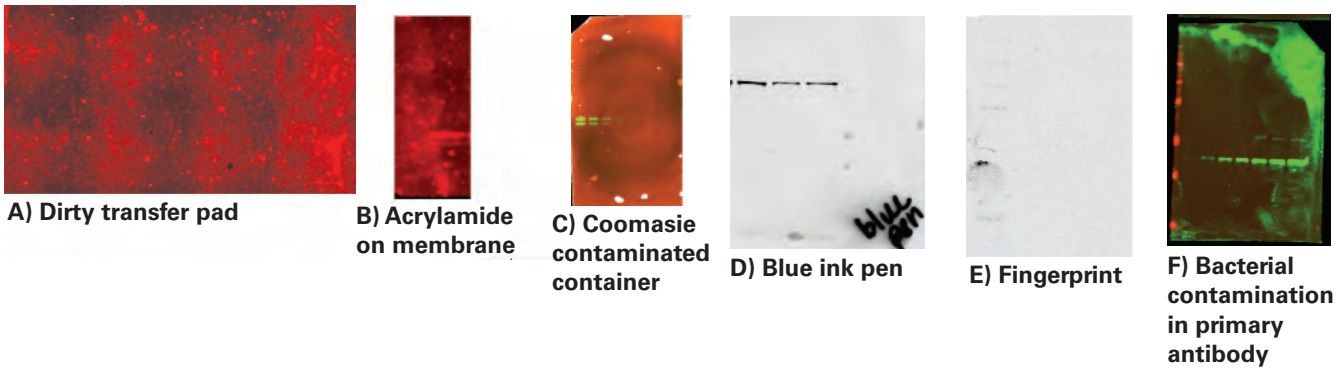


Figure 11. Examples of contamination events that may cause background on a Western blot.

Table 1.

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

III. Imaging Issues That Can Alter the Performance of a Western Blot

There are adjustments that can be made during the process of imaging a Western on any Odyssey® Imaging System that can greatly influence data acquired from the instrument.

A. Starting with a clean scan bed or imaging tray is critical. If you acquire an image and the area that doesn't have a membrane appears to have signal in either channel, the scan bed or imaging tray is contaminated. The contamination source may be as simple as dust or as complex as dye.

B. Air bubbles can result in reduced signal detection during imaging. Flatten the membrane with a roller to remove bubbles and excess liquid. See Figure 12.

C. A Western blot can be imaged either wet or dry on any Odyssey Imaging System. Typically, the signal is higher when a dry blot is imaged; however, background will increase.
NOTE: Once a blot is dry, or partially dried, stripping of the membrane for reuse is ineffective. See Figure 13.

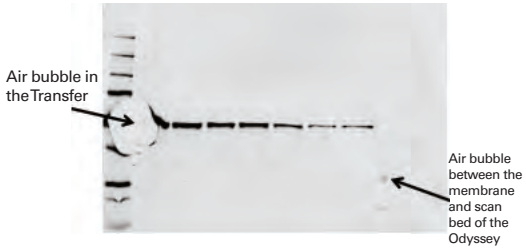


Figure 12. Examples of air bubbles in the transfer and on the Odyssey® Classic Infrared Imager scan bed.

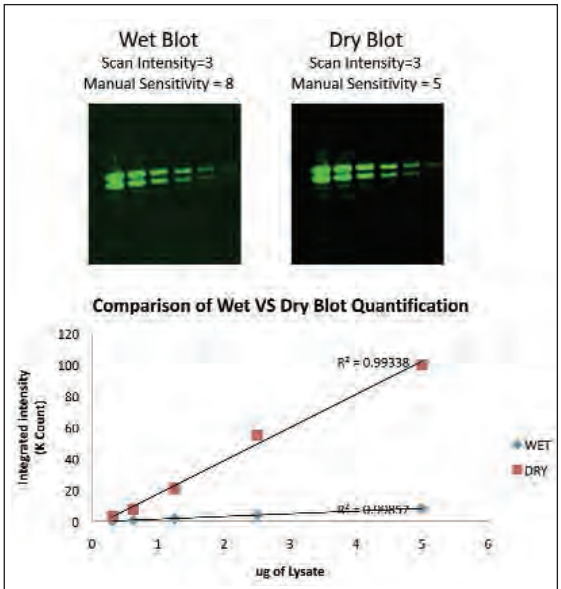


Figure 13. The same Western blot scanned wet and dry on an Odyssey Classic. The images are represented using the optimal display settings. Quantification is shown in the chart below the images.

Odyssey Classic, Odyssey CLx, and Odyssey Sa

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

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

It is important to note that saturated pixels (pixels that appear white in the image) cannot be accurately quantified. Signal saturation can also result in signal transfer to the alternate channel. For example, saturated signal in the 800 nm channel can be seen as 700 nm signal in the 700 channel scan (see Figure 16). This can be easily eliminated by scanning at a lower intensity.

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

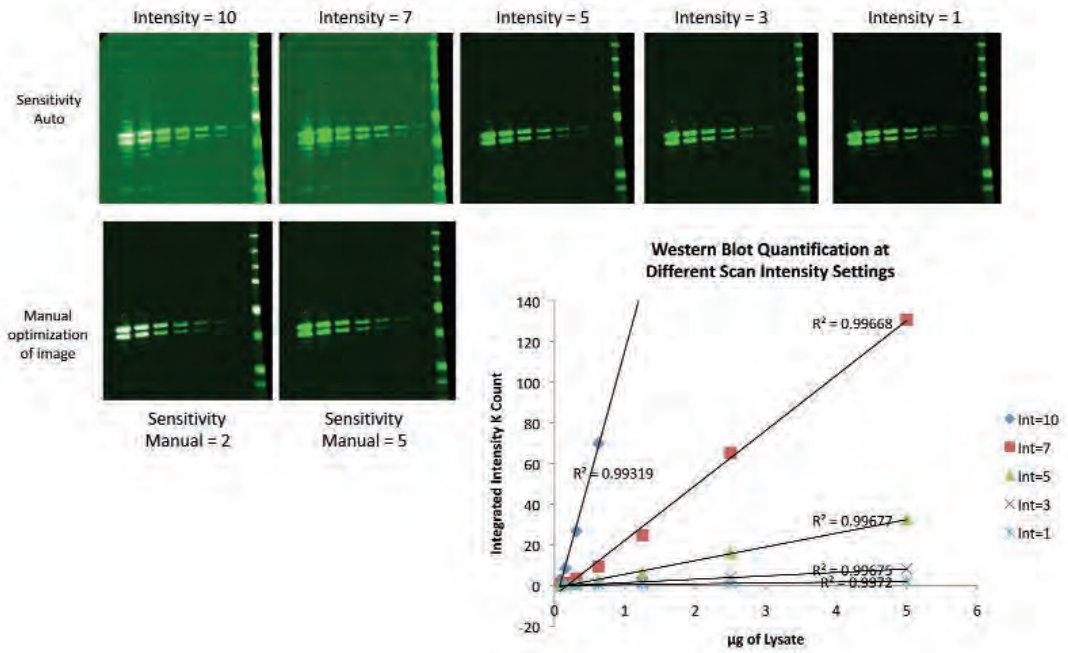


Figure 14. The same Western blot scanned at 5 different intensity settings on the Odyssey® Classic Infrared Imaging System. The top row of images are displayed using the Auto Sensitivity setting in the Odyssey Software. The bottom images were optimized using the Manual Sensitivity option for display.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

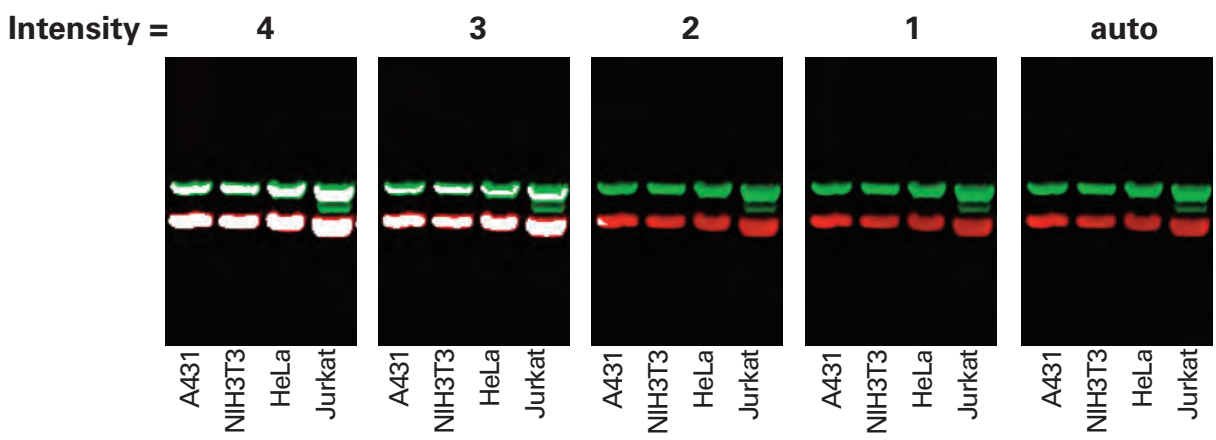


Figure 15. A single Western blot scanned on Odyssey CLx at decreasing Scan Intensity settings, and finally using AutoScan intensity. Pixel saturation appears in white. The antigen targets for each lysate sample are displayed in green (rabbit anti-Tubulin detected with IRDye® 800CW goat anti-rabbit) and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

IV. Software Adjustments for Image Optimization

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

- Blots that exhibit No Fluorescence
- Blots with Dim Bands

These software enhancements will only work on blots that are not experiencing binding chemistry problems.

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

No Fluorescence – Blots that unexpectedly exhibit no fluorescence can be enhanced by changing the sensitivity setting of the image from **Linear Auto** to **Linear Manual**. These settings can be changed from the **View** menu, then **Alter Image Display** menu. To enhance the image, simply click the **Linear Manual** radio button and adjust the slider. By manually adjusting the sensitivity settings, the most desirable image can be chosen. For details, see the *User Guide*.

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

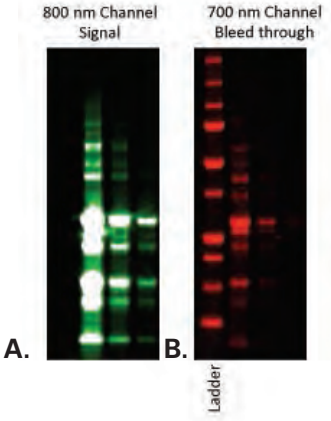
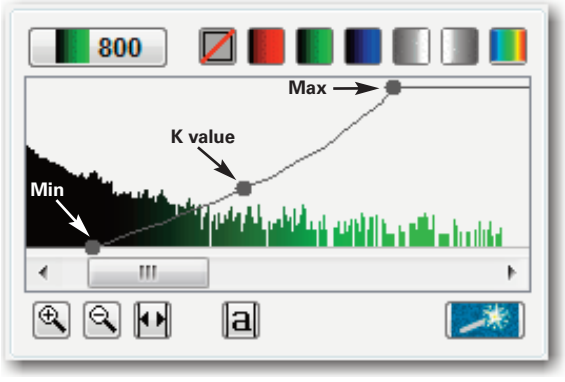


Figure 16. Saturated signal in the 800 nm channel (A) of the Odyssey Classic Infrared Imaging System can be visualized in the 700 nm channel (B). The only detection that should be seen in the 700 nm channel is the ladder on the far left of the image. Optimizing scan intensity can eliminate this.

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

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

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



V. Data Analysis Using the Odyssey® Classic

Odyssey Classic (ver. 3.x application software)

Background

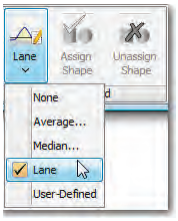
For accurate Western blot quantification, the **Background** setting must be applied effectively. The Background method sets the background calculation method for use in quantification, by measuring the intensity of the pixels selected as the background region. There are several methods for background subtraction, each unique to a specific need.

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

Image Studio (ver. 1.x – 2.x)

Background settings can be found in the **Background** group on the **Analyze** ribbon. To implement **User-Defined Background** selection in the Image Studio software, draw one or more shapes over an area of typical background. Select the shape(s) and click **Assign Shape** in the Background group in the Analyze ribbon. The background setting will change to **User-Defined**.

With the **Western Key**, the Background group on the Western and MPX™ Western Analysis ribbons includes the option of **Lane** background subtraction. This setting subtracts the background of the Lane from each Band. The same background settings used in Odyssey Classic 3.0 software can also be used in the Western and MPX Analysis ribbons.



VI. Data Analysis Using the Odyssey® CLx

Image Studio (ver. 1.x – 2.x)

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

VII. Data Analysis Using the Odyssey Sa

Application Software (ver. 1.x)

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

VIII. Data Analysis Using the Odyssey Fc

Image Studio (ver. 1.x – 2.x)

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

IX. Summary

There are many ways to maximize the performance of a Western blot. A fully optimized Western blot is the best place to start. LI-COR provides high-quality reagents for optimal Western blot detection. For a detailed protocol on how to do a Western blot with an Odyssey Family Imager, see the *Odyssey Western Blot Analysis* protocol.

X. Reference

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

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1. Introduction

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

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

2. Normalization of RNA silencing data from a Western blot

2.1 Western blot data

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

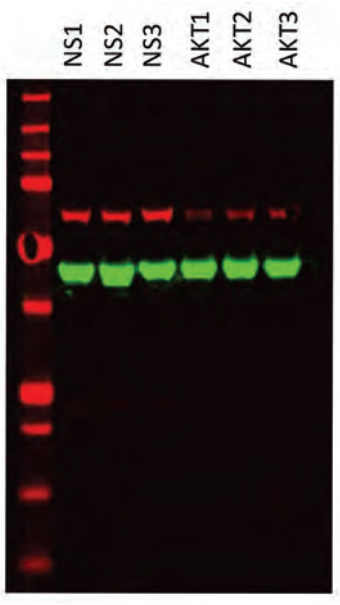


Figure 1. Two-color Western blot of HeLa cell lysates transfected with nonsense control siRNA (NS1-3) or AKT siRNA (AKT1-3). The proteins were detected with either mouse anti-pan actin and IRDye® 800CW Goat anti-Mouse IgG (LI-COR® P/N 926-32210) or rabbit anti-AKT and IRDye 680LT Goat anti-Rabbit IgG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

Table 1. Data analysis of AKT silencing in HeLa cells

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

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

2.2 Steps for normalization:

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

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

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

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

2.3 Using the geometric mean

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

Example: Data set values = 9, 4, 1

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

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

3. Housekeeping genes as normalization controls

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

4. Housekeeping genes and expression variability

4.1 Variability in tissue types

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

4.2 Variability caused by cell treatments

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

4.3 Variability caused by environmental changes

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

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

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

5. Choosing the correct normalization control

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

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

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

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

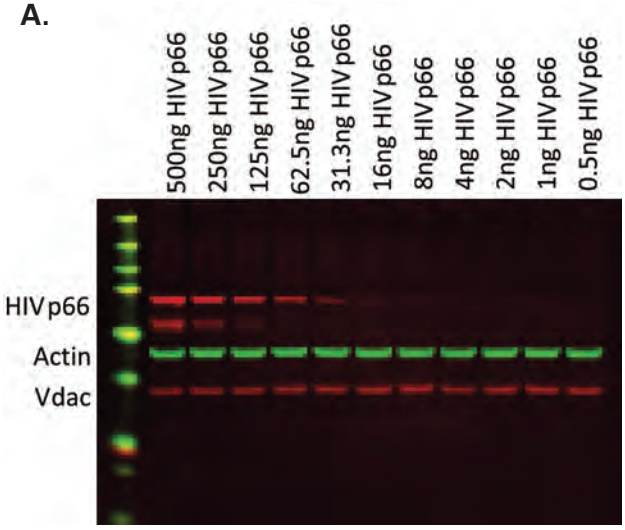
In all of these cases, it is important to run a pilot experiment under the same conditions you plan to use for the full experiment, to make sure that the expression of your normalization standard is NOT affected by the experimental protocol (i.e., chemical treatment, environmental changes, tissue choice).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

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

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

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

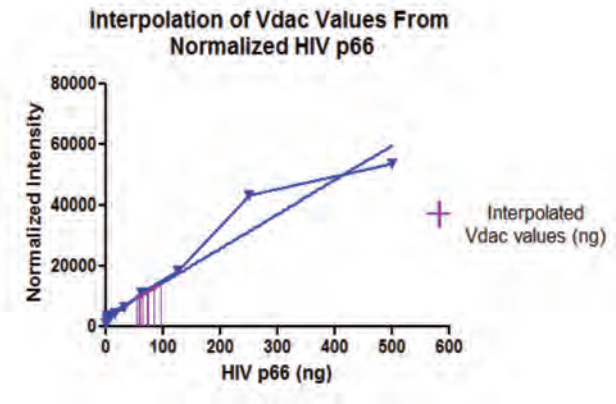
A.



B.

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

C.



D.

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

Figure 2.

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

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

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

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

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

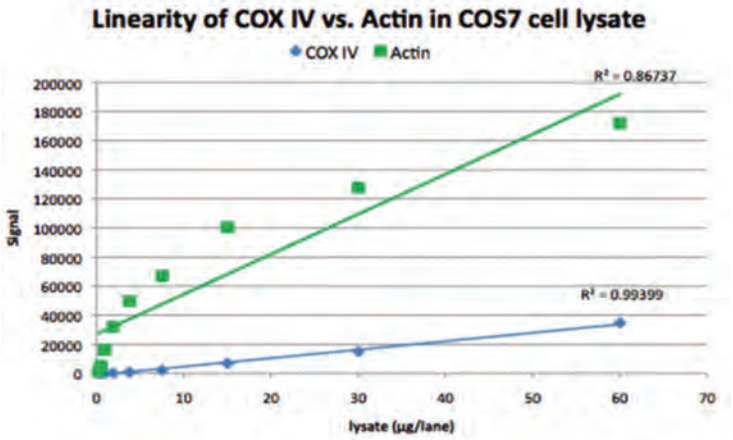


Figure 3. Detection of COX IV with COX IV Rabbit Monoclonal primary antibody (LI-COR® P/N 926-42214) is linear across a wide range, even when lysate is heavily loaded. COX IV and actin (Beta-Actin Rabbit Monoclonal; LI-COR P/N 926-42210) were detected in COS7 cell lysates, using IRDye® 800CW Goat anti-Rabbit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

6. Choosing the correct method of detection for normalization

6.1 Chemiluminescent detection (ECL)

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

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

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

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

6.2 NIR fluorescent detection

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

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

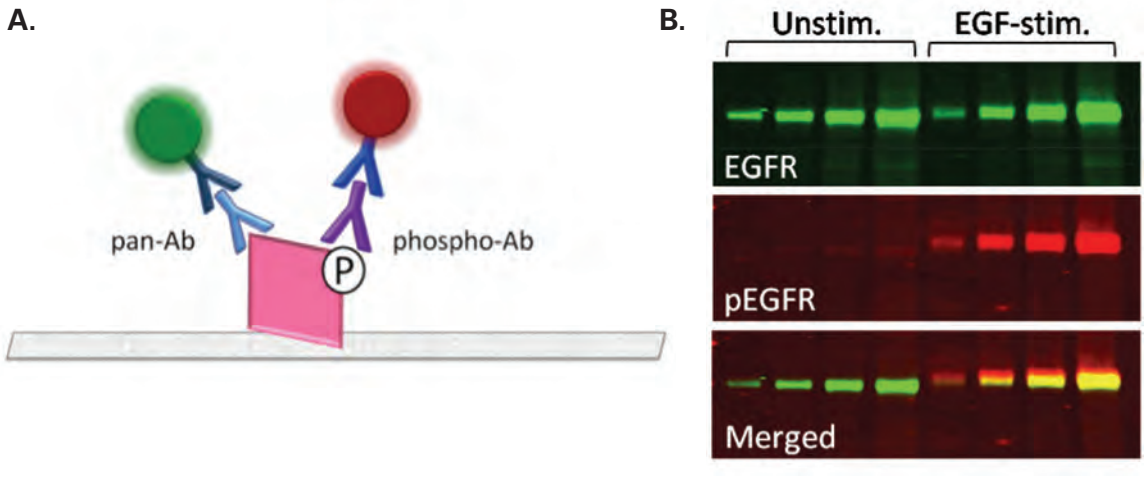


Figure 4.
A) Multiplex phosphorylation analysis combines a phospho-antibody with an antibody that recognizes the target protein regardless of its phosphorylation state (pan-antibody).
B) Multiplex phosphorylation analysis used to detect EGFR phosphorylation in EGF-stimulated A431 cells. This type of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

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

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

7. Conclusions

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

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Technical Note

Protein Electrotransfer Methods and the Odyssey® Infrared Imaging Systems

Developed for:

Aerius, and Odyssey Family of Imagers

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Published September 2008.
Revised October 2011. The most recent version of this technical note is posted at http://biosupport.licor.com/support

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 (LI-COR, P/N 928-40010)
- 10X PBS Buffer (LI-COR, P/Ns 928-40018 and 928-40020)
- 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.

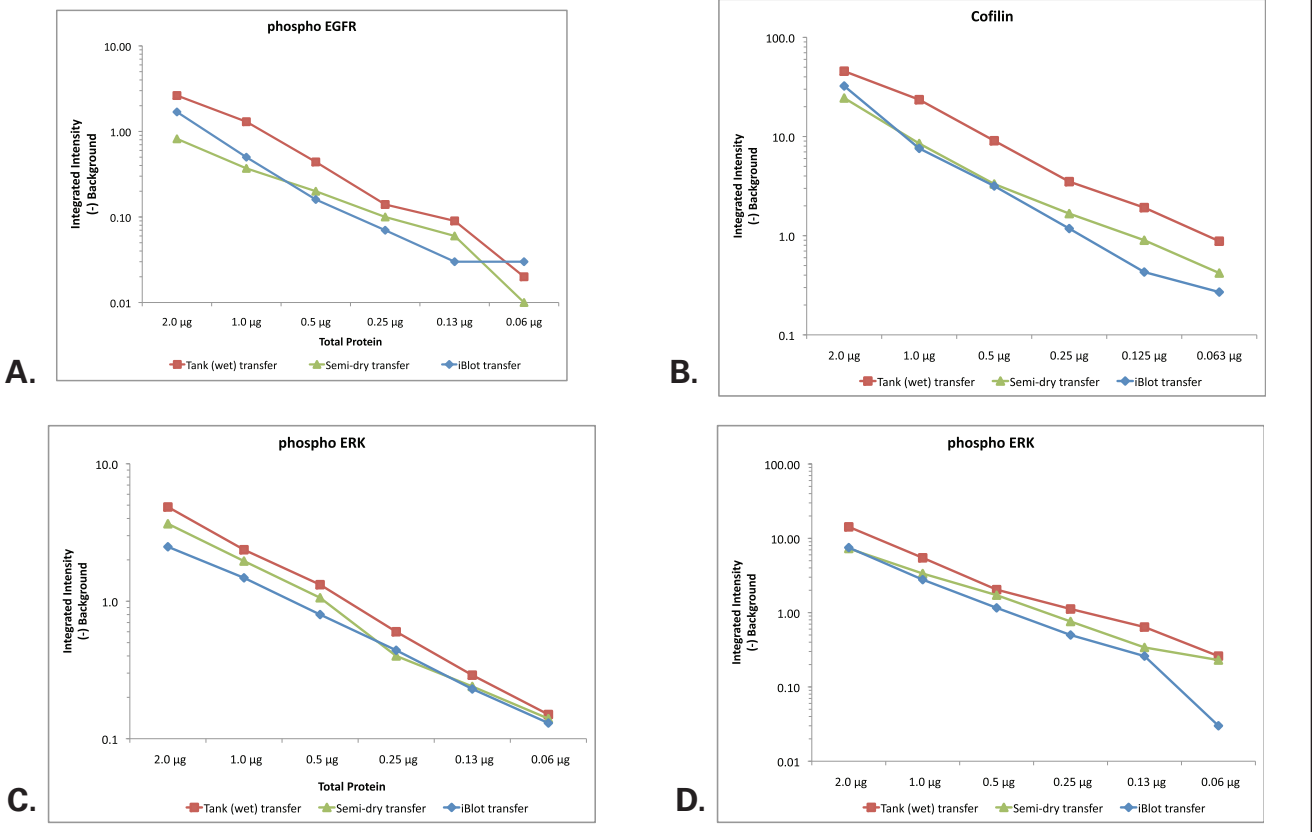

Figure 1. Western blot quantitation data comparing each of the three electrotransfer methods under varying conditions: (A) Phospho-EGFR data from NuPage 4-12% Bis-Tris gels transferred to Odyssey Nitrocellulose membranes. (B) Cofilin protein quantitation data from NuPage 4-12% Bis-Tris gels transferred 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.

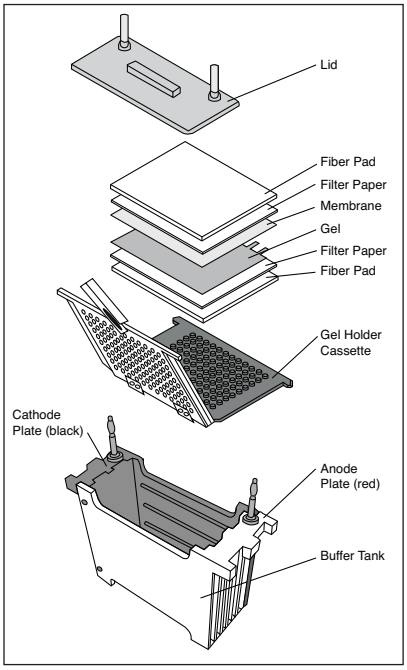

Figure 2. Diagram of tank transfer setup.

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 "Western Blotting Analysis" for details on performing Western Blot detection on the Odyssey Family of Imagers (available at biosupport.licor.com).

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

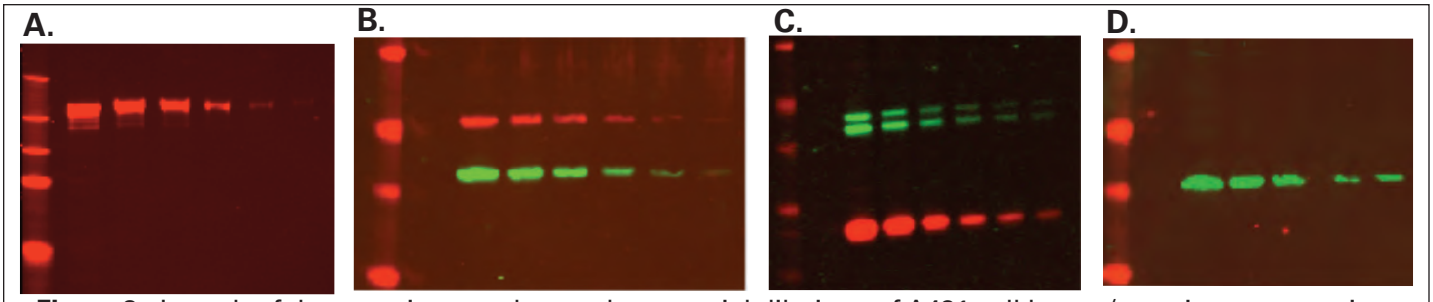

Figure 3. In each of the experiments shown above, serial dilutions of A431 cell lysate (protein concentration range 2.0 – 0.06 µg) were loaded onto polyacrylamide protein gels, electrophoresed and transferred to membrane using the Mini Trans-Blot® (Bio-Rad) wet tank transfer system. Detection was performed 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.

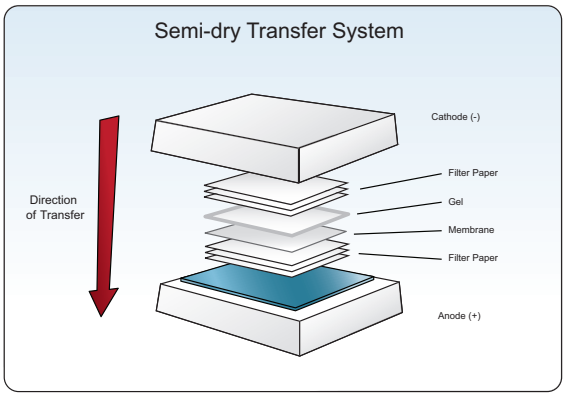

Figure 4. Diagram of semi-dry transfer setup.

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). The membrane and filter paper should be the same size as, or slightly larger than, the size of the gel(s) to be transferred for best results. 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 "Western Blotting Analysis" for details on performing Western Blot detection on the Odyssey Family of Imagers (available at biosupport.licor.com).

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

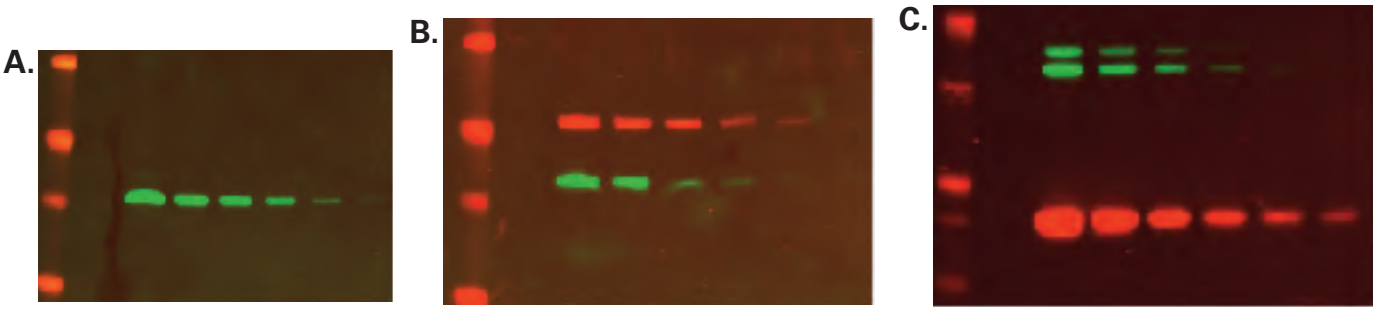

Figure 5. In each of the experiments shown above, serial dilutions of A431 cell lysate (protein concentration range 2.0 – 0.06 µg) were loaded onto polyacrylamide protein gels, electrophoresed and transferred to membrane using the Trans-Blot® SD (Bio-Rad) semi-dry transfer system. Detection was performed 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®)

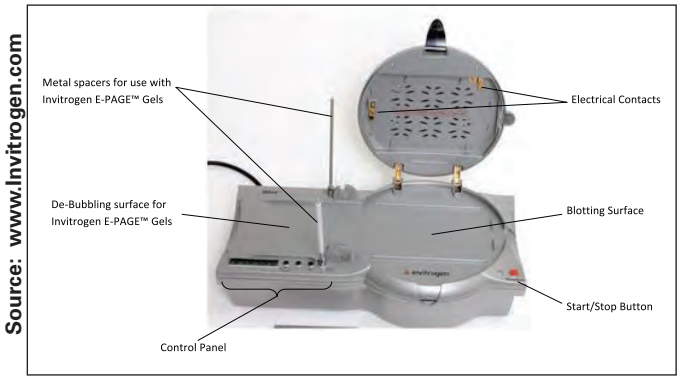

Figure 6. iBlot® Gel Transfer Device

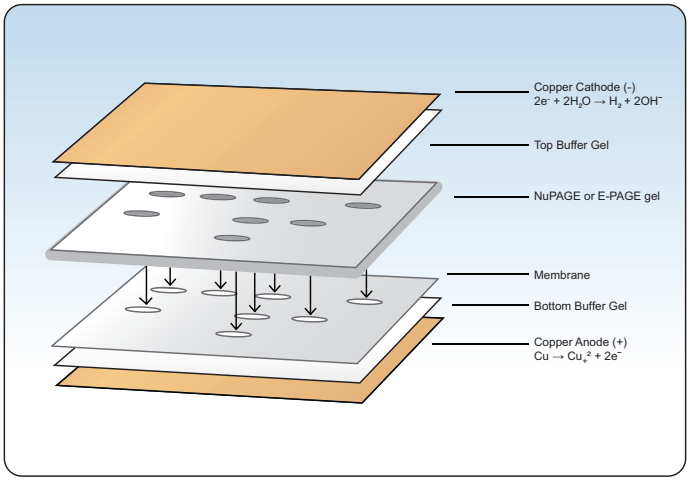

Figure 7. Gel/membrane sandwich with anode and cathode stacks

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 "Western Blotting Analysis" for details on performing Western blot detection on the Odyssey Family of Imagers (available at biosupport.licor.com). 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 pre-run 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

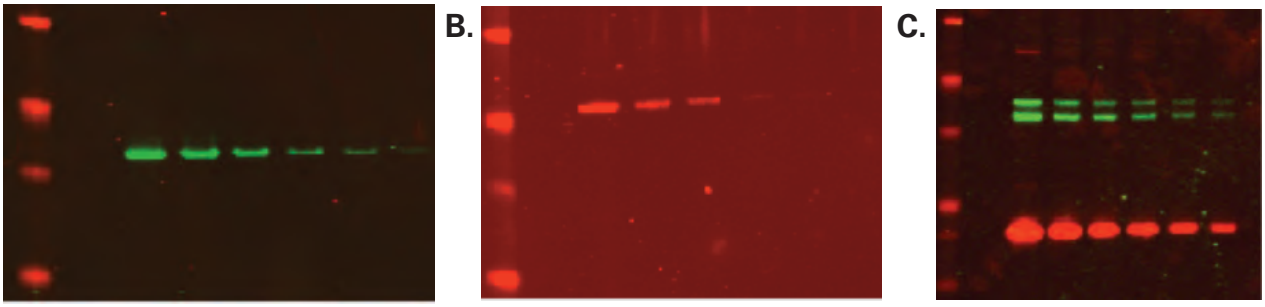

Figure 8. In each of the experiments shown above, serial dilutions of A431 cell lysate (protein concentration range 2.0 – 0.06 µg) were loaded onto polyacrylamide protein gels, electrophoresed and transferred to membrane using the iBlot Dry Blotting System (Invitrogen®). Detection was performed using 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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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.

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Published May 2009. Revised August 2011 and October 2011. The most recent version of this Technical Note is posted at http://biosupport.licor.com/support

Table of Contents

I. INTRODUCTION

The independent channels of the LI-COR® MPX (Multiplex) Blotter facilitate the ability to optimize blocking buffer, primary antibody dilution, and secondary antibody dilution in a single Western blot. Western blotting procedures that generate a blot of 7.0 × 8.5 cm are easily adaptable to the MPX format. The process fits into any laboratory's standard Western blot work flow. Both self-poured and pre-cast gels can be used to generate blots. Run electrophoresis and transfer to standard nitrocellulose or PVDF membrane under standard conditions. Clamp the blot into the MPX Blotter, which creates up to 24 independent channels. The range of usable channels per sample is relative to comb size. For Western Blot optimization, a single-well gel is all that is needed. For this application, any detection method can be used, including near infrared and chemiluminescence. The following is a general guideline for use with the Odyssey® Infrared Imaging System.

II. SUGGESTED MATERIALS

	Reagent/Supply	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
Blotting and Transfer	10X Tris Glycine (liquid or powder)	928-40010 or 928-40012
	Odyssey Nitrocellulose (7 x 8.5 cm or roll)	926-31090 or 926-31092
MPX Detection	LI-COR Blocking Buffer *Sample Pack:* • Odyssey Blocking Buffer • Casein Blocking Buffer	927-40050
	Commercial Milk Blocking Buffer	
	IRDye® Labeled Secondary Antibodies	Multiple P/Ns
	10X PBS (liquid or powder)	928-40018 or 928-40020
	MPX Membrane Cushion	921-00120
Imaging	Odyssey Infrared Imaging System	

III. GEL ELECTROPHORESIS & TRANSFER

- **Gel Preparation**

  There are a wide variety of gel matrices that are compatible with the MPX Blotter detection system. LI-COR® provides a solution for pouring gels with the NEXT GEL System (Amresco). The gel matrix can be used in your gel casting system with a single-well comb such as Bio-Rad (mini PROTEAN Comb, prep/2-D well, P/N 165-3361 1.0 mm, or P/N 165-3367, 1.5 mm) or from a vendor of the user's choice. Alternatively, pre-cast gels can be purchased and used. See Table 1 for a list of pre-cast gels available, and the number of usable ports compatible 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 can be used:

  Dilute the sample 1:4 in 4X Protein Sample Loading Buffer (LI-COR P/N 928-40004) with β-Mercaptoethanol. See pack insert at *www.licor.com/bio/reagents/buffers.jsp* for detailed instructions. 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 Molecular Weight Markers (LI-COR P/N 928-40000) is the recommended marker choice.

- **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. Once electrophoresis is complete, transfer proteins to Odyssey® Nitrocellulose Membrane (LI-COR® P/N 926-31092 or 926-31090) 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 in Figure 1. The marks help align the membrane once it is placed on the MPX Blotter. Allow the membrane to dry a minimum of one hour before proceeding with detection.
  Important: Ink from most pens will fluoresce on the Odyssey Imager.

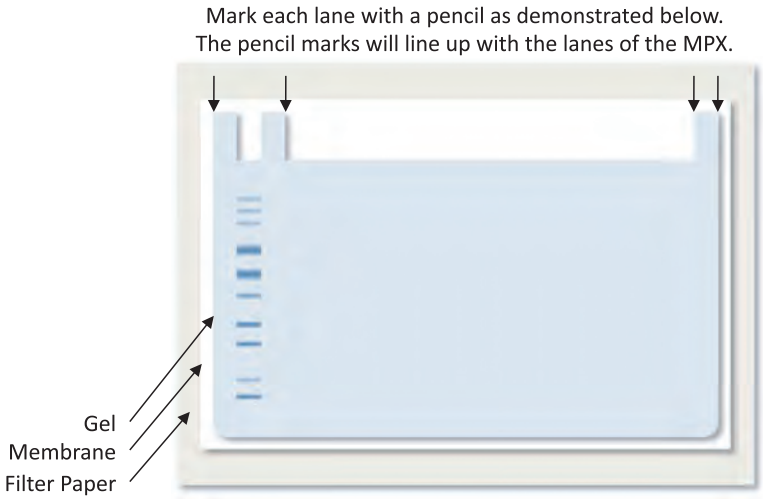

Figure 1. Diagram showing how to effectively mark the membrane for alignment into the MPX Blotter.

IV. MEMBRANE BLOCKING

- **Membrane Preparation**
  Cut the membrane into three individual blots as shown in Figure 2. Pre-wet membranes in PBS before proceeding with blocking.

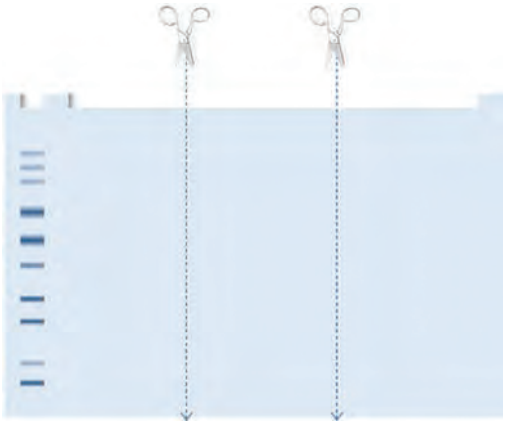

Figure 2. Diagram showing how to cut the membrane into three individual blots for blocking buffer optimization.

- **Blocking**
  Place the membranes into 3 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.

Example:

Membrane	Blocker
1	Odyssey® Blocking Buffer
2	Casein Blocking Buffer
3	Commercial Milk Blocking Buffer

V. ALIGNMENT IN MPX BLOTTER

For detailed instruction on use of the MPX Blotter, see MPX Blotter Multiplex Western Blotting Accessory User Guide at *http://biosupport.licor.com/docs/MPX_Blotter_UG_10644.pdf*

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

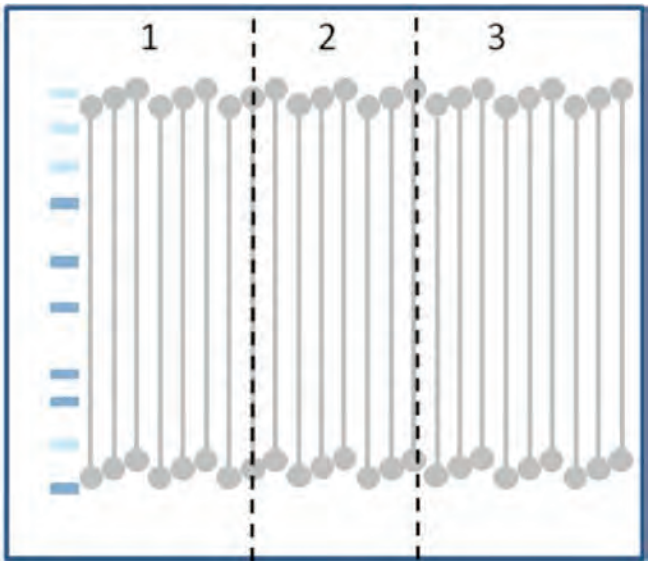

Figure 3. Diagram of how to place three individual blots into the MPX Blotter.

VI. PRIMARY & SECONDARY ANTIBODY APPLICATION

- **Primary Antibody Preparation**
  Two dilutions of primary antibody need to be made for each blocking buffer being evaluated. Dilutions should be chosen based on vendor recommendations. 500 µL of each dilution will be needed.

Example:

Membrane	Blocker	Primary Antibody Dilution	
1	Odyssey® Blocking Buffer	1:500	1:1,000
2	Casein Blocking Buffer	1:500	1:1,000
3	Commercial Milk Blocking Buffer	1:500	1:1,000

- **Primary Antibody Application**
  The primary antibody/blocker dilutions should be loaded into the MPX Blotter to correspond with the same blocked membrane. Apply 2 replicates of each primary antibody dilution; see Figure 4. Fill the unused channels with appropriate corresponding blocking buffer. Incubate for 1-4 hours at room temperature. Wash primary antibody from the channels thoroughly according to MPX Blotter manual instructions, using 1X PBS-T.

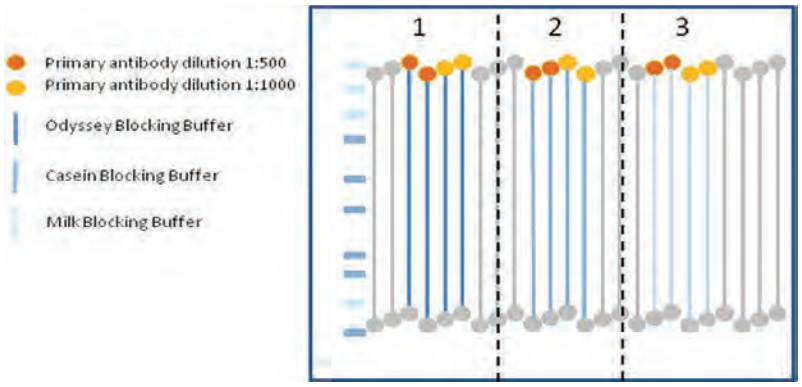

Figure 4. Diagram of how to place primary antibodies into the MPX Blotter.

- **Secondary Antibody Preparation**
  Two dilutions of secondary antibody need to be made for each blocking buffer being evaluated. Dilutions should be chosen based on vendor recommendations. For IRDye® conjugated secondary antibodies, we recommend 1:5,000 and 1:10,000 as a starting point. 500 µL of each antibody will be needed.

Example:

Membrane	Blocker	Secondary Antibody Dilution	
1	Odyssey Blocking Buffer	1:5,000	1:10,000
2	Casein Blocking Buffer	1:5,000	1:10,000
3	Commercial Milk Blocking Buffer	1:5,000	1:10,000

- **Secondary Antibody Application**
  The secondary antibody/blocker dilutions should be loaded into the MPX Blotter to correspond with the same blocked membrane. Add the secondary antibody dilutions to the primary antibody channels; see Figure 5. Fill the unused channels with the appropriate corresponding blocking buffer. Incubate 1 hour at room temperature. Wash secondary antibody from the channels thoroughly using 1X PBS-T, according to MPX Blotter manual instructions.

- Blots can be removed from the MPX Blotter and washed in 1X PBS-T for 5 minutes, followed by a 1X PBS rinse.

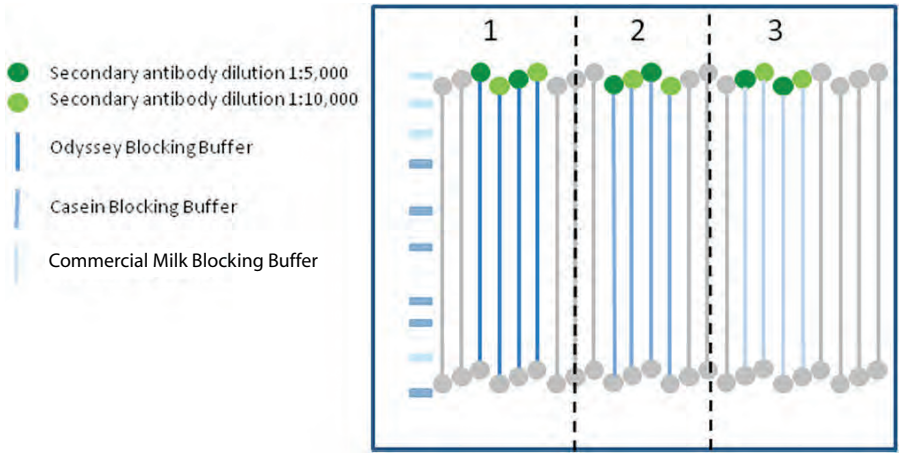

Figure 5. Diagram of how to place secondary antibodies into the MPX Blotter.

VII. IMAGING

Membranes can be imaged immediately. Use standard Western blot imaging settings on any Odyssey® Imaging System. Example data is shown in Figure 6.

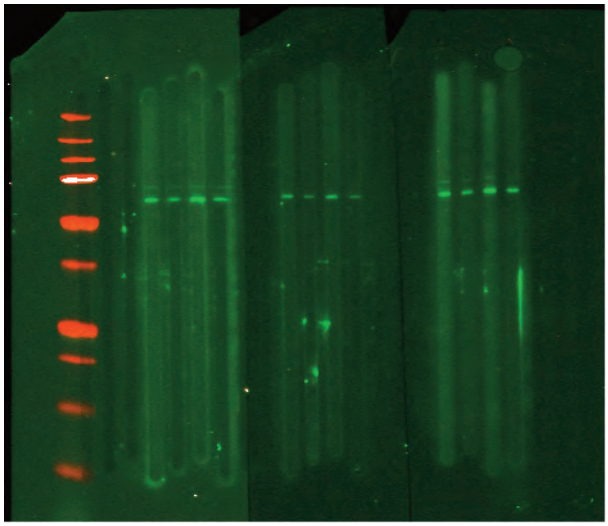

Figure 6. Akt antibody optimization using the MPX Blotter procedure.

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Doc # 988-12587
1011

Odyssey Western Blot Blocker Optimization

Developed for:

Aerius and Odyssey® Family of Imagers

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

Part Numbers:

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

LI-COR®

Published February 2009. Revised December 2010, October 2011, January 2012, and July 2013. The most recent version of this document is posted at http://biosupport.licor.com/support

Table of Contents

I. Required Reagents

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

- SDS (when using Immobilon-FL PVDF membrane)

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

II. Gel Preparation for Blocker Optimization

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

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

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

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

III. Western Blocker Optimization Method

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

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

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

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

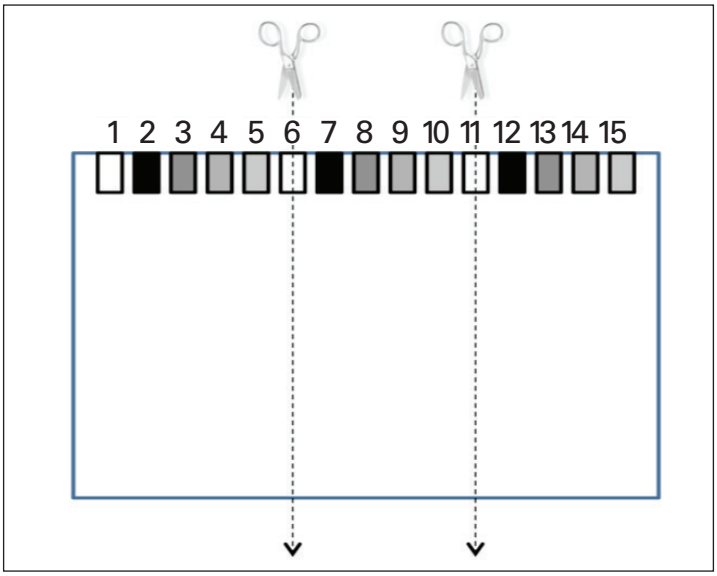

Figure 1. Cut Western blot along the Marker lanes into three individual optimization blots.

After cutting membrane, perform the following steps:

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

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

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

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

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

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

    * *Incubation times vary for different primary antibodies.*

5. Wash membranes:

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

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

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

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

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

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

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

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

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

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

Tips

- Follow the protocol carefully.

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

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Doc# 988-13828
07/13

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

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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.

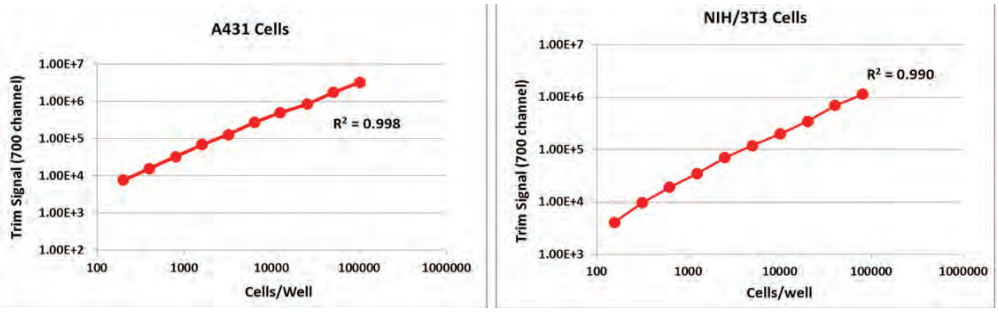

Figure 1. Linear Relationship between Fluorescence and Cell Number. Two-fold serial dilutions of A431 or NIH/3T3 cells were plated in 96-well plate. Cells were fixed, permeabilized, stained with CellTag 700 Stain (0.2 µM), and detected with Odyssey Classic Infrared Imaging System (Resolution: 169 µm; 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).

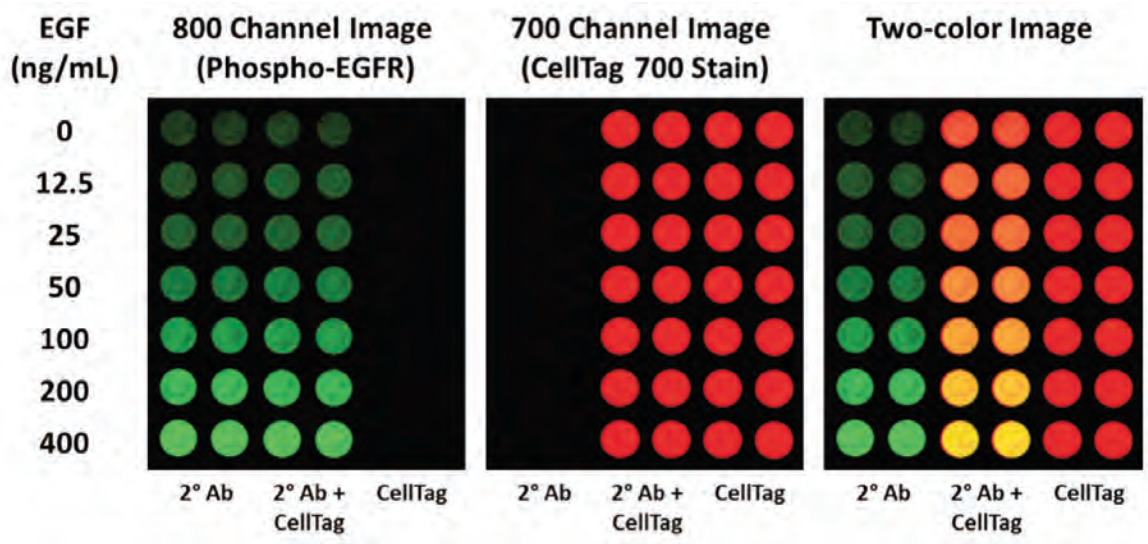

Figure 2. In-Cell Western Assay with CellTag 700 Stain in EGF-stimulated A431 Cells. EGF-stimulated A431cells were fixed and permeabilized. Phosphorylated EGFR was measured using rabbit anti-phospho-EGFR primary antibody followed by detection with IRDye® 800CW Goat anti-Rabbit IgG (LI-COR P/N 926-32211). 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.

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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.

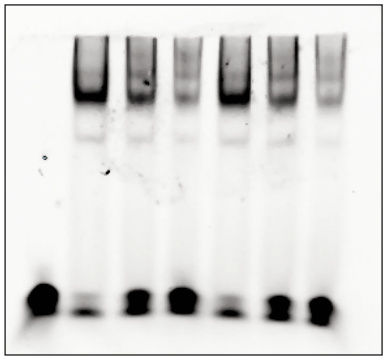
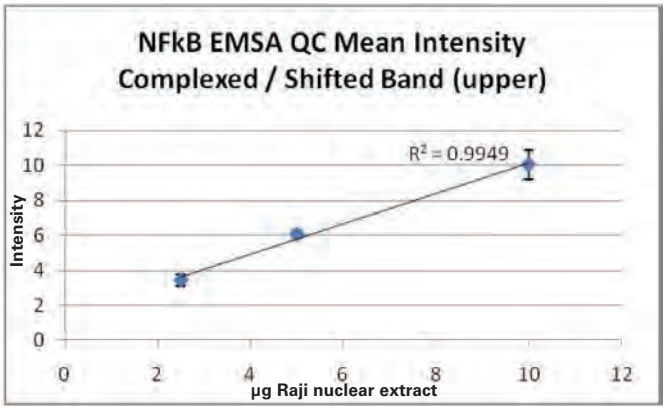


Figure 2. The uppermost shifted band in Lanes 2-7 of Figure 1 was analyzed to determine the level of NFkB binding to the IRDye 700 NFκB oligonucleotides.



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).

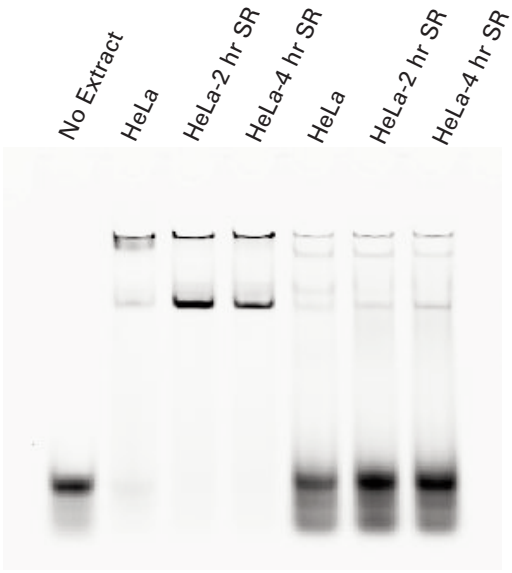


Figure 3. AP-1 EMSA using IRDye 700 end-labeled oligonucleotide duplex.
It is common to use unlabeled DNA duplex to determine binding specificity. Excess unlabeled DNA is added to the binding reaction; therefore, it competes with the labeled DNA for binding sites. If competition eliminates labeled DNA 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.

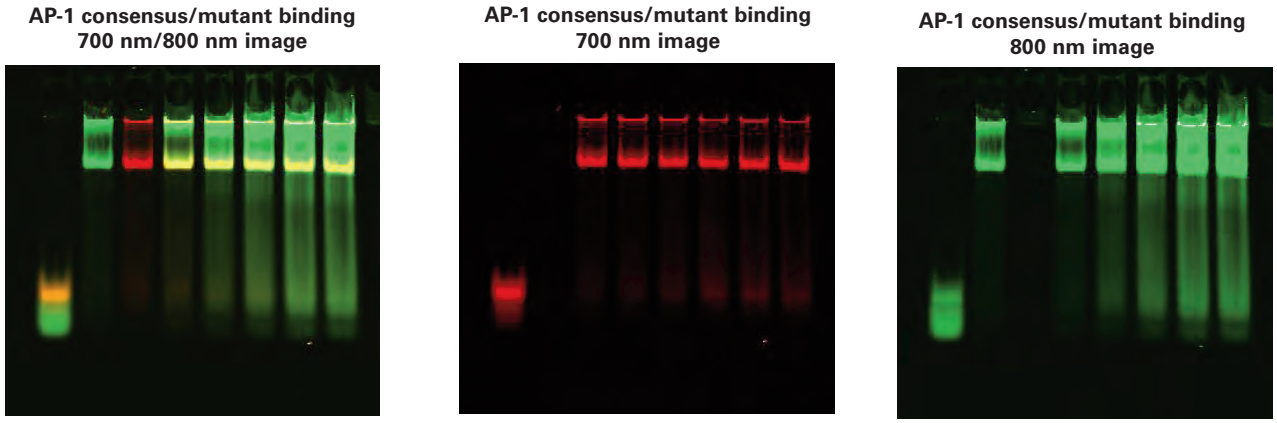


Figure 4. AP-1 EMSA using 2.5 µg HeLa 4-hour serum response nuclear extract to demonstrate binding specificity of AP-1 consensus DNA duplex. Binding specificity determination using Odyssey® two-color imaging.

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;

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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

LI-COR®
Experience Excellence

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

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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.

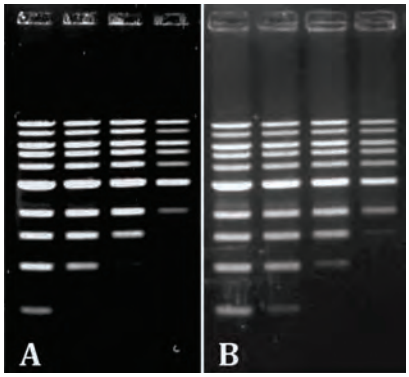

Figure 1. Two-fold dilutions of 1 kb ladder, from 1 µg to 0.125 µg, separated on a 1.2% agarose gel at 8V/cm in 1X TAE buffer for 1 hour. Panel A is the image of the gel obtained from the Odyssey Infrared Imaging System using an intensity of 5.0, gel face down. Panel B is the image of the gel acquired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

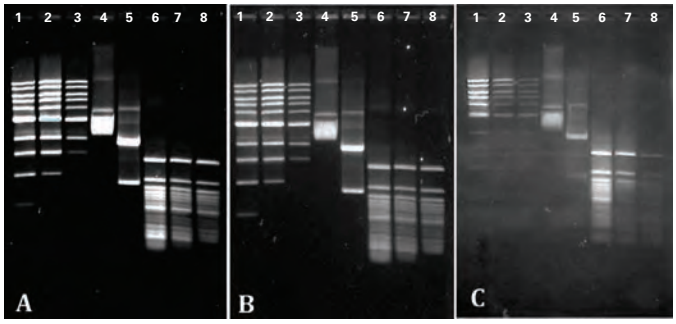

Figure 2. A 1.2% agarose gel was imaged using the Odyssey® Infrared Imaging System (panel A), Odyssey Fc Imaging System (panel B) or a UV transilluminator and the image captured using Polaroid 667 film (panel C). Lane 1) 1 µg 1 kb ladder; Lane 2) 0.5 µg 1 kb ladder; Lane 3) 0.25 µg 1 kb ladder; Lane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

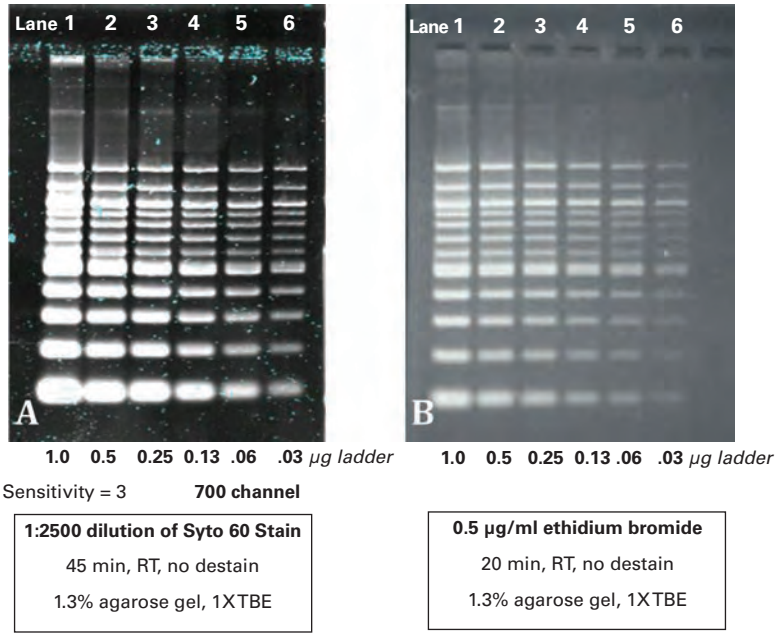

Figure 3. Panel A: Image of a Syto 60 stained gel using the Odyssey Infrared Imaging System. Panel B: Image of ethidium bromide-stained gel using a Polaroid camera.

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.

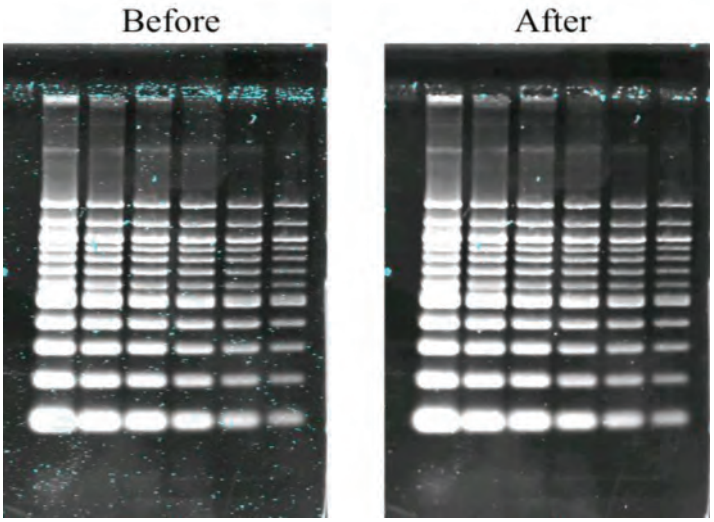

Figure 4. Image of agarose gel showing before and after using Odyssey Infrared Imaging System software's "FILTER" then "Noise Removal" function.

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.

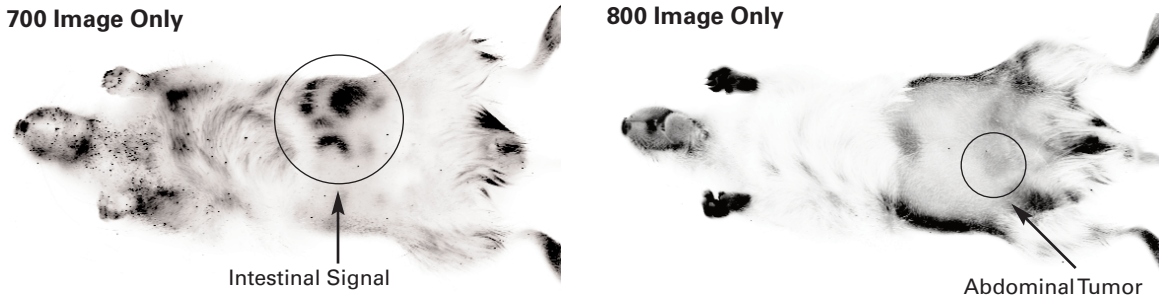


Figure 2. A typical mouse scan on the Odyssey Imager where an abdominal tumor was present. Scan parameters include resolution = 169 µm; quality = medium; focus offset = 2.0; intensity values = L1 for 700 channel and L3 for 800 channel.

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).

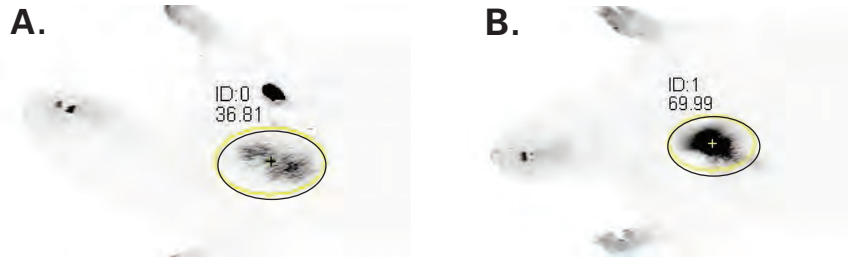
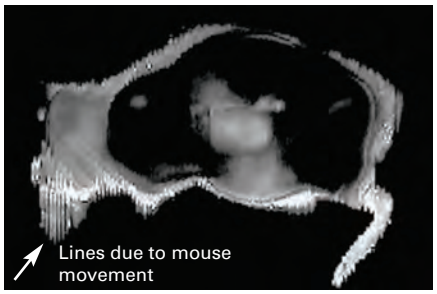


Figure 3. Signal from IRDye 800CW in the thoracic cavity when imaged on the Odyssey system before (Panel A) and after (Panel B) shaving.

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.

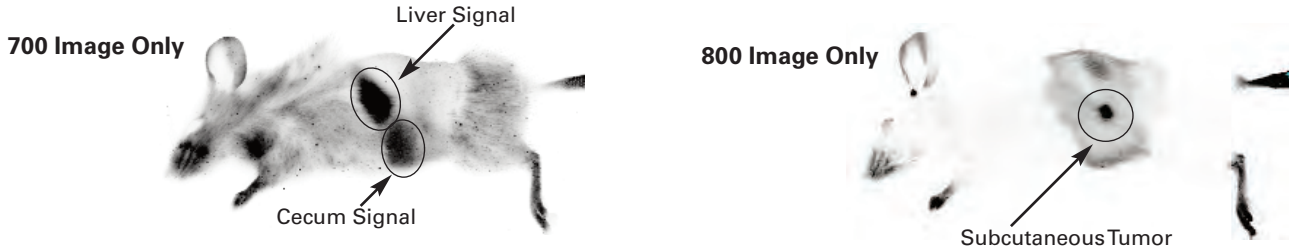
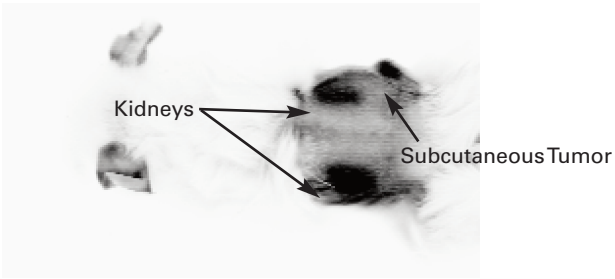


Figure 4. Liver and cecum signal present in the 700 nm channel and subcutaneous tumor (800 nm channel).

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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