Odyssey®

Infrared Imaging System

Western Blot Analysis

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Contents

I. Required Reagents

- Blotted nitrocellulose or PVDF membrane
- Odyssey® Blocking Buffer (LI-COR, Cat. #927-40000)
- Primary antibodies
- Infrared (IR)-labeled secondary antibodies (LI-COR)*
- Tween®-20
- PBS wash buffer
- Double distilled water
- Methanol for wetting of PVDF
- SDS (if desired)
- Other blocking buffers (if desired)

* IRDye™ 800CW-labeled secondary antibodies are also available from Rockland Immunochemicals, Inc. Alexa Fluor® 680-labeled secondary antibodies are available from Invitrogen Corporation.

Fluorescent Dyes Appropriate for Use with the Odyssey System

Dye	Sensitivity	Odyssey Channel
IRDye™ 800CW	+ + +	800
IRDye™ 800	+ + +	800
IRDye™ 680	+ + +	700
IRDye™ 700DX	+ +	700
Alexa Fluor® 680	+ + +	700
Alexa Fluor® 700	+ +	700
Alexa Fluor® 750	+ +	700/800 (not recommended; signal appears in both channels)
Alexa Fluor® 647	+	700
Cy®5.5	+ +	700
Cy®5	+	700

The most current information on dye compatibility can be found on the LI-COR web site.

II. Western Detection Methods

Nitrocellulose or PVDF membranes may be used for protein blotting, but nitrocellulose membrane is recommended for maximum performance. Pure cast nitrocellulose is generally preferable to supported nitrocellulose. Protein should be transferred from gel to membrane by standard procedures. Membranes should be handled only by their edges, with clean forceps.

After transfer, perform the following steps:

1.	Wet the membrane in PBS for several minutes. If using a PVDF membrane that has been allowed to dry, pre-wet briefly in 100% methanol and rinse with double distilled water before incubating in PBS. **Notes:** • Ink from most pens and markers will fluoresce on Odyssey. The ink may wash off and re-deposit elsewhere on the membrane, creating blotches and streaks. Mark with pencil or the provided Odyssey pen to avoid this problem. Use pencil for PVDF membrane (wetting in methanol will cause ink to run).
2.	Block the membrane in Odyssey Blocking Buffer for 1 hour. Be sure to use sufficient blocking buffer to cover the membrane (a minimum of 0.4 ml/cm^2 is suggested). **Notes:** • Membranes can be blocked overnight at 4°C if desired. • **DO NOT** add Tween-20 when blocking the membrane. The membrane should not be exposed to Tween-20 until blocking is completed, as high background will result. • Odyssey Blocking Buffer often yields higher and more consistent sensitivity and performance than other blockers. Nonfat dry milk or casein dissolved in PBS can also be used for blocking and antibody dilution, but be aware that milk may cause higher background on PVDF membranes. If using casein, a 0.1% solution in 0.2 X PBS buffer is recommended (Hammersten-grade casein is not required). • Milk-based reagents can interfere with detection when using anti-goat antibodies. They also deteriorate rapidly at 4°C, so diluted antibodies cannot be kept and re-used for more than a few days. • Odyssey Blocking Buffer can often be diluted at least 1:1 in PBS without loss of performance. • Odyssey Blocking Buffer can be saved and re-used. • Blocking solutions containing BSA can be used, but in some cases they may cause high membrane background. ***BSA-containing blockers are not generally recommended*** and should be used only when the primary antibody requires BSA as blocker.
3.	Dilute primary antibody in Odyssey Blocking Buffer. Optimum dilution depends on your antibody and should be determined empirically. A suggested starting range is 1:1000 to 1:5000. To lower background, add 0.1 - 0.2% Tween-20 to the diluted antibody before incubation. The optimum Tween-20 concentration will depend on your antibody. **Notes:** • Two-color detection requires primary antibodies raised in different host species, such as rabbit and mouse. For details, see ***III. Guidelines for Two Color Western Detection***.
4.	Incubate blot in primary antibody for 60 minutes or longer at room temperature with gentle shaking (optimum incubation times vary for different primary antibodies). Use enough antibody solution to completely cover the membrane.
5.	Wash membrane 4 times for 5 minutes each at room temperature in PBS + 0.1% Tween-20 with gentle shaking, using a generous amount of buffer.

6.	Dilute the fluorescently-labeled secondary antibody in Odyssey Blocking Buffer. Avoid prolonged exposure of antibody vial to light. Recommended dilution is 1:15,000 (suggested dilution range is 1:5000 to 1:25,000). Add Tween-20 to the diluted antibody as you did for the primary antibody. Add SDS if desired, see *Notes* below. **Notes:** • For detection of small amounts of protein, try using more secondary antibody (1:5000-1:10,000). • Be careful not to introduce contamination into the antibody vial. • Diluted secondary antibody can be saved and re-used. Store at 4°C and protect from light. However, for best sensitivity and performance, use freshly diluted antibody solution. • Adding **0.01% - 0.02% SDS** to the diluted secondary antibody (in addition to Tween-20) will substantially reduce membrane background, particularly when using PVDF. However, DO NOT add SDS during blocking or to the diluted primary antibody. See ***V. Optimization of Western Detection*** for more information about how and why to use SDS in the secondary antibody incubation.
7.	Incubate blot in secondary antibody for 30-60 minutes at room temperature with gentle shaking. Protect from light during incubation. **Notes:** • Allowing incubation to proceed more than 60 minutes may increase background.
8.	Wash membrane 4 times for 5 minutes each at room temperature in PBS + 0.1% Tween-20 with gentle shaking. Protect from light.
9.	Rinse membrane with PBS to remove residual Tween-20. The membrane is now ready to scan. **Notes:** • Scan in the appropriate channels (see ***I. Required Reagents*** for details). • Protect the membrane from light until it has been scanned. • Keep the membrane wet if you plan to strip and re-use it. Once a membrane has dried, stripping is ineffective. • Blots can be allowed to dry before scanning if desired. Signal strength may be enhanced on a dry membrane. The membrane can also be re-wetted for scanning. • The fluorescent signal on the membrane will remain stable for several months, or longer, if protected from light. Membranes may be stored dry or in PBS buffer at 4°C. • If signal on membrane is too strong or too weak, re-scan the membrane at a lower or higher scan intensity setting, respectively.

Molecular Weight Marker

If you loaded the Odyssey Prestained Molecular Weight Marker (LI-COR, Cat. #928-40000) on your gel before transfer, it will be visible in the 700 nm channel image and also faintly visible in the 800 nm channel image. If the marker is subjected to numerous freeze/thaw cycles, it may degrade. This is observed as multiple, high-molecular weight bands appearing in the 800 nm channel. If this occurs, discard the aliquot and use a fresh one.

Prestained blue molecular weight markers from other sources can also be used with Odyssey. Load 1/3 to 1/5 of the amount you would normally use for Western transfer. Too much marker can result in very strong marker bands that may interfere with visualization of sample lanes. If using multicolored markers, some bands may not be visualized with Odyssey.

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 banding 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 blocking solution for Odyssey detection.
- Addition of detergent such as Tween-20 can reduce membrane background and non-specific banding. Refer to ***V. Optimization of Western Detection*** for details.
- To avoid background speckles on blots, use high-quality double distilled water for buffers and rinse plastic dishes well before and after use. 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.
- After you handle membranes that have been incubating in antibody solutions, clean forceps thoroughly with distilled water and/or ethanol. If forceps are not cleaned after being dipped in antibody solutions, they can cause spots or streaks of background on the membrane that are difficult to wash away.
- When scanning, always clean the Odyssey scanning surface first to remove dust, residue, and smudges that may affect image quality or contaminate the membrane. If using a silicone mat over your membranes, carefully clean the surface of the mat that will touch the membrane; a dirty mat can deposit dust and residue. 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.
- If you plan to strip a Western blot, do not allow the membrane to dry. Stripping is ineffective once a membrane has dried, or even partially dried.

III. Guidelines for Two-Color Detection

Two different antigens can be detected simultaneously on the same blot using antibodies labeled with IR dyes that are visualized in different fluorescence channels (700 and 800 nm). Two-color detection requires careful selection of primary and secondary antibodies.

The following guidelines will help you design two color experiments:

- **The two primary antibodies must be derived from different host species** so 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).
- 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 a 700 channel dye, and the other with an 800 channel dye. For a list of fluorescent dyes and the Odyssey channels where they can be visualized, see ***I. Required Reagents***.
- Always use highly cross-adsorbed secondary antibodies for two color detection. Failure to use cross-adsorbed antibodies may result in cross-reactivity.

- For best results, avoid using primary antibodies from mouse and rat together for a two-color experiment. It is not possible to completely adsorb away cross-reactivity because the species are so closely related. If using 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. The secondary antibodies should not recognize immunoglobulins from other species that may be present in the sample.

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 dye-labeled secondary antibodies in the diluted antibody solution in step 6. Incubate simultaneously with membrane (step 7).

IV. Stripping the Membrane

PVDF membranes can be stripped. If a blot is to be stripped, DO NOT allow it to dry before, during, or after imaging (keep the blot as wet as possible). Stripping techniques used with other Western detection methods can often be used for Odyssey blots as well. Nitrocellulose membranes generally do not strip well.

Stripping buffer: 25 mM glycine pH 2.0 + 1-2% SDS.

Stripping Procedure

1.	Incubate blot in stripping buffer for 10-15 minutes at room temperature, with shaking.
2.	Replace with fresh stripping buffer and shake an additional 10-15 minutes.
3.	Wash in PBS + 0.1% Tween-20 for 5 minutes, with shaking.
4.	Rinse with PBS and quickly scan blot at low resolution (337 µm) to see if signal has been removed. If some signal remains, repeat stripping procedure as needed. Intense bands or strong antibody interactions may require additional incubations in stripping buffer. Frequent changes of stripping buffer are helpful.
5.	When ready to begin the next round of detection, re-block for 30-60 minutes and proceed with antibody incubations.

Tips

- Avoid overstripping the membrane, as target proteins may be lost from the blot during extended incubations. Scan membrane to determine when stripping is complete. If overstripping is a problem, try reducing the amount of SDS in the stripping buffer.
- Depending on the strength of the antibody-antigen interactions, you may need to increase the stringency. This can be achieved by increasing the SDS concentration from 1% to 1.5 - 2%, or by preheating the buffer to 65°C before use. Pour the warmed buffer onto the blot and shake at room temperature or perform stripping by shaking at 65°C in an oven or water bath. When using elevated temperature, check the blot frequently by scanning to avoid overstripping.

V. Optimization of Western Detection

When adapting your Western blotting protocols for Odyssey detection or using a new primary antibody, it is important that you determine the optimal antibody concentrations. Optimization allows you to achieve maximum sensitivity and consistency.

Three parameters should be optimized: primary antibody concentration, dye-labeled secondary antibody concentration, and detergent concentration in the diluted antibodies.

Primary Antibody Concentration

Primary antibodies vary widely in quality, affinity, and concentration. The correct working range for antibody dilution depends on the characteristics of your primary antibody and the amount of target antigen you want to detect. Suggested dilutions are 1:500, 1:1500, 1:5000, and 1:10,000 (start with the dilution factor you would normally use for chemiluminescent detection). Optimize your primary dilution to achieve maximum performance and conserve antibody.

Secondary Antibody Concentration

Optimal dilutions of dye-conjugated secondary antibodies should also be determined. Suggested starting dilutions to test are 1:5000, 1:10,000, and 1:20,000. The amount of secondary required varies depending on how much antigen is being detected – abundant proteins with strong signals require less secondary antibody.

Detergent Concentration

Addition of detergents to diluted antibodies can significantly reduce background on the blot. Optimal detergent concentration will vary, depending on the antibodies, membrane type, and blocker used. Keep in mind that some primaries do not bind as tightly as others and may be washed away by too much detergent. Never expose the membrane to detergent until blocking is complete, as this may cause high membrane background.

Tween-20:

- No Tween-20 should be present during the blocking step.
- Add Tween-20 to both the primary antibody and secondary antibody solutions when the antibodies are diluted in blocking buffer. A final concentration of 0.1 - 0.2% is recommended for nitrocellulose membranes, and a final concentration of 0.1% is recommended for PVDF membranes (higher concentrations of Tween-20 may actually cause increased background on PVDF).
- Wash solutions should contain 0.1% Tween-20.

SDS:

- Adding **0.01 - 0.02% SDS** to the diluted secondary antibody can dramatically reduce overall membrane background and also reduce or eliminate non-specific banding. It is critical to use only a very small amount, because SDS is an ionic detergent and can disrupt antigen-antibody interactions if too much is present at any time during the detection process.
- Addition of SDS is particularly helpful for reducing the higher overall background that is seen with PVDF membrane.

- ***DO NOT add SDS during the blocking step or to the diluted primary antibody.*** Presence of SDS during binding of the primary antibody to its antigen may greatly reduce signal. ***Add SDS only to the diluted secondary antibody.***
- When diluting the dye-labeled secondary antibody in blocking buffer, add **both** 0.1 - 0.2% Tween-20 and 0.01 - 0.02% SDS to the antibody solution.
- Wash solutions should contain 0.1% Tween-20, but no SDS.
- Some antibody-antigen pairs may be more sensitive to the presence of SDS and may require even lower concentrations of this detergent (less than 0.01%) for best performance. Titrate the amount of SDS to find the best level for the antibodies used.
- If high background is seen when using BSA-containing blocking buffers, adding SDS to the secondary antibody may alleviate the problem.

VI. General Tips

- Do not expose your membrane to Tween-20 until after it has been blocked. Presence of Tween-20 during blocking can cause strong background fluorescence on the blot.
- Milk-based blockers may contain IgG that can react with anti-goat antibodies. This can significantly increase background and reduce antibody titer.
- To stretch the Odyssey Blocking Buffer: recycle your blocking buffer for antibody dilution; dilute 1:1 in PBS; save excess used blocking buffer at 4°C for several days for re-use (use a separate container – do not return to the main bottle).
- Store the antibody vial at 4°C in the dark. Do not thaw and refreeze the vial, as this will affect antibody 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 secondary antibody incubations and washes.
- Use the narrowest well size possible for your loading volume to concentrate the target protein.
- The best transfer conditions, membrane, and blocking agent for your experiments will vary, depending on the antigen and antibody. If you have problems with high back ground or low signal level, a good first step is to try a different blocking solution.
- For maximum sensitivity, use nitrocellulose membrane for transfer.
- Small amounts of purified protein may not transfer well. Adding non-specific proteins of similar molecular weight can have a "carrier" effect and substantially increase transfer efficiency.
- For proteins <100 kDa, try blotting in standard 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).
- Soak the gel in transfer buffer for 10-20 minutes before setting up the transfer. Soaking the gel 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. Long blocking incubations, particularly with nonfat dry milk at 2% or higher, can cause loss of target protein from the membrane (*J. Immunol. Meth.* 122:129-135, 1989).

- To enhance signal, try extended primary antibody incubation at room temperature or overnight incubation at 4°C. Avoid extended incubations in secondary antibody.

VII. Imaging of Coomassie-Stained Protein Gels

Odyssey can also be used to document Coomassie™-stained gels. Coomassie™ Blue gel stains (both methanol-based and water-based) can be seen clearly in the 700 nm channel and faintly in the 800 nm channel. Imaging on the Odyssey System is often more sensitive than visual inspection. Water-based Coomassie stains yield the best sensitivity; performance may be further improved by destaining the gel overnight in water before imaging.

Gel Documentation Procedure:

1.	Thoroughly rinse the gel with de-staining solution or water to remove stain particulates. Stain particles on the gel surface will cause background speckles on the image.
2.	Place the gel on the Odyssey scanner surface, taking care not to trap bubbles underneath. Use of the silicone mat for Coomassie gels is not recommended, as it may be difficult to remove all traces of stain from the mat.
3.	Scan the gel in the 700 nm channel. The focus offset must be adjusted for gel imaging. The correct focus offset is 1/2 the thickness of the gel; for a 1 mm gel, set the focus offset to 0.5 mm.
4.	After removing the gel, **carefully clean the glass scanning surface to remove residual stain**.
5.	If speckles still appear on the gel image, wipe the gel gently with a gloved finger or wet lint-free tissue before scanning. Gel-drying solutions (for archival drying of gels between cellophane sheets) are effective for removal of speckles; wash in gel-drying solution for no more than 5 minutes to avoid loss of sensitivity. Alternatively, the filter/noise removal functions in Odyssey software can be used to eliminate speckles (see Odyssey User Guide).

VIII. Troubleshooting Guide

Problem	Possible Cause	Solution / Prevention
High background, uniformly distributed.	Membrane blocked in presence of Tween-20.	Do not expose any membrane to Tween-20 until after blocking.
	BSA used for blocking.	Blocking solutions containing BSA may cause high membrane background. Try adding SDS to reduce background, or switch to a different blocker.
	Not using optimal blocking reagent.	Compare different blocking buffers to find the most effective for your system; try blocking longer.
	Background on nitrocellulose.	Add Tween-20 to the diluted antibodies to reduce background. Try adding SDS to diluted secondary antibody.
	Background on PVDF.	Reduce Tween-20 in diluted antibodies to 0.1%. Add 0.01-0.02% SDS to diluted secondary antibody.
	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 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 is usually contaminated with IgG and will cross-react with anti-goat secondary antibodies.
	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.

Problem	Possible Cause	Solution / Prevention
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 or dishes.	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.
Weak or no signal.	Not using optimal blocking reagent.	Primary antibody may perform substantially better with a different blocker.
	Insufficient 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 hr at room temperature, or overnight at 4°C).
		Increase amount of primary or secondary antibody, optimizing for best performance.
		Try substituting a different dye-labeled secondary antibody.
		Primary or secondary antibody may have lost reactivity due to age or storage conditions.

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. Try using 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 detection.	Extended blocking times or high concentrations of detergent in diluted antibodies may cause loss of antigen from the blotted membrane.
	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:*** *methanol decreases pore size of 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:*** *presence of up to 0.05% SDS does improve transfer efficiency of some proteins*.
		Small proteins may pass through membrane during transfer ("blow-through"). Use membrane with smaller pore size or reduce transfer time.

Problem	Possible Cause	Solution / Prevention
Nonspecific or unexpected bands.	Antibody concentrations too high.	Reduce the amount of antibody used.
		Reduce 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 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 the two colors 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.	Check the fluorescent dye used. Fluorophores such as Alexa Fluor® 750 may appear in both channels and are not recommended for use with Odyssey.
		If signal in one channel is very strong (near or at saturation) it may generate a small amount of bleedthrough signal in the other channel. Minimize this by using a lower scan intensity setting in the problem channel.
		Reduce signal in further experiments by reducing the amount of protein loaded or antibody used.

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4308 Progressive Avenue • P.O. Box 4000 • Lincoln, Nebraska 68504 USA
Technical Support: 800-645-4260
North America: 800-645-4267
International: 402-467-0700 • 402-467-0819
LI-COR GmbH (Germany, Austria, Switzerland, Czech Republic, Hugary, Slovakia): +49 (0) 6172 17 17 771
LI-COR UK Ltd.: +44 (0) 1223 422104
www.licor.com