Odyssey® Western Blotting Kits RD (PBS)

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.

Part Numbers: 926-31081
926-31082
926-31083
926-31084

LI-COR®

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I. Required Reagents

- One of the following Odyssey Western Kits:
  - **Odyssey Western Blotting Kit I RD (LI-COR®, P/N 926-31081)**
    - IRDye 800CW Goat anti-Mouse Secondary Antibody (0.1 mg)
    - IRDye 680RD Goat anti-Rabbit Secondary Antibody (0.1 mg)
    - Odyssey Blocking Buffer (PBS) (500 mL)
    - Millipore Immobilon®-FL PVDF Membrane (0.45 µm, 10 x 10 cm)
  - **Odyssey Western Blotting Kit II RD (LI-COR, P/N 926-31082)**
    - IRDye 800CW Goat anti-Rabbit Secondary Antibody (0.1 mg)
    - IRDye 680RD Goat anti-Mouse Secondary Antibody (0.1 mg)
    - Odyssey Blocking Buffer (PBS) (500 mL)
    - Millipore Immobilon-FL PVDF Membrane (0.45 µm, 10 x 10 cm)
  - **Odyssey Western Blotting Kit III RD (LI-COR, P/N 926-31083)**
    - IRDye 800CW Goat anti-Mouse Secondary Antibody (0.1 mg)
    - IRDye 680RD Goat anti-Rabbit Secondary Antibody (0.1 mg)
    - Odyssey Blocking Buffer (PBS) (500 mL)
    - Odyssey Nitrocellulose Membrane (0.22 µm, 7 x 8.5 cm)
  - **Odyssey Western Blotting Kit IV RD (LI-COR, P/N 926-31084)**
    - IRDye 800CW Goat anti-Rabbit Secondary Antibody (0.1 mg)
    - IRDye 680RD Goat anti-Mouse Secondary Antibody (0.1 mg)
    - Odyssey Blocking Buffer (PBS) (500 mL)
    - Odyssey Nitrocellulose Membrane (0.22 µm, 7 x 8.5 cm)
- Primary antibodies (when using the above Odyssey Western Kits, the primary antibodies must be from Rabbit or Mouse host species)
- Tween® 20
- PBS buffer (LI-COR, P/N 928-40020)
- Methanol (when using Western Blotting Kit I or II)
- SDS (when using Western Blotting Kit I or II)
- Western Blot Incubation Box (appropriate for blot size)
  - Small (7.2 x 4.9 x 3.0 cm) (LI-COR, P/N 929-97101, 929-97105, 929-97110)
  - Medium (8.9 x 6.5 x 2.8 cm) (LI-COR, P/N 929-97201, 929-97205, 929-97210)
  - Large (11.5 x 8.8 x 2.8 cm (LI-COR, P/N 929-97301, 929-97305, 929-97310)
  - X-Large (15.2 x 10.1 x 3.1 cm) (LI-COR, P/N 929-97401, 929-97405, 929-97410)
- Odyssey Pen (LI-COR, P/N 926-71804) (optional)

II. Western Detection Method

Western blots should be prepared using standard transfer procedures and the Millipore Immobilon®-FL PVDF or the Odyssey Nitrocellulose membranes included in the Odyssey Western Blotting Kit. Allow blots to dry for at least 1 hour before proceeding with detection. Dry blots can be stored between filter paper at room temperature overnight.

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

NOTE: Do not write on any membranes with a regular ink pen or marker as the ink will fluoresce on Odyssey Imaging Systems. Mark the nitrocellulose membrane with pencil or the Odyssey Pen (P/N 926-71804). Use only pencil on the PVDF membrane, as the ink from the Odyssey Pen will dissolve in the methanol used to pre-wet the PVDF membrane.

Continue with the immunoassay::

1. ***For Immobilon-FL PVDF membrane:***
   - Pre-wet 1 minute in 100% methanol
   - Rinse with ultra pure water
   - Wet in 1X PBS for 2 minutes

   For Odyssey nitrocellulose membrane:
   - Wet in 1X PBS for 2 minutes
     NOTE: Leave the membrane immersed in 1X PBS until the next step.

2. Place the membrane in a Western Blot Incubation Box and block the membrane in Odyssey Blocking Buffer (PBS) for 1 hour with gentle shaking.

   Use the amount of liquid per box indicated below for this and the following steps.
   - 5 mL Odyssey Blocking Buffer (PBS) for Small Box
   - 10 mL Odyssey Blocking Buffer (PBS) for Medium Box
   - 15 mL Odyssey Blocking Buffer(PBS) for Large Box
   - 20 mL Odyssey Blocking Buffer (PBS) for X-Large Box

3. Prepare primary antibody dilution:
   - Prepare the primary antibody diluent by adding Tween® 20 to Odyssey Blocking Buffer (PBS) for a final concentration of 0.2%.
   - Dilute the primary antibody in the diluent (Odyssey Blocking Buffer (PBS) with 0.2% Tween 20) using the vendor's recommended dilution for Western blot applications for that primary antibody. Dilutions may range from 1:200 – 1:5,000, depending on the primary antibody.

4. Incubate the blot in the diluted primary antibody (see step 2 for volume) for 1 – 4 hours* at room temperature or overnight at 4°C with gentle shaking.

   **Optimal incubation times vary for different primary antibodies*

5. Wash membranes:
   - Carefully pour off the primary antibody solution.
   - Rinse the membrane with 1X PBS-T (0.1% Tween 20).
   - Cover the blot with 1X PBS-T (see step 2 for volume).
   - Shake on a platform shaker at room temperature for 5 minutes.
   - Carefully pour off the wash solution.
   - Repeat 3 additional times.

6. Prepare secondary antibodies provided in the Western Blotting Kit:
   - Reconstitute contents of each vial of secondary antibody with 0.1 mL sterile distilled water.
   - Mix gently by inverting, and allow to rehydrate for at least 30 minutes before use.
   - If solution is not completely transparent after standing at room temperature, centrifuge the product.
   - Store reconstituted antibody at 4 °C. Reconstituted antibody is stable for up to 3 months at 4 °C when stored undiluted as directed.

7. Dilute secondary antibody in the appropriate diluent listed below:
   – For IRDye® 800CW secondary antibodies and IRDye 680RD secondary antibodies, the suggested dilution range is 1:5,000 to 1:25,000.

   Secondary antibody diluent for Immobilon®-FL PVDF membrane
   - Add Tween® 20 to a final concentration of 0.2% and SDS to a final concentration of 0.01 – 0.02% in Odyssey Blocking Buffer

   Secondary antibody diluent for Odyssey nitrocellulose membrane
   - Add Tween 20 to a final concentration of 0.2% in Odyssey Blocking Buffer

     NOTE: Do not incubate for longer than 60 minutes, as the background may increase.

8. **Protect the membrane from light during washes.**
   To wash the membranes:
   - Carefully pour off the secondary antibody solution.
   - Rinse the membrane with 1X PBS-T.
   - Cover the blot with 1X PBS-T (see step 2 for volume).
   - Shake vigorously on a platform shaker at room temperature for 5 minutes.
   - Carefully pour off the wash solution.
   - Repeat 3 additional times.

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

10. Image on an instrument from the Odyssey Family of imagers. Protect from light prior to imaging.

    Store the membranes at 4 °C protected from light. Store dry for several months or in PBS buffer for a few weeks.

    NOTE: If you plan to strip and reprobe a Western blot, do not allow the completed Western blot to dry. The only time the membrane should be allowed to dry is immediately following the transfer step. Stripping is less effective on completed Western blots that have been allowed to dry.

Optimization Tips

- ***Follow the protocol carefully.***
- 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 the primary antibody has worked well in the past using chemiluminescent detection, try that same blocking solution for Odyssey near-infrared fluorescent detection.

- 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.
- Never perform Western incubations or washes in dishes that have been used for Coomassie staining.
- Membranes should be handled only by their edges, with clean forceps.
- Always pour off antibody solution and washes from the same corner of the box to ensure complete removal of previous solutions.
- After handling membranes that have been incubating in antibody solutions, clean forceps thoroughly with methanol, then rinse with distilled water.

III. 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 in step 3. Incubate simultaneously with the membrane (step 4). The primary antibodies must be from two different host species.
- Combine the two IRDye secondary antibodies in the diluted antibody solution in step 6. Incubate simultaneously with the membrane (step 7).

Two-color detection requires careful selection of primary and secondary antibodies. The following guidelines provide further 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).
- 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.
- 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.

IV. 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 1X Tris-glycine buffer (LI-COR, P/N 928-40012) 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).

V. Troubleshooting Guide

Problem	Possible Cause	Solution / Prevention
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 Odyssey Blocking Buffer (PBS) (P/N 927-40000), Odyssey Blocking Buffer (TBS) (P/N 927-50000), and Casein Blocking Buffer (P/N 927-40200).
	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. Note that excess Tween 20 (0.5-1%) may decrease signal.
	Cross-reactivity of antibody with	Use Odyssey Blocking Buffer (PBS) instead contaminants in blocking buffer. 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.

Problem	Possible Cause	Solution / Prevention
High background, uniformly distributed.	Membrane contamination.	Always handle membranes carefully and with forceps. Do not allow membrane to dry. Use clean dishes, bags, or trays for incubations.
Uneven, blotchy, or speckled background	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 before each use with methanol. Dust, lint, and residue will cause speckles.
	Incompatible marker or pen used to mark membranes.	Use only pencil to mark membrane. (Odyssey Pen can also be used on nitrocellulose.)
Weak or no signal	Not using optimal blocking reagent.	Primary antibody may perform substantially better with a different blocker. LI-COR offers Odyssey Blocking Buffer (PBS) (P/N 927-40000), Odyssey Blocking Buffer (TBS) (P/N 927-50000), and Casein Blocking Buffer (P/N 927-40200).
	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 or unexpired antibodies.

Problem	Possible Cause	Solution / Prevention
Weak or no signal *(Continued)*	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.
	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. *NOTE: 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. *NOTE: The presence of up to 0.05% SDS improves transfer efficiency of some proteins.*
	Proteins not retained on membrane during transfer.	Small proteins may pass through membrane during transfer ("blow-through"). Use a membrane with a smaller pore size or reduce the transfer time.
Non-specific or unexpected bands	Antibody concentrations too high.	Reduce the amount of antibody used. Reduce the 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 primary and secondary 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**).

Problem	Possible Cause	Solution / Prevention
Non-specific or unexpected bands *(Continued)*		Use only highly cross-adsorbed secondary antibodies.
		There is always potential for cross-reactivity in two-color experiments. Use less secondary antibody to minimize this.
		Always test primary and secondary antibody sets 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 into another channel.	Reduce signal in future experiments by reducing the amount of protein loaded or antibody used.

VI. Kit Replacement Reagents & More

Kit Replacement Reagents	LI-COR® P/N
• IRDye® 800CW Goat anti-Mouse Secondary Antibody, 0.1 mg	925-32210
• IRDye 680RD Goat anti-Rabbit Secondary Antibody, 0.1 mg	925-68071
• IRDye 800CW Goat anti-Rabbit Secondary Antibody, 0.1 mg	925-32211
• IRDye 680RD Goat anti-Mouse Secondary Antibody, 0.1 mg	925-68070
• Odyssey Blocking Buffer (PBS), 500 mL	927-40000
• Odyssey Block Buffer (TBS) 500 mL	927-50000
• Casein Blocking Buffer, 500 mL	927-40200
• Odyssey Nitrocellulose Membrane	926-31090 926-31092
• Blocking Buffer & Membrane Kit: 1 bottle of Odyssey Blocking Buffer (PBS), and 1 roll of Millipore Immobilon®-FL PVDF membrane	926-31098
• Blocking Buffer & Membrane Kit: 10 bottles of Odyssey Blocking Buffer (PBS), and 1 roll of Millipore Immobilon-FL PVDF membrane.	829-31080

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LI-COR®

Global Headquarters, Serving the United States • 4647 Superior Street • Lincoln, NE 68504
Phone: +1-402-467-0700 • Toll free: 800-645-4267 • Fax: +1-402-467-0819 • biosales@licor.com

Regional Offices
LI-COR GmbH, Germany, Serving Europe, Africa, and the Middle East.
LI-COR Biosciences GmbH • Siemensstraße 25A • 61352 Bad Homburg • Germany
Phone: +49 (0) 6172 17 17 771 • Fax: +49 (0) 6172 17 17 799 • bio-eu@licor.com

LI-COR Ltd., United Kingdom, Serving Denmark, Finland, Iceland, Ireland, Norway, Sweden, and UK.
LI-COR Biosciences UK Ltd. • St. John's Innovation Centre • Cowley Road • Cambridge CB4 0WS • United Kingdom
Phone: +44 (0) 1223 422104 • Fax: +44 (0) 1223 422105 • bio-eu@licor.com

LI-COR Distributor Network: www.licor.com/bio/distributors

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