Near-Infrared (NIR) Western Blot Detection (nitrocellulose membrane)

LI-COR Biosciences

Abstract

Infrared fluorescence detection with Odyssey Family Imaging Systems provides a quantitative two-color detection method for Western blots.

This protocol is designed to help you achieve success with NIR Western blot detection methods, using the **nitrocellulose** membrane.

Read the entire protocol carefully before beginning your optimization experiments.

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Guidelines

Developed for:

Odyssey® Classic,

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

I. Required Reagents

- Blotted nitrocellulose (LI-COR, P/N 926-31090/926-31092) or Immobilon®-FL PVDF mem- brane (LI-COR, P/N 926-31099 or 926-31100)

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

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

II. Quick Start Hints and Tips

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

1. Store the IRDye secondary antibody vial in darkness at 4 °C. Minimize exposure to light and

take care not to introduce contamination into the vial. Dilute immediately prior to use. If particulates are seen in the antibody solution, centrifuge before use.

2. The best transfer conditions, membrane, and blocking agent for experiments will vary, depending on the antigen and antibody.
3. Do not write on blot with a pen or Sharpie® marker. Ink from most pens and markers will fluoresce in the 700 nm channel of all Odyssey® Family Imaging Systems. The ink may wash off and re-deposit elsewhere on the membrane, causing increased background. Use a pencil to mark membranes. Ink from the Odyssey Pen does not fluoresce and can be used to mark nitrocellulose membranes. If the Odyssey Pen is used for PVDF membranes, the ink will dissolve and wash off when the blot is wetted in methanol.
4. Let the membrane dry after transfer for 1 hour or overnight, to maximize protein retention on the membrane.
5. Handle blot with clean forceps only.
6. Before using forceps, incubation trays, and the Odyssey scanning surface or sample tray (if applicable), clean with 100% methanol to remove any residual dye signal from previous use. Rinse with a small volume of distilled water, followed by isopropanol. Dry with a lint- free wipe.
7. When processing Western blots, do not use dishes/boxes that have ever been used for Coomassie staining. The Odyssey imagers are very sensitive to Coomassie (which is a strongly-fluorescent dye), and use of dishes with small traces of Coomassie will add a tremendous amount of background in the 700 nm channel. (Maintain the same buffer system throughout the Western blot process. For example, if you block your blot in Odyssey Blocking Buffer (PBS), use PBS-based buffers throughout the protocol.)
8. Do not include detergents during the blocking step.
9. For 1-color blots, use IRDye® 800CW secondary antibody for detection of the protein for best sensitivity.
10. For 2-color blots:

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

11. If you are using PVDF, add 0.01% SDS to the diluted secondary antibody. Do not add SDS if using nitrocellulose membrane.
12. Incubate with secondary antibodies in the dark for one hour with gentle shaking. The incubation box can be covered with aluminum foil.

III. Western Blot Detection Methods

This protocol is designed to help you achieve success with NIR Western blot detection methods.

Read the entire protocol carefully before beginning your optimization experiments.

Membrane Guidelines

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

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

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

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

See 'STEPS' for protocol

IV. Guidelines for Two-Color Detection

Two different antigens can be detected simultaneously on the same blot using IRDye® secondary antibodies. When performing a two-color blot, use the standard Western blot protocol with the following modifications:

- Combine the two primary antibodies in the diluted antibody solution (Step 3). Incubate simultaneously with membrane (Step 4, Section III). The primary antibodies must be from two different host species.

- Combine the two IRDye® secondary antibodies in the diluted antibody solution (Step 14). Incubate simultaneously with membrane (Step 15).

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

- The two primary antibodies must be derived from different host species so that they can be

discriminated by secondary antibodies of different specificities (for example, primary antibodies from rabbit and mouse will be discriminated by anti-rabbit IgG and anti-mouse IgG secondary antibodies, respectively).

- If the two primary antibodies are mouse monoclonals from different IgG subclasses (IgG1, IgG2a, or IgG2b), IRDye Subclass-Specific secondary antibodies can be used for multiplex detection. The same subclasses cannot be combined in a two-color Western blot (for example, two IgG1 primary antibodies). For details, refer to *Western Blot and In-Cell Western™ Assay Detection Using IRDye Subclass-Specific Antibodies* (www.licor.com/subclass)

- Anti-Goat secondary antibodies cannot be multiplexed with Goat-derived secondary antibodies (Example: Donkey anti-Goat and Goat anti-Rabbit). The secondary antibodies will cross-react.

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

- In general, it is recommended that the IRDye® 800CW secondary antibody (800 nm channel) be used to detect the lower-abundance protein target and IRDye 680RD secondary antibody (700 nm) to detect the more abundant protein.

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

V. Adapting Western Blot Protocols for Odyssey® Imaging Systems

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

Primary Antibody Concentration

Primary antibodies vary widely in quality, affinity, and concentration. The correct working range for antibody dilution depends on the characteristics of the primary antibody and the amount of target antigen to detect. Start with the vendor’s dilution recommendation for Western blotting or with the dilution normally used for chemiluminescent detection.

Secondary Antibody Concentration

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

Detergent Concentration

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

Tween® 20:

- Blocking buffer – do not add Tween 20 during blocking.
- Diluted primary and secondary antibodies should contain Tween 20.

  Use a final concentration of 0.1 - 0.2% Tween 20 for PVDF or nitrocellulose membranes.
- Wash solutions should contain 0.1% Tween 20.

SDS:

When using PVDF membrane, addition of SDS will dramatically reduce overall membrane background in the 700 nm channel. It is critical to use only a very small amount, because SDS is an ionic detergent and can disrupt antibody-antigen interactions if too much is present at any time during the protocol.

SDS **should not** be used with nitrocellulose membranes.

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

- SDS is optional when using IRDye 680RD antibodies with PVDF membrane, but is essential when using IRDye 680LT antibodies with PVDF.

- Wash solutions **should not** contain SDS.

Materials

Blotted nitrocellulose 926-31090/926-31092 by LI-COR

Immobilon®-FL PVDF membrane 926-31099 926-31100 by LI-COR

Odyssey® Blocking Buffer (PBS) 927-40000 927-40100 by LI-COR

Odyssey Blocking Buffer (TBS) 927-50000 927-50100 by LI-COR

IRDye® 800CW, 680RD, or 680LT by LI-COR

Protocol

Preparing the membrane

Step 1.

After membrane transfer and you have removed the membrane from the transfer stack, allow the membrane to air dry. This takes about 1 hour at room temperature, depending on lab conditions.

DURATION

01:00:00

Preparing the membrane

Step 2.

After you have air dried, the membrane, wet it in 1X PBS or TBS for 2 minutes, or until fully hydrated (using the appropriate buffer system).

DURATION

00:02:00

Preparing the membrane

Step 3.

Place membrane in incubation box and block the membrane in Odyssey Blocking Buffer (PBS or TBS) for 1 hour with gentle shaking. Be sure to use sufficient blocking buffer to cover the membrane (a minimum of 0.4 mL/cm^2 is suggested).

DURATION

01:00:00

NOTES

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For a detailed Western blot blocker optimization protocol, see Odyssey Western Blot Blocker Optimization (www.licor.com/optimize)

Primary antibody incuba

Step 4.

Primary antibody diluent: Odyssey Blocking Buffer (PBS or TBS) + 0.2% Tween® 20 (final concentration).

Primary antibody incubation

Step 5.

Dilute primary antibody in Odyssey Blocking Buffer + 0.2% Tween 20, using the vendor's recommended dilution for Western blot applications. Dilutions typically range from 1:200 - 1:5,000, depending on the primary antibody.

Primary antibody incubation

Step 6.

Use enough antibody solution to completely cover the membrane.

NOTES

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

Primary antibody incubation

Step 7.

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

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*Optimal incubation times vary for different primary antibodies.

Primary antibody incubation

Step 8.

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

Wash membranes

Step 9.

Pour off primary antibody solution. (wash 1/4)

Wash membranes

Step 10.

Rinse membrane with appropriate buffer – 1X TBS-T (0.1% Tween® 20) or 1X PBS-T (0.1% Tween 20). (wash 1/4)

Wash membranes

Step 11.

Cover blot with 1X TBS-T (0.1% Tween 20) or 1X PBS-T (0.1% Tween 20). (wash 1/4)

Wash membranes

Step 12.

Shake vigorously on platform shaker at room temperature for 5 minutes. (wash 1/4)

DURATION

00:05:00

Wash membranes

Step 13.

Pour off wash solution. (wash 1/4)

Wash membranes

Step 14.

Rinse membrane with appropriate buffer – 1X TBS-T (0.1% Tween® 20) or 1X PBS-T (0.1% Tween 20). (wash 1/4)

Wash membranes

Step 15.

Cover blot with 1X TBS-T (0.1% Tween 20) or 1X PBS-T (0.1% Tween 20). (wash 2/4)

Wash membranes

Step 16.

Shake vigorously on platform shaker at room temperature for 5 minutes. (wash 2/4)

DURATION

00:05:00

Wash membranes

Step 17.

Pour off wash solution. (wash 2/4)

Wash membranes

Step 18.

Rinse membrane with appropriate buffer – 1X TBS-T (0.1% Tween® 20) or 1X PBS-T (0.1% Tween 20). (wash 3/4)

Wash membranes

Step 19.

Cover blot with 1X TBS-T (0.1% Tween 20) or 1X PBS-T (0.1% Tween 20). (wash 3/4)

Wash membranes

Step 20.

Shake vigorously on platform shaker at room temperature for 5 minutes. (wash 3/4)

DURATION

00:05:00

Wash membranes

Step 21.

Pour off wash solution. (wash 3/4)

Wash membranes

Step 22.

Rinse membrane with appropriate buffer – 1X TBS-T (0.1% Tween® 20) or 1X PBS-T (0.1% Tween 20). (wash 4/4)

Wash membranes

Step 23.

Cover blot with 1X TBS-T (0.1% Tween 20) or 1X PBS-T (0.1% Tween 20). (wash 4/4)

Wash membranes

Step 24.

Shake vigorously on platform shaker at room temperature for 5 minutes. (wash 4/4)

DURATION

00:05:00

Wash membranes

Step 25.

Pour off wash solution. (wash 4/4)

IRDye® secondary antibody incubation

Step 26.

Secondary antibody diluent:

To blocking buffer, add Tween 20 to a final concentration of 0.2%. Do not add SDS.

NOTES

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NOTE: A suggested dilution range for secondary antibodies is typically 1:10,000 to 1:40,000. Recommended dilutions can be found on the secondary antibody pack insert. Use 1:20,000 as a suggested starting point if using LI-COR IRDye secondary antibodies.

IRDye® secondary antibody incubation

Step 27.

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

DURATION

01:00:00

Wash membranes

Step 28.

Protect membrane from light during washes.

Pour off secondary antibody solution. (wash 1/4)

Wash membranes

Step 29.

Rinse membrane with appropriate buffer – 1X TBS-T (0.1% Tween® 20) or 1X PBS-T (0.1% Tween 20). (wash 1/4)

Wash membranes

Step 30.

Cover blot with 1X TBS-T (0.1% Tween 20) or 1X PBS-T (0.1% Tween 20). (wash 1/4)

Wash membranes

Step 31.

Shake vigorously on platform shaker at room temperature for 5 minutes. (wash 1/4)

DURATION

00:05:00

Wash membranes

Step 32.

Pour off wash solution. (wash 1/4)

Wash membranes

Step 33.

Rinse membrane with appropriate buffer – 1X TBS-T (0.1% Tween® 20) or 1X PBS-T (0.1% Tween 20). (wash 2/4)

Wash membranes

Step 34.

Cover blot with 1X TBS-T (0.1% Tween 20) or 1X PBS-T (0.1% Tween 20). (wash 2/4)

Wash membranes

Step 35.

Shake vigorously on platform shaker at room temperature for 5 minutes. (wash 2/)

DURATION

00:05:00

Wash membranes

Step 36.

Pour off wash solution. (wash 2/4)

Wash membranes

Step 37.

Rinse membrane with appropriate buffer – 1X TBS-T (0.1% Tween® 20) or 1X PBS-T (0.1% Tween 20). (wash 3/4)

Wash membranes

Step 38.

Cover blot with 1X TBS-T (0.1% Tween 20) or 1X PBS-T (0.1% Tween 20). (wash 3/4)

Wash membranes

Step 39.

Shake vigorously on platform shaker at room temperature for 5 minutes. (wash 3/4)

DURATION

00:05:00

Wash membranes

Step 40.

Pour off wash solution. (wash 3/4)

Wash membranes

Step 41.

Rinse membrane with appropriate buffer – 1X TBS-T (0.1% Tween® 20) or 1X PBS-T (0.1% Tween 20). (wash 4/4)

Wash membranes

Step 42.

Cover blot with 1X TBS-T (0.1% Tween 20) or 1X PBS-T (0.1% Tween 20). (wash 4/4)

Wash membranes

Step 43.

Shake vigorously on platform shaker at room temperature for 5 minutes. (wash 4/4)

DURATION

00:05:00

Wash membranes

Step 44.

Pour off wash solution. (wash 4/4)

Step 45.

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

Image with an Odyssey® Family Imaging System

Step 46.

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

Image with an Odyssey® Family Imaging System

Step 47.

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

Dried blots kept in the dark can be imaged up to 3 years later without loss of signal.

Image with an Odyssey® Family Imaging System

Step 48.

If signal on membrane is too strong or too weak, adjust the imaging parameters to optimize the image.

- Odyssey Classic: Re-image the membrane at a lower or higher scan intensity setting, respectively.
- Odyssey Fc: Adjust image acquisition time.
- Odyssey CLx: Use the AutoScan function to improve the dynamic range of the image.