In-Gel Western Detection Using Near-Infrared Fluorescence

Developed for:

Aerius, and 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.

LI-COR®

Published January 2006, revised June 2012. The most recent version of this protocol is posted at https://www.licor.com/bio/support/

Contents

I. Reagents

Required Reagents:

- IRDye® Infrared Dye labeled secondary antibodies (LI-COR)*
- NEXT GEL™ (AMRESCO, P/N M255, M256, M257), Bis-Tris acrylamide gels, Tris-Glycine gels, or equivalent, for electrophoresis
- 50% isopropanol + 5% acetic acid (made with ultrapure water)
- Blocking buffer (5% BSA)
- Primary antibodies
- Tween® 20 detergent
- PBS buffer
- Ultrapure water

Optional Reagents:

- Odyssey Blocking Buffer (LI-COR, P/N 927-40000)

*Go to www.licor.com/bio for the current list of LI-COR IRDye Conjugates

II. Description

Western blot detection of proteins requires separation of protein mixtures by electrophoresis, followed by transfer of the separated proteins to nitrocellulose or PVDF membranes for detection. The Odyssey Systems allow you to detect target proteins while still embedded in the gel, without transfer to a membrane using near-infrared secondary antibodies, such as LI-COR IRDye conjugates.

Using near-infrared fluorescence detection methods for In-Gel Westerns makes this a powerful technique. It saves time, reduces cost, and eliminates variables introduced by the transfer step or subsequent blocking of the membrane. In-Gel Western detection can be performed with standard

Odyssey reagents – no special kit is required. After electrophoresis, the gel is fixed briefly in a solution of isopropanol and acetic acid. Following a wash step to remove the alcohol, the gel is incubated in diluted antibodies and washed in a method similar to an ordinary Western blot. After washing, the wet gel is ready to scan on Odyssey. There is no substrate to apply, no plastic wrap, and no film exposures. In addition, two-color Western detection of two different protein targets can be performed within the gel.

In-Gel detection can enable faster results and eliminates inconsistencies due to transfer. In-Gel detection eliminates the problem of target proteins not transferring well (for example, large proteins or glycoproteins that are retained in the gel, or small proteins that may pass through the membrane during transfer). Near-infrared In-Gel Westerns also offer unparalleled sensitivity in the low-picogram range with the Odyssey Infrared Imaging System. This technique provides a very useful tool for protein detection and research; however, it is important to note that In-Gel detection may not be quantitative.

NOTE: Imaging area of the Odyssey Infrared Imaging System is 25 x 25 cm. For the Odyssey Fc Imaging System, it is 10 x 12 cm.

III. Electrophoresis

1. Separate the proteins of interest by electrophoresis.

   NOTES:
   - Gel type will affect the success and sensitivity of In-Gel Western detection. Best results can be obtained with AMRESCO NEXT GEL® (for self-poured gels) or NuPAGE® Bis-Tris pre-cast gels. The performance of different pre-cast gels may vary. Other gel types can be used but may require optimization.
   - Gel thickness and acrylamide percentage affect the ability of antibody molecules to penetrate the gel. We generally recommend that gel percentage be 12% or less, with a thickness of 1 - 1.5 mm.

IV. In-Gel Western Detection Protocol

2. After electrophoresis, separate the two plates and cut away any stacking gel present at the top of the gel using a scalpel or razorblade.

   NOTE: The stacking gel will exhibit high background when the gel is imaged. Notch one corner of the gel for orientation, if desired.

3. Incubate the gel in 50% isopropanol + 5% acetic acid (prepared with ultrapure water) for 15 minutes. Use enough solution that the gel is completely covered and can move freely. Shake gently.

   IMPORTANT: Always use clean gloves and incubation trays when handling the gel to avoid high background. Handle the gel gently. Squeezing or pressing can cause splotches or fingerprints to appear in the image.

4. Remove isopropanol/acetic acid and wash the gel in ultrapure water for 15 minutes with gentle shaking. Use enough water so that the gel is completely submerged and can move freely. The gel may curl and/or float to the surface; gently flatten or turn it over, making sure it is completely covered. Residual alcohol on the gel surface can cause diffuse bands.

   TIP: If desired, stop at this point and store the gel overnight in water at 4 °C.

5. No blocking step is required before antibody incubations.
   - Dilute primary antibody to the desired concentration in 5% BSA, Odyssey Blocking Buffer, or PBS (5% BSA is recommended).
   - Include 0.1% Tween® 20 in the diluted antibody solution.
   - Since In-Gel detection is not as sensitive as a standard Western blot, more primary antibody than usual may be needed. Make sure the gel is completely covered with antibody solution.
   - Incubate gel for 1 hour with gentle shaking.

6. Primary antibody incubation can be extended to several hours, or carried out overnight at 4 °C. Extended incubation will increase signal.

7. Wash the gel 3 times for 10 minutes in PBS + 0.1% Tween 20 with gentle shaking, using a generous amount of wash buffer.

8. - Dilute secondary antibody at 1:1000 – 1:5000 in the appropriate diluent with 0.1% Tween 20.
   - Incubate gel in secondary antibody solution for 1 hour with gentle shaking, and protect from light.
   - Use enough antibody solution to completely cover gel.

9. Wash the gel 3 times for 10 minutes in PBS + 0.1% Tween 20 with gentle shaking, using a generous amount of wash buffer.

10. Wash the gel for 5 minutes in PBS.

11. Place the gel on the imaging surface. For Odyssey Classic, CLx, and Sa, set the focus offset to 1/2 the gel thickness (e.g., for a 1 mm gel, set the focus offset to 0.5 mm).

12. If image background is high, the background may be reduced by soaking the gel several hours or overnight in PBS and re-scanning. Store the gel at 4 °C and protect from light. Gels can be kept in PBS at 4 °C for several days, if desired.

V. Guidelines for Two-Color In-Gel Western Detection

It is absolutely critical that primary and secondary antibodies be carefully selected for two-color detection or cross-reactivity will result. The following guidelines should be used when selecting primary and secondary antibodies for two color detection:

a. All secondary antibodies must be highly cross-adsorbed to eliminate cross-reactivity.

b. The two primary antibodies used must be derived from different host species* so they can be discriminated by secondary antibodies of different specificities; example: rabbit anti-protein X + mouse anti-protein Y primary antibodies.

   *The exception to this is when using IRDye® subclass-specific antibodies. IRDye Goat anti-Mouse IgG, Goat anti-Mouse IgG_{2a}, and Goat anti-Mouse IgG_{2b} allow for two-color detection using primary antibodies derived from the same species (mouse).

c. The two secondary antibodies used must be derived from the same host species so they will not react against one another. The secondary antibodies should not recognize immunoglobulins from other species that may be present in the sample; example: goat anti-rabbit IgG + goat anti-mouse IgG.

d. One secondary antibody should be labeled with IRDye 800CW, and the other with IRDye 680RD, IRDye 680LT, or other commercially available near-infrared dyes.

e. Always perform preliminary blots with each antibody alone to determine the expected banding pattern for each, before combining them in a two-color experiment. Slight cross-reactivity may occur, particularly if the antigen is very abundant, and can complicate interpretation of your blot. If cross-reactivity is a problem, load less protein or reduce the amount of antibody.

f. For best results, avoid using primary antibodies from mouse and rat together for a two-color experiment. Because the species are so closely related, it is not possible to completely adsorb away cross-reactivity. Substantial cross-reactivity between bands may occur. 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.

Protocol Modifications for Two-Color Detection

For two-color detection, follow **IV. In-Gel Western Detection Protocol**, with the following modifications:

1. Use two labeled secondary antibodies that are labeled with dyes that can be detected in two different channels; example: IRDye 680RD (700 nm) and IRDye 800CW (800 nm).
2. Make sure that antibody specificities and hosts are appropriate and will not cross-react.
3. Combine the two primary antibodies in antibody diluent in step 5, and incubate simultaneously with the gel.
4. Combine the two IRDye-labeled secondary antibodies in the antibody diluent in step 8. Incubate simultaneously with the gel.

VI. Optimization

The In-Gel detection protocol may require optimization for each target protein or gel type. Sensitivity of In-Gel Westerns may be lower than standard Western blots. (Transfer to a membrane concentrates the target protein, whereas in gels, protein is dispersed through the thickness of the gel.)

Use the following guidelines for optimization:

- Optimization of primary and secondary antibody dilutions, as well as amounts of Tween® 20, may be needed to achieve maximum signal and minimum background. Recommended Tween 20 concentration is 0.1%.
- Try different buffers for dilution of the antibodies, including PBST alone, Odyssey Blocking Buffer (LI-COR, P/N 927-40000), or milk. Changing the buffer solution may dramatically improve performance.
- To avoid background issues, use high-quality ultrapure water. Rinsing previously-used incubation boxes or trays with methanol can reduce background contamination on gels.
- For experiments utilizing streptavidin labeled with IRDye® Infrared Dyes, add 0.01% SDS in addition to Tween® 20 in the antibody diluents and wash buffer.

VII. Troubleshooting Guide

Problem	Possible Cause	Solution / Prevention
High background	Stacking gel is still present.	Cut the stacking gel away after electrophoresis.
	Too much antibody.	Reduce concentration of secondary antibody.
	Uneven gel background may result from insufficient solution volumes for incubations.	Use enough solution at each step (fixation, washes and antibody incubations) to completely immerse the gel.
	Pressing or squeezing gel during fixation and staining can cause splotchy background.	Handle gel gently, with gloved hands, and by the edges whenever possible.
	Gel was not thoroughly washed.	Use plenty of wash buffer to allow gel to move freely. Do not allow the gel to stick to bottom of container.
		Extend wash times or increase number of washes. Background may decrease if the gel is allowed to soak in PBS several hours to overnight at room temperature (protect from light).
	Contaminated scanning surface.	Before each use, apply methanol or ethanol followed by ultrapure water, and wipe with lint-free tissues to remove residual dye. Remove any visible smears with isopropanol. Use canned air to remove any lint or dust.

Problem	Possible Cause	Solution / Prevention
Weak or no signal	Not enough antibody.	Increase amount of primary and/or secondary antibody. Extend primary antibody incubation to overnight at 4 °C.
		Remember that In-Gel detection is not as sensitive as blot detection; adjust sample loading and antibody concentrations accordingly.
	Antibody dilution buffer is not optimal for your primary antibody.	Try a different dilution buffer; this can significantly affect performance of some primary antibodies.
		Suggested buffers include 3-5% BSA, Odyssey Blocking Buffer and PBS or TBS (all with 0.1% Tween® 20). Other blockers (milk, casein, commercial blockers) and Tween 20 concentrations can also be tested.
	Gel type is not optimal.	AMRESCO NEXT GEL®s or NuPAGE® Bis-Tris pre-cast gels are recommended for In-Gel detection. Other commercial gel sources and homemade gels can be used, but may show reduced sensitivity and require further optimization.
	Antibody did not penetrate gel sufficiently or evenly.	Acrylamide percentage was too high. Try a lower percentage or a gradient gel.
		Increase volume for antibody incubations so that gel is completely immersed in antibody solution.
		Make sure gel is adequately fixed. Some monoclonal antibodies may be sensitive to residual acid in the gel; in this situation, eliminate acetic acid from the fix or extend the water wash step.
	Gel was left in isopropanol/acetic acid too long.	This may cause protein to be lost from the gel. Fix for 15 minutes only.
Fuzzy or irregularly shaped bands.	Gel type is not optimal.	We recommend AMRESCO NEXT GELs or NuPAGE Bis-Tris pre-cast gels for In-Gel detection. Other commercial gel sources and homemade gels can be used, but may show reduced sensitivity and require further optimization.

Problem	Possible Cause	Solution/Prevention
	Gel is overloaded.	Try loading less protein; bands can appear "blobby" if the amount of target protein in the band is too high.
	Inadequate fixation of gel.	If problem persists when gel is fixed according to the protocol, try adjusting isopropanol or acetic acid concentrations. Fixing in isopropanol alone (no acetic acid) can cause irregularly shaped bands.
Non-specific or unexpected bands	Antibody concentration too high.	Reduce amount of antibody used or reduce incubation times.
	Cross-reactivity between antibodies in a two-color experiment.	Antibodies must be chosen carefully. Read **V. Guidelines for Two-Color Western Detection**.
	Antibody dilution buffer is not optimal for primary antibody.	Try a different dilution buffer; this can significantly affect performance of some primary antibodies.
		Suggested buffers include 3-5% BSA, Odyssey Blocking Buffer, and PBS or TBS (all with 0.1% Tween® 20).
	Bleedthrough between 700 nm and 800 nm channels.	If signal is extremely strong (saturated) in one channel, it may faintly appear in the other channel. Re-scan gel at a lower intensity or repeat using less antibody or protein.

LI-COR is an ISO 9001 registered company. © 2012 LI-COR Inc. LI-COR, IRDye, Smart and Odyssey are trademarks or registered trademarks of LI-COR, Inc. in the United States and other countries. All other trademarks belong to their respective owners. The Odyssey Infrared Imaging System and IRDye reagents are covered by U.S. patents, foreign equivalents, and patents pending.

LI-COR®

4647 Superior Street • P.O. Box 4000 • Lincoln, Nebraska 68504 USA
Technical Support: 800-645-4260 • North America: 800-645-4267
International: 402-467-0700 • Fax: 402-467-0819

LI-COR GmbH Germany, Serving Europe, Middle East, and Africa: +49 (0) 6172 17 17 771
LI-COR UK Ltd. UK, Serving UK, Ireland, and Scandinavia: +44 (0) 1223 422104
All other countries, contact LI-COR Biosciences or a local LI-COR distributor:
http://www.licor.com/distributors

www.licor.com/bio

Doc# 988-12954
11/12