Protocol

Fluorescent Western Blot Detection

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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 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), Intercept Protein-Free Blocking Buffer (PBS or TBS)
- Intercept T20 Antibody Diluent
- Primary antibodies
- IRDye® 800CW, 680RD, or 680LT secondary antibodies or VRDye™ 490 secondary antibodies or VRDye™ 549 secondary antibodies

Note: The Odyssey M Imager is the only Odyssey Imager that can detect VRDye secondary antibodies.

- Tween® 20
- PBS or TBS
- Methanol for wetting PVDF
- Ultrapure water
- SDS
III. Quick Start Hints and Tips

Fuorescence detection with Odyssey Imagers provides a quantitative detection method for Western blots. The following section includes 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 to maximize protein retention on the membrane. They can be dried using any of the following options.

- Place the membrane on a piece of dry filter paper. Leave the membrane and filter paper on the benchtop for approximately 1 hour.
- Place the membrane on a piece of dry filter paper. Leave the membrane and filter paper in a 37 °C oven for approximately 10 minutes.
- Place the membrane between two pieces of dry filter paper, and place the membrane and filter papers into a protected place overnight. A drawer or a cabinet shelf may suffice.

For weak or low abundance targets, 800 nm channel detection is recommended for best results.

To use Revert™ 520 Total Protein Stain or Revert™ 700 Total Protein Stain for normalization, rewet the membrane (see "Wet Membrane" on the facing page) and see its pack insert for more information.

**Handling Antibodies**

- Store the IRDye® secondary antibody or VRDye™ 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.
- Incubate membranes with secondary antibodies in the dark for 1 hour with gentle shaking.
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 fluorescent Western blot success. Background can result from membrane autofluorescence, blocking buffer, or non-specific binding of antibodies.

Using Detergents

- Avoid adding detergent during the blocking step.

- Use Intercept T20 Antibody Diluent or blocking buffer with 0.2% Tween® 20 to the primary and secondary antibody solution.

- If you are using PVDF, add 0.01 – 0.02% 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 been used with any protein stain (e.g., Coomassie stain), as it may introduce background in the visible channels.

- Before using forceps, incubation trays, and the Odyssey Imager scanning surface or sample tray (if applicable), rinse with a small volume of distilled water, followed by 70% ethanol or isopropanol and 100% methanol (optional) to remove any residual dye signal from previous use. 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 TBS or PBS for 5 minutes or until fully hydrated (using the appropriate buffer system).

For Immobilon®-FL PVDF Membranes

1. Wet for 30 seconds in 100% methanol.
2. Wet in 1X TBS or PBS for 5 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](http://licor.com/optimize)) for more information.

**Step 3. Dilute Primary Antibody**

1. Use Intercept T20 Antibody Diluent as the primary antibody diluent or prepare your own by adding 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](http://licor.com/oneblot)) for more information on optimizing and validating antibodies.

**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 (0.2% Tween 20) or 1X PBS-T (0.2% 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 steps 3 to 5 for a total of 4 washes.

Step 6. Dilute Secondary Antibody

Dilute secondary antibody in the appropriate diluent for the membrane you’re using. For IRDye® 800CW secondary antibodies, IRDye® 680RD secondary antibodies, or IRDye® 680LT secondary antibodies, the recommended starting dilution is 1:20,000. For VRDye secondary antibodies, the recommended starting dilution is 1:10,000. The following includes recommendations on antibody dilution buffers.

Secondary Antibody Diluent for Nitrocellulose Membranes

Use Intercept T20 Antibody Diluent. Alternatively, 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

Use Intercept T20 Antibody Diluent and SDS. Alternatively, 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

- 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.

Note: Protect membrane from light during incubation.
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 steps 3 to 5 for a total of 4 washes.

Step 9. Rinse Membrane

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

- Membranes can be stored at 4 °C in TBS or PBS for short periods. Always protect membranes from light.
- Membranes can be stored dry at room temperature for prolonged storage. Always protect membranes from light.
- 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® Imager.

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 Multiplexed Detection

Multiple antigens can be detected simultaneously on the same blot using IRDye® secondary antibodies and VRDye™ secondary antibodies. When performing a blot, use the standard Western blot protocol with the following modifications:

- Combine the primary antibodies in the antibody diluent. Incubate simultaneously with the membrane. The primary antibodies must be from different host species or subclasses if the targets are close in molecular weight.

- Combine the IRDye secondary antibodies and VRDye secondary antibodies in the antibody diluent.

- Incubate simultaneously with the membrane.

Multiplexed detection requires careful selection of primary and secondary antibodies. A comprehensive course is available on the Lambda U® Western Blot Education portal (lambdau.net) to help you select appropriate antibodies. The following guidelines provide information that will help you successfully design multiplexed experiments:

- **The 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 (e.g., 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 (e.g., 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 (e.g., donkey anti-goat and goat anti-rabbit). The secondary antibodies will cross-react.

- Before combining primary antibodies in a multiplexed 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 secondary antibody, IRDye 680LT secondary antibody, or VRDye secondary antibodies. The other must be labeled with IRDye 800CW secondary antibody or VRDye secondary antibodies.
• It is generally 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. If using an instrument that can also detect visible fluorescence, use those channels to detect the internal loading control using Revert 520 Total Protein Stain (preferred) or VRDye 490 secondary antibodies in the 490 nm channel or VRDye 549 secondary antibodies in the 520 nm channel.

• Always use highly cross-adsorbed secondary antibodies for multiplexed 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 multiplexed 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 secondary antibodies should be derived from the same host species (e.g., donkey anti-mouse and donkey anti-goat) to eliminate the chance of the secondary antibodies reacting against one another.

• High abundant targets, such as housekeeping proteins, may perform better in the 490 or 520 channels. Consequently, their working range can be identified and combined with lower abundant targets in the 700 and 800 channels using VRDye secondary antibodies.

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 or VRDye™ 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 and VRDye 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**

- Avoid adding Tween® 20 during the blocking step.
- Diluted primary and secondary antibodies should contain Tween 20. Use Intercept T20 Antibody Diluent. Alternatively, use a final concentration of 0.2% Tween 20 for PVDF and nitrocellulose membranes.
- Wash solutions should contain 0.2% 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 Intercept T20 Antibody Diluent. If using Intercept T20 Antibody Diluent, add 0.01 – 0.02% SDS.