Components

Western Blotting Kit V RD (TBS/PVDF), PN 926-35010

- IRDye® 800CW Goat anti-Mouse Secondary Antibody (0.1 mg)
- IRDye 680RD Goat anti-Rabbit Secondary Antibody (0.1 mg)
- Intercept® (TBS) Blocking Buffer (500 mL)
- Millipore Immobilon®-FL PVDF Membrane (0.45 µm, 10 x 10 cm)

Western Blotting Kit VI RD (TBS/PVDF), PN 926-35011

- IRDye 800CW Goat anti-Rabbit Secondary Antibody (0.1 mg)
- IRDye 680RD Goat anti-Mouse Secondary Antibody (0.1 mg)
- Intercept (TBS) Blocking Buffer (500 mL)
- Millipore Immobilon-FL PVDF Membrane (0.45 µm, 10 x 10 cm)

Western Blotting Kit VII RD (TBS/NITRO), PN 926-35014

- IRDye 800CW Goat anti-Mouse Secondary Antibody (0.1 mg)
- IRDye 680RD Goat anti-Rabbit Secondary Antibody (0.1 mg)
- Intercept (TBS) Blocking Buffer (500 mL)
- Odyssey® Nitrocellulose Membrane (0.22 µm, 7 x 8.5 cm)

Western Blotting Kit VIII RD (TBS/NITRO), PN 926-35015

- IRDye 800CW Goat anti-Rabbit Secondary Antibody (0.1 mg)
- IRDye 680RD Goat anti-Mouse Secondary Antibody (0.1 mg)
- Intercept (TBS) Blocking Buffer (500 mL)
- Odyssey Nitrocellulose Membrane (0.22 µm, 7 x 8.5 cm)

Other Required Reagents

- Primary antibodies

  When using an Western Blotting Kit, the primary antibodies must be from Rabbit or Mouse host species.
- Tween® 20
- TBS buffer
- Methanol (when using Western Blotting Kit V or VI)
- SDS (when using Western Blotting Kit V or VI)
- Western Blot Incubation Box (appropriate for blot size)
  - Small (7.2 x 4.9 x 3.0 cm) (LI-COR, PN 929-97101, 929-97105, 929-97110)
  - Medium (8.9 x 6.5 x 2.8 cm) (LI-COR, PN 929-97201, 929-97205, 929-97210)
  - Large (11.5 x 8.8 x 2.8 cm (LI-COR, PN 929-97301, 929-97305, 929-97310)
  - X-Large (15.2 x 10.1 x 3.1 cm) (LI-COR, PN 929-97401, 929-97405, 929-97410)
- Odyssey Pen (LI-COR, PN 926-71804) (optional)

Western Blot Detection Protocol

Prepare your Western blot using standard blotting procedures and the membrane provided in your Western Blotting Kit. After transfer, allow the blot to dry for an hour before beginning the detection protocol. Dry blots can be stored between pieces of filter paper overnight at room temperature.

Note: *Only handle membranes by the edges with clean forceps. Be careful not to touch the membrane with your hands or gloves.*

Note: *Do not write on membranes with regular ink pens or markers, because the ink will fluoresce on Odyssey Imaging Systems. 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. You can write on nitrocellulose membranes with pencil or the Odyssey Pen (PN 926-71804).*

Incubation Box Volumes

Use the following volumes for blocking and washing steps.

- 5 mL for a Small Box.
- 10 mL for a Medium Box.
- 15 mL for a Large Box.
- 20 mL for an X-Large Box.

Step 1. Wet Membrane

For Nitrocellulose Membranes

Wet in 1X TBS for 5 minutes or until fully hydrated.

For Immobilon®-FL PVDF Membranes

1. Wet for 30 seconds in 100% methanol.
2. Wet in 1X TBS for 5 minutes.

Step 2. Block the Membrane

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

See the *Incubation Box Volumes* section for recommended volumes.

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 (see the *Incubation Box Volumes* section for recommended volumes).

   Depending on the primary antibody, dilutions may range from 1:200 – 1:5,000.

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).
3. Cover blot with 1X TBS-T (see the *Incubation Box Volumes* section for recommended volumes).
4. Shake vigorously on platform shaker at room temperature for 5 minutes.
5. Pour off wash solution.
6. Repeat 3 additional times.

Step 6. Reconstitute Secondary Antibody

1. Combine the contents of each vial of secondary antibody with 0.1 mL of sterile distilled water.
2. Gently mix by inverting.
3. Allow the solution to stand at room temperature for at least 30 minutes before use.

   Note: *If the solution is not completely transparent after standing at room temperature, centrifuge the solution.*

When stored as recommended, this product is stable for 3 months.

Step 7. 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 8. 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 9. Wash Membrane

Note: *Protect membrane from light during washes.*

1. Carefully pour off secondary antibody solution.
2. Rinse the membrane with 1X TBS-T (0.1% Tween 20).
3. Cover blot with 1X TBS-T (see the *Incubation Box Volumes* section for recommended volumes).
4. Shake vigorously on platform shaker at room temperature for 5 minutes.
5. Pour off wash solution.
6. Repeat 3 additional times.

Step 10. Rinse Membrane

Rinse the membrane with 1X TBS 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 11. 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.

Optimization

- Follow the protocol carefully.
- No single blocking buffer 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 the primary antibody has worked well in the past using chemiluminescent detection, try that same blocking solution for near-infrared fluorescent detection on an Odyssey Imaging System.
- To avoid background speckles on blots, use high-quality ultra pure water for buffers. Rinsing previously-used incubation boxes with methanol can reduce background contamination of future blots.
- Always use a consistent buffering system for wash buffers and blocking buffers. (e.g. Intercept (TBS) Blocking Buffer, use TBS as the base for wash buffers; for Intercept (PBS) Blocking Buffer, use PBS as the base for wash buffers).
- Never perform Western incubations or washes in dishes that have been used for Coomassie staining.
- Only handle membranes by the edges with clean forceps. Be careful not to touch the membrane with your hands or gloves.

  After handling membranes that have been incubating in antibody solutions, clean forceps thoroughly with methanol, then rinse with distilled water.
- Always pour off antibody solution and washes from the same corner of the box to ensure complete removal of previous solutions.
- Do not wrap the membrane in plastic when scanning.

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., IgG_1, IgG_{2a}, or IgG_{2b}), IRDye® Subclass-Specific secondary antibodies can be used for multiplex detection. The same subclasses cannot be combined in a two-color Western blot (e.g., two IgG_1 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.

General Tips

- Store the IRDye secondary antibody vials at 4 °C in the dark. Do not freeze antibodies, as this will affect performance. 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, mix gently by inversion and centrifuge before use.
- Protect membrane from light during IRDye secondary antibody incubations and washes.
- Use gels that contain the narrowest well size possible to minimize load volume and concentrate the target protein.
- The best transfer conditions, membrane, and blocking agent for each experiment will vary, depending on the antigen, sample type, and antibody.
- For proteins <100 kDa, the recommended transfer buffer is 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). For proteins >100 kDa, decrease the methanol concentration in the transfer buffer to 10%. For transfers to PVDF membrane, methanol can be eliminated from the transfer buffer.
- Soak the gel in transfer buffer for 5-10 minutes before setting up the transfer. Soaking equilibrates the gel and removes SDS so that it will not be carried over into the transfer tank.
- To maximize retention of transferred proteins on the membrane, allow the membrane to air-dry completely after transfer (approximately 1-2 hours).

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

Troubleshooting

High background, uniformly distributed

Inefficient or ineffective blocking	Blocking solutions containing BSA may cause high membrane background. Try switching to a different blocker. LI-COR offers Intercept (PBS) Blocking Buffer, Intercept (TBS) Blocking Buffer, and Casein Blocking Buffer.
Background on nitrocellulose	Increase amount of Tween® 20 added to the diluted antibodies, staying in the range of 0.1 - 0.2%. Add SDS to diluted secondary antibody, staying in the range of 0.01 - 0.02%.
Background on PVDF	Reduce Tween 20 in diluted antibodies to 0.1%. Add 0.01 - 0.02% SDS to diluted secondary antibody.
Membrane autofluorescence	Scan an unused dry membrane to check for autofluorescence using the same image acquisition parameters as the high background Western blot.
Streptavidin conjugate	Add SDS to diluted streptavidin conjugate, staying in the range of 0.02-0.1% (v/v) for nitrocellulose and PVDF membranes.
Antibody concentrations too high	Optimize primary and secondary antibody dilutions.
Insufficient washing	Increase number of washes and buffer volume. Make sure that 0.1% Tween 20 is present in wash buffer and increase if needed. Excess Tween 20 (0.5-1%) may decrease signal.
Cross-reactivity of antibody with contaminants in blocking buffer	Use Intercept (TBS) Blocking Buffer instead of milk. Milk-based blockers may contain IgG that can cross-react with anti-goat antibodies. This can significantly increase background and reduce antibody titer. Milk-based blockers may also contain endogenous biotin or phospho-epitopes that can cause higher background.
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.

Uneven, blotchy, or speckled background

Problem	Solution
Blocking multiple membranes together in small volume	If multiple membranes are being blocked together 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-probed. If using PVDF, remember to first pre-wet in 100% methanol.
Contaminated forceps, dishes, or transfer equipment	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, silicone mat, or Odyssey Fc Imaging tray	Clean imaging surface, mat, or tray carefully with methanol before each use. Dust, lint, and residue will cause speckles.
Incompatible marker or pen used to mark membranes	Use a pencil to write on the membrane. You can write on nitrocellulose membranes with pencil or the Odyssey Pen (PN 926-71804).

Non-specific or unexpected bands

Problem	Solution
Antibody concentration too high	Reduce the amount of antibody used. Reduce the antibody incubation times. Increase Tween 20 in antibody diluent. 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 primary and secondary antibodies in a two-color experiment	Check the source and specificity of the primary and secondary antibodies (see the Guidelines for Two-Color Detection section). Use only highly cross-adsorbed secondary antibodies. There is always potential for cross-reactivity in two color experiments. Using less secondary antibody to minimize this. Always test primary and secondary antibody 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.
Bleed through of signal from one channel to another	Reduce signal in future experiments by reducing the amount of protein loaded or the antibody used.

Weak or No Signal

Not using optimal blocking reagent	Primary antibody may perform substantially better with a different blocker. LI-COR offers Intercept (PBS) Blocking Buffer and Intercept (TBS) Blocking Buffer, and Casein Blocking Buffer.
Insufficient amount of 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 hrs at room temperature, or overnight at 4 °C). Increase amount of primary or secondary antibody, optimizing for best performance.
Primary or secondary antibody may have lost reactivity due to age or storage conditions	Use fresh, unexpired antibodies.
Too much detergent	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.
Insufficient antigen loaded	Load more protein on the gel. Use 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 incubations	Reduce blocking times or decrease high concentrations of detergent in diluted antibodies.
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, especially on nitrocellulose. Methanol decreases the pore size of the 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. The presence of up to 0.05% SDS improves transfer efficiency of some proteins. Small proteins may pass through membrane during transfer (“blow-through”). Use a membrane with a smaller pore size or reduce the transfer time.

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