In-Gel Western Detection Using Near-Infrared Fluorescence

LI-COR Biosciences

Abstract

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.

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.

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Guidelines

I. Reagents

Required Reagents:

- IRDye® Infrared Dye-labeled secondary antibodies (LI-COR)*
- NEXT GELTM (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)

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- 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

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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

See 'STEPS'

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

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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-proteinY primary antibodies.

**The exception to this is when using IRDye® subclass-specific antibodies. IRDye Goat anti-Mouse IgG, Goat anti-Mouse IgG2a, and Goat anti-Mouse IgG2b 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 800 CW, 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 crossreactivity 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 ('STEPS'), with the following modifications:

- 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).
- Make sure that antibody specificities and hosts are appropriate and will not cross-react.
- Combine the two primary antibodies in antibody diluent in step 5, and incubate simultaneously with the gel.
- Combine the two IRDye-labeled secondary antibodies in the antibody diluent in step 8.

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- 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 ofTween® 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 toTween® 20 in the antibody diluents and wash buffer.

Protocol

Electrophoresis

Step 1.

Separate the proteins of interest by electrophoresis.

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Margaret Dentlinger 21 Dec 2016

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Gel type will affect the success and sensitivity of In-Gel Western detection. Best resultscan 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.

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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.

In-Gel Western Detection Protocol

Step 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.

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

In-Gel Western Detection Protocol

Step 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.

DURATION
00:15:00

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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 finger- prints to appear in the image.

In-Gel Western Detection Protocol

Step 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.

DURATION
00:15:00

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

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In-Gel Western Detection Protocol

Step 5.

No blocking step is required before antibody incubations.

In-Gel Western Detection Protocol

Step 6.

Dilute primary antibody to the desired concentration in 5% BSA, Odyssey Blocking Buffer, or PBS (5% BSA is recommended).

In-Gel Western Detection Protocol

Step 7.

Include 0.1% Tween® 20 in the diluted antibody solution.

In-Gel Western Detection Protocol

Step 8.

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.

In-Gel Western Detection Protocol

Step 9.

Incubate gel for 1 hour with gentle shaking.

DURATION

01:00:00

In-Gel Western Detection Protocol

Step 10.

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

In-Gel Western Detection Protocol

Step 11.

Wash the gel for 10 minutes in PBS + 0.1%Tween 20 with gentle shaking, using a generous amount of wash buffer. (1/3)

DURATION

00:10:00

In-Gel Western Detection Protocol

Step 12.

Wash the gel for 10 minutes in PBS + 0.1%Tween 20 with gentle shaking, using a generous amount of wash buffer. (2/3)

DURATION

00:10:00

In-Gel Western Detection Protocol

Step 13.

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

DURATION

00:10:00

In-Gel Western Detection Protocol

Step 14.

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Dilute secondary antibody at 1:1000 – 1:5000 in the appropriate diluent with 0.1%Tween 20.

In-Gel Western Detection Protocol

Step 15.

Incubate gel in secondary antibody solution for 1 hour with gentle shaking, and protect from light.

DURATION

01:00:00

NOTES

Margaret Dentlinger 17 May 2017

Use enough antibody solution to completely cover gel.

In-Gel Western Detection Protocol

Step 16.

Wash the gel for 10 minutes in PBS + 0.1%Tween 20 with gentle shaking, using a generous amount of wash buffer. (1/3)

DURATION

00:10:00

In-Gel Western Detection Protocol

Step 17.

Wash the gel for 10 minutes in PBS + 0.1%Tween 20 with gentle shaking, using a generous amount of wash buffer. (2/3)

DURATION

00:10:00

In-Gel Western Detection Protocol

Step 18.

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

DURATION

00:10:00

In-Gel Western Detection Protocol

Step 19.

Wash the gel for 5 minutes in PBS.

DURATION

00:05:00

In-Gel Western Detection Protocol

Step 20.

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).

In-Gel Western Detection Protocol

Step 21.

If image background is high, the background may be reduced by soaking the gel several hours or overnight in PBS and re-scanning.

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Step 22.
Store the gel at 4 °C and protect from light. Gels can be kept in PBS at 4 °C for several days, if desired.

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