

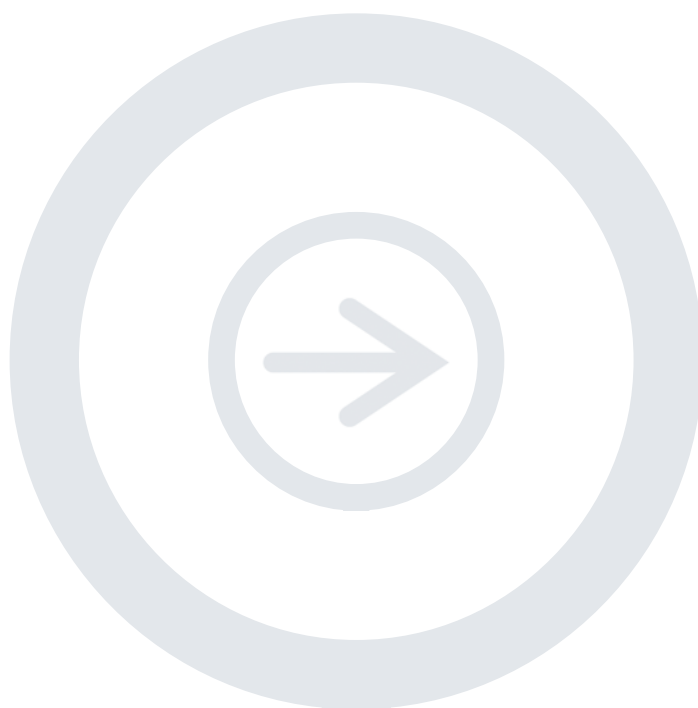
Odyssey®

Infrared Imaging System

Odyssey Western Blot Kit

Western Blotting Kits

Part Numbers: 926-31050
926-31052
926-31058
926-31060



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I. Required Reagents

- One of the following IRDye® Western Kits:
 - ◆ IRDye Western Kit I (LI-COR, Cat. #926-31050)
 - IRDye 800CW Goat Anti-Mouse Secondary Antibody
 - IRDye 680 Goat Anti-Rabbit Secondary Antibody
 - Odyssey® Blocking Buffer
 - Millipore Immobilon® FL PVDF Membrane
 - ◆ IRDye Western Kit II (LI-COR, Cat. #926-31052)
 - IRDye 800CW Goat Anti-Rabbit Secondary Antibody
 - IRDye 680 Goat Anti-Mouse Secondary Antibody
 - Odyssey Blocking Buffer
 - Millipore Immobilon FL PVDF Membrane
 - ◆ IRDye Western Kit III (LI-COR, Cat. #926-31058)
 - IRDye 800CW Goat Anti-Mouse Secondary Antibody
 - IRDye 680 Goat Anti-Rabbit Secondary Antibody
 - Odyssey Blocking Buffer
 - Odyssey Nitrocellulose Membrane
 - ◆ IRDye Western Kit IV (LI-COR, Cat. #926-31060)
 - IRDye 800CW Goat Anti-Rabbit Secondary Antibody
 - IRDye 680 Goat Anti-Mouse Secondary Antibody
 - Odyssey Blocking Buffer
 - Odyssey Nitrocellulose Membrane
- Primary antibodies (when using the above IRDye Western Kits the primary antibodies must be from Rabbit or Mouse host species)
- Tween® 20
- PBS buffer (LI-COR, Cat. #928-40018 or 928-40020)
- Methanol (when using IRDye Western Kit I or II)
- SDS (when using IRDye Western Kit I or II)
- Western Blot Incubation Box (appropriate for blot size)
 - ◆ Small (7.3 x 5.1 x 3.0 cm) (LI-COR, Cat. #929-97101, 929-97105, 929-97110)
 - ◆ Medium (8.9 x 6.6 x 2.9 cm) (LI-COR, Cat. #929-97201, 929-97205, 929-97210)
 - ◆ Large (11.6 x 8.9 x 2.9 cm) (LI-COR, Cat. #929-97301, 929-97305, 929-97310)
 - ◆ X-Large (15.2 x 10.2 x 3.2 cm) (LI-COR, Cat. #929-97401, 929-97405, 929-97410)

II. Western Detection Method

Western blot should be prepared using standard blotting procedures using the Millipore Immobilon® FL PVDF or Odyssey Nitrocellulose Membrane contained in the IRDye® Western Kit. Allow blot to dry for at least 1 hour before proceeding with detection. Dry blots can be stored at room temperature between filter paper overnight.

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

Note: Do not write on membrane with an ink pen or marker as they will fluoresce on the Odyssey Imager. Mark with pencil to avoid this problem. Use pencil for PVDF membrane as wetting in methanol will cause ink to run.

After transfer, perform the following steps:

1.	<p>For Immobilon FL PVDF membrane:</p> <ul style="list-style-type: none"> • Pre-wet 1 minute in 100% methanol • Rinse with ultra pure water • Wet in 1X PBS for 2 minutes <p>For Odyssey nitrocellulose membrane:</p> <ul style="list-style-type: none"> • Wet in 1X PBS for 2 minutes
2.	<p>Place membrane in Western Blot Incubation Box and block the membrane in Odyssey Blocking Buffer for 1 hour with gentle shaking.</p> <ul style="list-style-type: none"> • 5 ml Odyssey Blocking Buffer for Small Box • 10 ml Odyssey Blocking Buffer for Medium Box • 15 ml Odyssey Blocking Buffer for Large box • 20 ml Odyssey Blocking Buffer for X-Large box
3.	<p>Prepare primary antibody dilution:</p> <ul style="list-style-type: none"> • Prepare the primary antibody diluent. Add Tween® 20 to Odyssey Blocking Buffer for a final concentration of 0.2% Tween 20. • Dilute primary antibody in Odyssey Blocking Buffer 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 – 1:5000, depending on the primary antibody. • Primary antibody dilution volumes as follows: <ul style="list-style-type: none"> 5 ml Odyssey Blocking Buffer for Small Box 10 ml Odyssey Blocking Buffer for Medium Box 15 ml Odyssey Blocking Buffer for Large box 20 ml Odyssey Blocking Buffer for X-Large box
4.	<p>Incubate blot in diluted primary antibody for 1 to 4 hours* at room temperature, or overnight at 4°C with gentle shaking.</p> <p><i>*incubation times vary for different primary antibodies</i></p>
5.	<p>Wash membranes:</p> <ul style="list-style-type: none"> • Pour off primary antibody solution • Rinse membrane with 1 X PBS-T (0.1% Tween 20) • Cover blot with 1 X PBS-T (0.1% Tween 20) using same volumes indicated above for Western blot incubation boxes

Continued

	<ul style="list-style-type: none"> • Shake vigorously on platform shaker at room temperature for 5 minutes • Pour off wash solution • Repeat 3 additional times
6.	<p>Dilute secondary antibody provided in Western Kit 1:10,000* in the appropriate diluent listed below:</p> <p>Secondary antibody diluent for Immobilon® FL PVDF membrane</p> <ul style="list-style-type: none"> • Add Tween 20 to a final concentration of 0.2% and SDS to a final concentration of 0.01% in Odyssey Blocking buffer <p>Secondary antibody diluent for Odyssey nitrocellulose membrane</p> <ul style="list-style-type: none"> • Add Tween 20 to a final concentration of 0.2% in Odyssey Blocking buffer <p><i>*suggested dilution range is 1:5000 to 1:25,000 and may require optimization</i></p>
7.	<p>Protect membrane from light during incubation. Incubate blot in diluted secondary antibody for 30-60 minutes at room temperature with gentle shaking.</p>
8.	<p>Protect from light during washes</p> <p>Wash membranes:</p> <ul style="list-style-type: none"> • Pour off secondary antibody solution • Rinse membrane with 1X PBS-T (0.1% Tween 20) • Cover blot with 1X PBS-T (0.1% Tween 20) using same volumes indicated above for Western blot incubation boxes • Shake vigorously on platform shaker at room temperature for 5 minutes • Pour off wash solution • Repeat 3 additional times
9.	<p>Rinse membrane with 1X PBS to remove residual Tween 20. The membrane can be scanned wet or dry.</p>

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 different nonspecific 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 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 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 distilled water and/or methanol, then rinse with distilled water.

- ◆ Always clean the Odyssey scanning surface prior to scanning to remove dust, buffer residue, and smudges that may affect image quality or contaminate the membrane. The scanning surface can be wiped with methanol, rinsed with a small volume of water, followed by a final rinse with isopropanol to help prevent blot contamination from the scan bed. Pressurized “canned air” may be helpful in removing dust and lint.
- ◆ If using a silicone mat to cover your membranes, carefully clean the surface of the mat that will touch the membrane with isopropanol. Blot the mat dry and avoid rubbing or wiping the mat with tissue, as this creates more lint and leads to speckling.
- ◆ Do not wrap the membrane in plastic when scanning.

III. Guidelines for Two-Color Detection

Two different antigens can be detected simultaneously on the same blot using IRDye® labeled 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 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 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 680 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 secondaries reacting against one another.

IV. General Tips

- ◆ Store the IRDye® secondary antibody vials at 4°C in the dark. Do not thaw and refreeze 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, 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 Part #928-40010 or 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%.
- ◆ Soak the gel in transfer buffer for 10-20 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	BSA used for blocking.	Blocking solutions containing BSA may cause high membrane background. Try switching to a different blocker.
	Not using optimal blocking reagent.	Compare different blocking buffers to find the most effective for your system; try blocking longer. Try diluting Odyssey blocker 1:1 in 1X PBS.
	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.

Problem	Possible Cause	Solution / Prevention
High background, uniformly distributed (Continued)	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 contaminants in blocking buffer.	Use Odyssey 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.		
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 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-used.
		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 or silicone mat.	Clean scanning surface and mat carefully before each use. Dust, lint, and residue will cause speckles.	
Incompatible marker or pen used to mark membrane.	Use only pencil or Odyssey pen to mark membranes.	

Problem	Possible Cause	Solution / Prevention
Weak or no signal.	Not using optimal blocking reagent.	Primary antibody may perform substantially better with a different blocker. Try diluting Odyssey blocker 1:1 in 1X PBS.
	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.
	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. Note: <i>methanol decreases pore size of gel and can hamper transfer of large proteins.</i>
		SDS in transfer buffer may interfere with binding of transferred proteins, especially for low molecular weight proteins. Try reducing or eliminating SDS. Note: presence of up to 0.05% SDS does improve transfer efficiency of some proteins.

Problem	Possible Cause	Solution / Prevention
Weak or no signal (Continued)		Small proteins may pass through membrane during transfer (“blow-through”). Use membrane with smaller pore size or reduce transfer time.
Nonspecific or unexpected bands.	Antibody concentrations too high.	<p>Reduce the amount of antibody used.</p> <p>Reduce antibody incubation times.</p> <p>Increase Tween® 20 in diluted antibodies.</p> <p>Add or increase SDS in diluted secondary antibodies.</p>
	Not using optimal blocking reagent.	Choice of blocker may affect background bands. Try a different blocker.
	Cross-reactivity between 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).
		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. Sheep and goat antibodies may exhibit the same behavior.	
	Bleedthrough of signal from one channel into other channel.	Reduce signal in further experiments by reducing the amount of protein loaded or antibody used.

VI. Kit Replacement Reagents and More

Kit Replacement Reagents	LICOR Part #
• IRDye® 800CW Goat Anti-Mouse Secondary Antibody926-32210
• IRDye 680 Goat Anti-Rabbit Secondary Antibody926-32221
• IRDye 800CW Goat Anti-Rabbit Secondary Antibody926-32211
• IRDye 680 Goat Anti-Mouse Secondary Antibody926-32220
• Odyssey Blocking Buffer927-40000
• Odyssey Nitrocellulose Membrane926-31090 926-31092
• Blocking Buffer & Membrane Kit: 10 bottles of Odyssey Blocking Buffer, and 1 roll of Millipore Immobilon® FL PVDF membrane829-31080

Other Western Products	LICOR Part #
• IRDye 800CW Donkey Anti-Mouse Secondary Antibody926-32212
• IRDye 800CW Donkey Anti-Rabbit Secondary Antibody926-32213
• IRDye 800CW Donkey Anti-Goat Secondary Antibody926-32214
• IRDye 800CW Goat Anti-Rat Secondary Antibody926-32219
• IRDye 800CW Donkey Anti-Chicken Secondary Antibody926-32218
• IRDye 680 Donkey Anti-Mouse Secondary Antibody926-32222
• IRDye 680 Donkey Anti-Rabbit Secondary Antibody926-32223
• IRDye 680 Donkey Anti-Goat Secondary Antibody926-32224
• IRDye 680 Goat Anti-Rat Secondary Antibody926-32229
• IRDye 680 Donkey Anti-Chicken Secondary Antibody926-32228

Other Western Products (Continued)LICOR Part #

- 10 X Tris-Glycine Buffer928-40010
928-40012
- 10 X Tris-Glycine-SDS Buffer928-40014
928-40016
- 10 X PBS Buffer928-40018
928-40020
- 2X Protein Sample Loading Buffer928-40004
- Protein Markers928-40000
- IRDye® Blue Protein Stain928-40002
- Western Blot Incubation Boxes
 - Small (7.3 x 5.1 x 3.0 cm)929-97101
929-97105
929-97110
 - Medium (8.9 x 6.6 x 2.9 cm)929-97201
929-97205
929-97210
 - Large (11.6 x 8.9 x 2.9 cm)929-97301
929-97305
929-97310
 - X-Large (15.2 x 10.2 x 3.2 cm)929-97401
929-97405
929-97410
- NewBlot™ Stripping Buffer for nitrocellulose928-40030
- NewBlot Stripping Buffer for PVDF928-40032
- SMART™ Gel 7.5%928-40040
928-40041
- SMART Gel 10%928-40042
928-40043
- SMART Gel 12.5%928-40044
928-40045
- MPX™ Blotting System921-00000

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