Protocol

In-Gel Western Detection Using Near-Infrared Fluorescence

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I. Introduction

In traditional Western blotting, certain target proteins do not transfer well. Large proteins or glycoproteins may get stuck in the gel, or small proteins may pass through the membrane. An In-Gel Western assay directly detects proteins in the polyacrylamide gel—without the need for transfer or blocking. Performing an In-Gel Western saves time, reduces cost, and eliminates variables introduced during the transfer step of a traditional Western blot.

In-Gel Western assays provide a useful tool for protein detection and research. Detection can be performed using an Odyssey® Imaging System and standard LI-COR reagents, such as IRDye® conjugates. No special kit is required. Two-color detection enables targeting of two different proteins in the same gel.

II. Reagents

Required Reagents

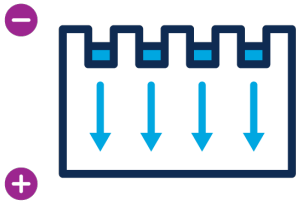
- IRDye Secondary Antibodies (licor.com/antibodies)
- VWR NEXT GEL® (VWRVM255, M256, or M257), Bis-Tris acrylamide gels, Tris-Glycine gels, or equivalent, for electrophoresis
- 50% isopropanol + 5% acetic acid solution (made with ultrapure water)
- Blocking buffer (5% BSA)
- Primary antibodies
- Tween® 20 detergent
- PBS or TBS buffer
- Ultrapure water

Optional Reagents

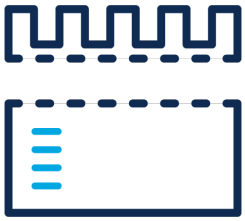
- Intercept® Blocking Buffer (licor.com/intercept)

III. Protocol Overview

Step 1. Perform electrophoresis

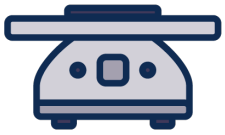


Step 2. Remove stacking gel

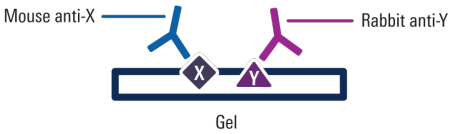


Step 3. Fix proteins in gel

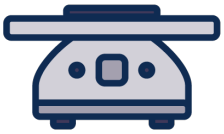
Step 4. Wash



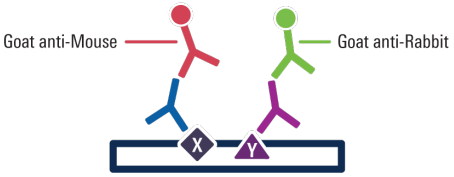
Step 5. Incubate in primary antibody



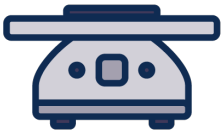
Step 6. Wash



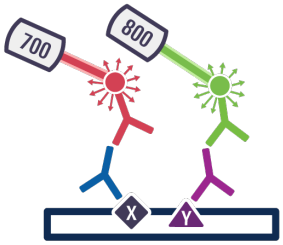
Step 7. Incubate in secondary antibody



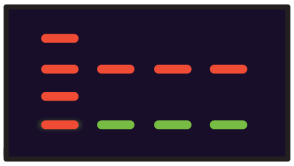
Step 8. Wash



Step 9. Excite with infrared lasers



Step 10. Acquire image using Odyssey® Imager and analyze data



IV. Protocol

This protocol highlights the main steps of an In-Gel Western assay.

Step 1. Perform Electrophoresis

Separate the proteins by electrophoresis. The type of gel used will affect the success and sensitivity of In-Gel Western detection. Optimal results can be obtained with VWR NEXT GEL® (for self-poured gels) or NuPAGE® Bis-Tris precast gels. The performance of different precast gels may vary. Other gel types can be used but may require optimization.

> **Note:** 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 mm.

> **Note:** Low molecular weight proteins may run near the front of blue Laemmli protein loading buffers. Depending on the molecular weight of your target protein, switching to LI-COR 4X Protein Loading Buffer buffer (licor.com/loadingbuffer) may improve detection of low molecular weight proteins.

Step 2. Remove Stacking Gel

After electrophoresis, separate the two plates and cut away any stacking gel above the highest molecular weight marker using a clean scalpel or razor blade. You can also notch one corner of the gel to help with orientation. The stacking gel exhibits high background when the gel is imaged.

> **Important:** Do not allow the gel to dry throughout the protocol. Always use clean gloves and incubation trays when handling the gel to avoid high background. Handle the gel gently as squeezing or pressing can cause splotches or fingerprints to appear in the image.

Step 3. Fix Proteins in Gel

Incubate the gel in 50% isopropanol + 5% acetic acid solution (prepared with ultrapure water) for 15 minutes with gentle shaking. Use enough solution to cover the gel completely and to allow the gel to move freely.

Step 4. Wash Gel

Pour off isopropanol/acetic acid solution and wash the gel in ultrapure water for 15 minutes with gentle shaking. Use enough water to cover the gel completely and to allow the gel to 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.

> **Note:** If needed, you can stop at this point and store the gel overnight in water at 4 °C.

Step 5. Dilute Primary Antibody

Dilute the primary antibody to the desired concentration in 3-5% BSA in PBS or TBS, Intercept® Blocking Buffer, or your blocking buffer of choice. Include 0.1% Tween® 20 in the diluted antibody solution.

Due to gel thickness, In-Gel Western detection is not as sensitive as a standard Western blot. Optimization of the primary antibody concentration may be needed.

Step 6. Incubate Gel in Diluted Primary Antibody

Incubate the gel in diluted primary antibody solution for at least 2 hours or up to overnight at 4 °C with gentle shaking to increase the signal. Use enough solution to cover the gel completely and to allow the gel to move freely.

Step 7. Wash Gel

Carefully pour off the primary antibody solution. Wash the gel 3 times for 10 minutes each time in PBS/TBS + 0.1% Tween 20 with gentle shaking. Use enough wash solution to cover the gel completely and to allow the gel to move freely.

Step 8. Dilute Secondary Antibody

Dilute the secondary antibody at 1:5000-1:10,000 using the same blocking buffer + 0.1% Tween 20 used in Step 5.

Step 9. Incubate Gel in Diluted Secondary Antibody

> **Note:** Protect the gel from light from this point on.

Incubate the gel in the diluted secondary antibody solution for at least 2 hours with gentle shaking. Use enough solution to cover the gel completely and to allow the gel to move freely.

Step 10. Wash Gel

> **Note:** Protect the gel from light during the wash.

Carefully pour off secondary antibody solution. Wash the gel 3 times for 10 minutes each time in PBS/TBS + 0.1% Tween® 20 with gentle shaking. Use enough solution to cover the gel completely and to allow the gel to move freely. Then wash the gel for 5 minutes in PBS/TBS.

> **Note:** Background from secondary antibodies is often high in an In-Gel Western. You may want to increase the wash to several hours or overnight to reduce the background.

Step 11. Scan Gel

> **Note:** Protect the gel from light prior to scanning.

Scan the gel on an Odyssey® Imaging System. Place the gel on the imaging surface. For Odyssey M, use the gel workflow to determine the resolution setting. For Odyssey DLx, CLx, Classic, and Sa (discontinued), set the resolution to 169 µm, the quality to lowest, and the focus offset to 1/2 the gel thickness (e.g., for a 1 mm gel, set the focus offset to 0.5 mm).

If the image background is high, soaking the gel for several hours or overnight in PBS/TBS may reduce the background. Store the gel at 4 °C and protect from light. Gels can be kept in PBS/TBS at 4 °C for several days.

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

> **Important:** It is absolutely critical that primary and secondary antibodies be carefully selected for two-color detection or cross-reactivity will result in spurious bands.

The following guidelines should be used when selecting primary and secondary antibodies for two-color detection:

- All secondary antibodies must be highly cross-adsorbed to eliminate cross-reactivity.
- The two primary antibodies used must be derived from different host species* so they can be discriminated by secondary antibodies of different specificity. For 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_{2a} and Goat anti-Mouse IgG_{2b} allow for two-color detection using primary antibodies derived from the same species (mouse) but must be evaluated for specificity.
- The two secondary antibodies used must not react against one another and should not recognize immunoglobulins from other species that may be present in the sample. For example: goat anti-rabbit IgG + goat anti-mouse IgG would be a correct pairing, but a goat anti-Rabbit IgG and a Rabbit anti-mouse IgG would not.
- One secondary antibody should be labeled with IRDye 800CW and the other with IRDye 680RD, IRDye 680LT, or other commercially available near-infrared dyes.
- Always perform preliminary gels 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 results. If cross-reactivity is a problem, load less protein or reduce the amount of antibody.
- 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 gels first with each individual antibody to be certain of expected band sizes.

Protocol Modifications for Two-Color Detection

The following modifications are necessary for two-color detection:

- Use two spectrally distinct secondary antibodies that are labeled with dyes that can be detected in two different channels. For example: IRDye® 680RD (700 nm) and IRDye 800CW (800 nm).
- Make sure that antibody specificity and hosts are appropriate and will not cross-react.
- Combine the two primary antibodies in the antibody diluent in Step 5, and incubate simultaneously with the gel.
- Combine the two secondary antibodies in the antibody diluent in Step 8, and incubate simultaneously with the gel.

VI. Optimization

The In-Gel Western detection protocol may require optimization for each target protein or gel type. Sensitivity of In-Gel Westerns is lower than standard Western blot. This is because transfer to a membrane concentrates the target protein. 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 or Intercept® Blocking Buffer. Changing the buffer solution may dramatically improve performance. Just be sure to use the same buffer for both primary and secondary antibodies.
- 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 secondary antibody diluents and wash buffer.

VII. Troubleshooting

Below are some steps you can take if you run into problems with your In-Gel Western assay.

Problem: High background

Possible Cause	Solution / Preventative Measure
Stacking gel is still present	Cut the stacking gel away after electrophoresis.
Too much antibody	Reduce the 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 the gel to move freely. Do not allow the gel to stick to the bottom of the container. Extend wash times or increase number of washes. Background may decrease if the gel is allowed to soak in PBS/TBS several hours or even overnight at room temperature.
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: Weak or no signal

Possible Cause	Solution / Preventative Measure
Not enough antibody	Increase the amount of primary and/or secondary antibody. Extend primary antibody incubation to overnight at 4 °C. Remember that In-Gel Western detection is not as sensitive as blot detection because the protein is dispersed through the thickness of the gel. 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, Intercept® Blocking Buffer, and PBS/TBS (all with 0.1% Tween® 20). Other blockers (casein, commercial blockers) and Tween 20 concentrations can also be tested.
Gel type is not optimal	VWR NEXT GEL® or NuPAGE® Bis-Tris precast gels are recommended for In-Gel Western detection. Other commercial gel sources and homemade gels can be used, but may show reduced sensitivity and may 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 the gel is completely immersed in antibody solution. Make sure the gel is adequately fixed. Some monoclonal antibodies may be sensitive to residual acid in the gel. In this situation, extend the water wash step for up to overnight.

Problem: Fuzzy or irregularly shaped bands

Possible Cause	Solution / Preventative Measure
Gel type is not optimal	We recommend VWR NEXT GEL® or NuPAGE® Bis-Tris precast gels for In-Gel Western detection. Other commercial gel sources and homemade gels can be used, but may show reduced sensitivity and may require further optimization.
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 the problem persists when the 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.

Problem: Non-specific or unexpected bands

Possible Cause	Solution / Preventative Measure
Antibody concentration too high	Reduce the amount of primary antibody used or reduce incubation times.
Cross-reactivity between antibodies in a two-color experiment	Antibodies must be carefully selected using the "Guidelines for Two-Color In-Gel Western Detection" on page 8.
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, Intercept® Blocking Buffer, and PBS/TBS (all with 0.1% Tween® 20). Other blockers (casein, commercial blockers) and Tween 20 concentrations can also be tested.
Bleed-through between 700 nm and 800 nm channels	If signal is extremely strong in one channel, it may faintly appear in the other channel. Re-scan the gel at a lower intensity or repeat using less antibody or protein.

VIII. Protocol Checklist

Below is a checklist you can remove or print to guide you through the protocol.

- ☐ Perform electrophoresis
- ☐ Remove stacking gel
- ☐ Fix proteins in gel

  Incubate in 50% isopropanol + 5% acetic acid solution (prepared with ultrapure water) for 15 minutes with gentle shaking.

- ☐ First wash

  Wash in ultrapure water for 15 minutes with gentle shaking.

- ☐ Dilute primary antibody
- ☐ Incubate in primary antibody

  Incubate for at least 2 hours or up to overnight at 4 °C with gentle shaking.

- ☐ Second wash

  Wash 3 times for 10 minutes each time in PBS/TBS + 0.1% Tween® 20 with gentle shaking.

- ☐ Dilute secondary antibody
- ☐ Incubate in secondary antibody

  Incubate for at least 2 hours with gentle shaking.

- ☐ Third wash

  Wash 3 times for 10 minutes each time in PBS/TBS + 0.1% Tween 20 with gentle shaking. Then wash for 5 minutes in PBS/TBS

- ☐ Scan gel

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