

Protocol

Near-Infrared Western Blot Detection



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Visit us on protocols.io! Explore an interactive version of this protocol at bit.ly/NIRWesternDetection_Nitro or bit.ly/NIRWesternDetection_PVDF.

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I. Introduction

The Western blot detection protocol covered in this document begins **after the transfer step** and continues to the **imaging step**.

Hints are provided before the protocol to help you get started. Guidelines for two-color Western blot detection and adapting your protocol for an Odyssey® Imager are provided after the protocol.

II. Required Reagents

You can use TBS-based or PBS-based buffers with this protocol.

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

- Blotted nitrocellulose (LI-COR, PN 926-31090 or 926-31092) or Immobilon®-FL PVDF membrane (LI-COR, PN 926-31099 or 926-31100)
- Intercept® Blocking Buffer (PBS or TBS) or Intercept Protein-Free Blocking Buffer (PBS or TBS)
- Primary antibodies
- IRDye® 800CW, 680RD, or 680LT secondary antibodies
- Tween® 20
- PBS or TBS
- Methanol for wetting PVDF
- Ultrapure water
- SDS

III. Quick Start Hints and Tips

Infrared fluorescence detection with Odyssey® Imaging Systems provides a quantitative two-color detection method for Western blots. Following, you will find some basic tips to help you get started.

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

Before Beginning This Protocol

- Let the membrane dry after transfer for 1 hour or overnight, to maximize protein retention on the membrane.
- For weak or low abundance targets, 800 nm channel detection is recommended for best results.
- Be sure to keep your buffer system consistent throughout the protocol for blocking, antibody dilutions, and washes. For example, if you use a TBS-based buffer system, choose Intercept[®] (TBS) Blocking Buffer. If you use a PBS-based buffer system, choose Intercept[®] (PBS) Blocking Buffer.

Handling Antibodies

- 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.
- Incubate with secondary antibodies in the dark for one hour with gentle shaking. The incubation box can be covered with aluminum foil.
- Dilute immediately prior to use. If particulates are seen in the antibody solution, centrifuge before use.

Handling Membranes

- Do not write on membranes with regular ink pens or markers, because the ink will fluoresce on Odyssey Imaging Systems. You can write on nitrocellulose membranes with pencil or the Odyssey Pen (PN 926-71804). Use only a pencil to write on PVDF membranes, because the ink from the Odyssey Pen will dissolve in the methanol used to wet the PVDF membrane.
- Only handle membranes by the edges with clean forceps. Be careful not to touch the membrane with your hands or gloves.
- A low-background membrane is essential for NIR Western blot success. Background can result from membrane autofluorescence or from non-specific binding of antibodies. Polyvinylidene fluoride (PVDF) and nitrocellulose membranes are typically used for Western blotting applications. There are many brands and vendors for both types of membrane.

Test Membrane Background

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

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

Using Detergents

- It is generally best to not add detergent in this protocol before the blocking step is complete.
- If you are using PVDF, add 0.01% SDS to the diluted secondary antibody.
- Do not add SDS if using a nitrocellulose membrane.

Keeping Equipment Clean

- 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.
- 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.

IV. Western Blot Detection Protocol

For the best results, read the entire protocol carefully before beginning your experiments.

- To learn more about optimizing your Western blots, read *Good Westerns Gone Bad* (licor.com/GWGBIR).
- To learn more about gel transfer, read *Protein Electrotransfer Methods and the Odyssey Infrared Imaging Systems* (licor.com/proteintransfer).

Step 1. Wet Membrane

For Nitrocellulose Membranes

Wet in 1X PBS or TBS for 2 minutes or until fully hydrated (using the appropriate buffer system).

For Immobilon®-FL PVDF Membranes

1. Wet for 1 minute in 100% methanol.
2. Rinse with ultra pure water.

3. Wet in 1X PBS or TBS for 2 minutes (using the appropriate buffer system).

Step 2. Block the Membrane

Place the membrane in an incubation box and block the membrane with Intercept Blocking Buffer for 1 hour at room temperature with gentle shaking.

Be sure to use sufficient blocking buffer to cover the membrane (a minimum of 0.4 mL/cm² is suggested).

See the *Western Blot Blocker Optimization Protocol* ([licor.com/optimize](https://www.licor.com/optimize)) for more information.

Step 3. Dilute Primary Antibody

1. Prepare the primary antibody diluent: Add Tween[®] 20 to Intercept Blocking Buffer for a final concentration of 0.2% Tween 20.
2. Dilute primary antibody in antibody diluent using the vendor's recommendations.
 - Depending on the primary antibody, dilutions may range from 1:200 – 1:5,000.
 - Use enough antibody solution to completely cover the membrane.

See *One Blot Western Optimization Using the MPX™ Blotting System* ([licor.com/oneblot](https://www.licor.com/oneblot)) for more information.

Step 4. Incubate Blot in Diluted Primary Antibody

Incubate the blot for 1 - 4 hours at room temperature or overnight at 4 °C with gentle shaking.

Note: Optimal incubation times vary for different primary antibodies.

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

Step 5. Wash Membrane

1. Carefully pour off primary antibody solution.
2. Rinse the membrane with 1X TBS-T (0.1% Tween 20) or 1X PBS-T (0.1% Tween 20).
3. Cover blot with 1X TBS-T or 1X PBS-T.

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

Step 6. Dilute Secondary Antibody

Dilute secondary antibody in the appropriate diluent for the membrane you're using. For IRDye® 800CW secondary antibodies and IRDye 680RD secondary antibodies, the recommended starting dilution is 1:20,000.

Secondary Antibody Diluent for Nitrocellulose Membranes

Add Tween® 20 to a final concentration of 0.2% in Intercept Blocking Buffer. Do **not** add SDS.

Secondary Antibody Diluent for Immobilon®-FL PVDF

Add Tween 20 to a final concentration of 0.2% and SDS to a final concentration of 0.01 - 0.02% in Intercept Blocking Buffer.

Step 7. Incubate Blot in Secondary Antibody

Note: Protect membrane from light during incubation.

- Incubate blot in diluted secondary antibody for **1 hour** at room temperature with gentle shaking.
- Do not incubate for longer than **1 hour**, because the background may increase.

Step 8. Wash Membrane

Note: Protect membrane from light during washes.

1. Carefully pour off secondary antibody solution.
2. Rinse the membrane with 1X TBS-T or 1X PBS-T.
3. Cover blot with 1X TBS-T or 1X PBS-T.
4. Shake vigorously on platform shaker at room temperature for 5 minutes.

5. Pour off wash solution.
6. Repeat 3 additional times.

Step 9. Rinse Membrane

Rinse the membrane with 1X PBS or TBS to remove residual Tween 20.

- Store membrane at 4 °C protected from light. Store dry for several months.
- If you plan to strip and reprobe the Western blot, do not allow the completed Western blot to dry. The stripping process is less effective on Western blots that have been allowed to dry.

Step 10. Scan Membrane

Note: Protect the membrane from light prior to scanning.

Scan the membrane on an Odyssey Imaging System.

The membrane can be scanned wet or dry. Scanning the membrane dry can add signal intensity, but can also lead to increased background.

V. 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. Incubate simultaneously with the membrane. The primary antibodies must be from two different host species.
- Combine the two IRDye secondary antibodies in the diluted antibody solution.

Incubate simultaneously with the membrane.

Two-color detection requires careful selection of primary and secondary antibodies. A comprehensive course is available on the LambdaU Western Blot Education portal (lambdau.net) to help you select appropriate antibodies. The following guidelines provide information that 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, primaries from rabbit and mouse will be discriminated by anti-rabbit and anti-mouse secondary antibodies, respectively).
- If the two primary antibodies are mouse monoclonals from different IgG subclasses (IgG₁, IgG_{2a}, or IgG_{2b}), IRDye Subclass-Specific secondary antibodies can be used for multiplex detection. The same subclasses cannot be combined in a two-color Western blot (for example, two IgG₁ primary antibodies).

See *Western Blot and In-Cell Western™ Assay Detection Using IRDye Subclass-Specific Antibodies* (licor.com/subclass) for more information.

- 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.
- 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 your blot, particularly if the antigen is very abundant. If cross-reactivity is a problem, load less protein or reduce the amount of antibody.
- One secondary antibody must be labeled with IRDye® 680RD and the other with IRDye 800CW.

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.
- For best results, avoid using primary antibodies from mouse and rat together in a two-color experiment. The two species are so closely related it is not possible to completely adsorb away all cross-reactivity. If there is no other option but to use mouse and rat together, it is crucial to run single-color blots first with each individual antibody to be certain of expected band sizes.
- If possible, the two secondary antibodies should be derived from the same host species (for example, goat anti-mouse and goat anti-rabbit) to eliminate the chance of the secondary antibodies reacting against one another.

VI. Adapting Western Blot Protocols for Odyssey® Imagers

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. Parameters such as primary

antibody concentration, IRDye secondary antibody concentration, detergent concentration in diluted antibodies, and blocking buffer should be optimized.

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 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.

Tween® 20

- Blocking buffer – It is best to 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 and nitrocellulose membranes.

- Wash solutions should contain 0.1% Tween 20.

SDS

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

Important: SDS should not be used with nitrocellulose membranes.

- Do **not** add SDS to the blocking reagent during blocking.
- Diluted primary antibodies should **not** contain SDS.
- Wash solutions should **not** contain SDS.

- When using a PVDF membrane, your secondary antibody diluent should contain a final concentration of 0.01 – 0.02% SDS and 0.1 – 0.2% Tween® 20.



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