Western Blot and In-Cell Western™ Assay Detection Using IRDye® Subclass Specific Antibodies

Developed for:

Odyssey® Family of Imagers

*Please refer to your manual to confirm that this protocol is appropriate for the applications compatible with your model of Odyssey Imager.*
I. Introduction

This Technical Note is a guide to using IRDye Subclass Specific antibodies for Western blotting and In-Cell Western™ Assay (ICW) applications. For more detailed descriptions of Western blotting and/or ICW techniques, refer to Western Blot Analysis and In-Cell Western Kits I and II on the LI-COR® Biosciences website (www.licor.com).

IRDye Goat anti-Mouse IgG1, Goat anti-Mouse IgG2a, and Goat anti-Mouse IgG2b, allow for two-color detection using primary antibodies derived from the same species (mouse). IRDye Subclass Specific antibodies react with the heavy (gamma) chain only of the primary antibody. In mice, there are five unique subclasses of IgG; IgG1, IgG2a, IgG2b, IgG2c and IgG3. Each subclass is based on small differences in amino acid sequences in the constant region of the heavy chains so antibodies directed against a particular subclass will not recognize antibodies directed against other subclasses. For example, IRDye goat anti-mouse IgG1 recognizes mouse gamma 1; it will not recognize mouse gamma 2a, 2b, 2c or gamma 3. All other LI-COR IRDye secondary antibodies are whole IgG (H + L) and react with the heavy (gamma) and light (kappa or lambda) chains of the primary antibody. Figure I demonstrates the differences in detection between the IRDye antibodies.

![Figure I](image-url)

*Figure I.* Western blot detection of various purified subclasses. Each lane was loaded with 50 ng of antibody. Blots were detected with IRDye Subclass Specific antibodies or IRDye whole IgG.
Antibody Subclasses may also be designated by their light chains. There are two types of light chains, kappa (κ) or lambda (λ). In mice, 95% of light chains are kappa and 5% are lambda. These subclasses still contain the heavy (gamma) portion of the antibody so IRDye Subclass Specific antibodies still recognize them. If the subclass of the primary antibody is unknown, LI-COR® whole IgG secondary antibodies may be used since they recognize most mouse IgG subclasses.

II. Suggested Materials

This section is intended as a guideline; other materials may be substituted, if desired.

- Proteins transferred to a nitrocellulose or PVDF membrane (for Western blot only)
- Cells that have been fixed and permeabilized on a 96 well plate (for ICW only)
- Odyssey® Blocking Buffer
- 10X PBS
- 20% Tween® 20
- SDS (if using PVDF membrane)
- Suggested mouse primary antibodies for normalization:
  - Beta-Actin Mouse mAb IgG2b (LI-COR P/N 926-42212)
  - Alpha-Tubulin Mouse mAb IgG1 (LI-COR P/N 926-42213)
- One or two of the following IRDye secondary antibodies

<table>
<thead>
<tr>
<th>Description</th>
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<tr>
<td>IRDye 800CW Goat anti-Mouse IgG1 Specific</td>
<td>926-32350</td>
</tr>
<tr>
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<td>926-32351</td>
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III. Western Blot Detection

IRDye Subclass Specific antibodies are easily incorporated into the detection step of any Western blot protocol. The sample protocol provided below, optimized for LI-COR reagents, is recommended. After protein transfer to the membrane is complete, perform the following steps for one- or two-color detection:

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 ultrapure water before incubating in PBS.

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/cm² is suggested).

3. Dilute primary antibody in Odyssey Blocking Buffer. Optimum dilution depends on the antibody and should be determined empirically. A suggested starting range can usually be found in the product information from the vendor. To lower background, add Tween® 20 to the diluted antibody at a final concentration of 0.1 – 0.2% prior to incubation. **Note:** *If performing two-color detection, dilute primary antibodies together in the same buffer.*
4. Incubate blot in primary antibody solution for a minimum of 60 minutes 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 IRDye Subclass Specific antibody in Odyssey® Blocking Buffer. Avoid prolonged exposure of the antibody vial to light. Recommended dilution can be found in the pack insert for the IRDye conjugate. Add the same amount of Tween 20 to the diluted secondary antibody as was added to the primary antibody. **Note:** **If performing two-color detection, dilute secondary antibodies simultaneously in the same buffer.** Adding SDS to the diluted secondary antibody at a final concentration of 0.01% - 0.02% will substantially reduce membrane background when using PVDF membrane.

7. Incubate blot in secondary antibody solution for 30-60 minutes at room temperature with gentle shaking. Protect from light during incubation.

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 (no detergent) to remove residual Tween 20. The membrane is now ready to image.

**IV. Two-Color Western Blot Considerations**

Two different antigens can be detected simultaneously on the same blot using IRDye Subclass Specific OR IRDye whole IgG antibodies 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 with the design of two-color experiments:

- If the two primary antibodies are monoclonals (mouse) and are IgG1, IgG2a or IgG2b, IRDye Subclass Specific secondary antibodies must be used. The same subclasses cannot be combined in a two-color Western blot (for example, two IgG1 primary antibodies).

- If the two primary antibodies are derived from different host species (for example, primary antibodies from mouse and chicken), IRDye whole IgG secondary antibodies derived from the same host and labeled with different IRDye fluorophores must be used (for example, IRDye 800CW Donkey anti-mouse and IRDye 680LT Donkey anti-chicken).

- 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 non-specific background bands.

Figures II (A-C) and III (A-C) demonstrate two-color Western blot detection using (A) IRDye Subclass Specific antibodies and (B) IRDye whole IgG antibodies, respectively. IRDye Subclass Specific secondary antibodies should NOT be used in combination with IRDye whole anti-mouse IgG secondary antibodies for two-color detection. IRDye whole anti-mouse IgG secondary antibodies and IRDye Subclass Specific secondary antibodies both recognize the gamma chain of the primary antibody, causing detection in both channels (C). IRDye Subclass Specific antibodies can be used in combination with IRDye whole goat anti-rabbit secondary antibodies.
Figure II. Western blot analysis of PTEN expression in mouse PTEN transfected 293T whole cell lysate (Lane 2) and non-transfected 293T lysate (Lane 3). Both lysates were loaded with 2 μg total protein per lane. LI-COR® Molecular Weight Marker is loaded in Lane 1 (LI-COR P/N 928-40000).

A. Blot was probed with mouse anti-PTEN (IgG2b) and mouse anti-GAPDH (IgG1) for normalization. The blot was detected with IRDye 800CW GAM IgG2b (LI-COR P/N 926-32352) and IRDye 680LT GAM IgG1 (LI-COR P/N 926-68050).

B. Blot was probed with mouse anti-PTEN (IgG2b) and chicken anti-GAPDH for normalization. The blot was detected with IRDye 800CW DAM (LI-COR P/N 926-32212) and IRDye 680LT DAC (LI-COR P/N 926-68028).

C. Blot was probed with mouse anti-PTEN (IgG2b) and mouse anti-GAPDH (IgG1) for normalization. The blot was detected with IRDye 800CW GAM (LI-COR P/N 926-32210) and IRDye 680LT GAM IgG1 (LI-COR P/N 926-68050).

Note: Apparent MW differences in GAPDH between lanes 2 and 3 could be due to post-translational differences (e.g., glycosylation, nitrosylation, glutathionylation) between cell lines. Colell, A., et.al., Cell Death and Differentiation (2009) 16, 1573-1581.

Figure III. Illustrations of detection mechanisms from the corresponding Western blots in Figure II. The yellow bands on blot C in Figure II are the cross-reactivity that occurs from combining IRDye 680LT GAM IgG1 with IRDye 800CW GAM for two-color detection.

Two-color Western blot detection can be achieved by multiplexing LI-COR® mouse primary antibodies and IRDye Subclass Specific antibodies. Figure IV demonstrates two-color detection utilizing the LI-COR mouse primaries and IRDye Subclass Specific secondaries.
V. ICW Detection and Considerations

In-Cell Western™ assays commonly use primary and secondary antibodies for normalization in the 700 channel. For example, if phospho-ERK is the target of interest, an antibody against total ERK (or against a housekeeping protein) can be used to normalize for variations in cell number. IRDye Subclass Specific antibodies can be incorporated into the detection step of any ICW protocol. A recommended protocol is provided below. After cells have been fixed and permeabilized, perform the following steps:

1. Using a multi-channel pipettor, block cells by adding 150 μL of Odyssey® Blocking Buffer to each well. **Add the solution carefully by pipetting down the sides of the wells to avoid detaching the cells.**

2. **Allow blocking for 1.5 hours at room temperature with gentle shaking on a plate shaker.**

3. Dilute desired primary antibodies in Odyssey Blocking Buffer or other appropriate blocker. As a general guideline, 1:50 to 1:200 dilutions are recommended, depending on the primary antibody. If the antibody supplier provides dilution guidelines for immunofluorescent staining, start with that recommended range.
   - **It is important to include control wells that DO NOT contain primary antibody. These wells will be treated with secondary antibody only, and should be used to correct for background staining in the data analysis.**
   - a. Remove blocking buffer from step 1.
   - b. Add 50 μL of Odyssey Blocking Buffer to the control wells and 50 μL of the desired diluted primary antibodies in Odyssey Blocking Buffer to the rest of the wells.

4. Incubate with primary antibody solution for 2 hours at room temperature with gentle shaking, or overnight at 4°C with no shaking.

5. **Wash the plate five times with 1X PBS + 0.1% Tween® 20 for 5 minutes at room temperature with gentle shaking, using a generous amount of buffer:**
   - a. Prepare Tween Washing Solution by adding 5 mL of 20% Tween 20 to 995 mL of 1X PBS.
   - b. Using a multi-channel pipettor, add 200 μL of Tween Washing Solution. **Make sure to carefully add the solution down the sides of the wells to avoid detaching the cells from the well bottom.**
   - c. Allow wash to shake gently on a plate shaker for 5 minutes.
   - d. Repeat washing steps 4 more times.

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**Figure IV.** Two-color Western blot detection using LI-COR® mouse primary antibodies. Two different blots were prepared using HeLa and COS7 whole cell lysates. Both lysates were loaded at 5 μg total protein per lane.

A. The blot was probed with Beta-Actin Mouse mAb IgG2b (LI-COR P/N 926-42212) and p53 mouse mAb IgG2b. The blot was detected with IRDye 800CW GAM IgG2a and IRDye 680LT GAM IgG2b.

B. The blot was probed with Alpha-Tubulin Mouse mAb IgG1 (LI-COR P/N 926-42213) and ERK2 mouse mAb IgG2b. The blot was detected with IRDye 680LT GAM IgG1 and IRDye 800CW GAM IgG2b.
6. Dilute the IRDye labeled Subclass Specific antibodies in Odyssey® Blocking Buffer or other appropriate blocker. The recommended dilution range is 1:200 to 1:1,200. The optimal dilution for your assay should be determined empirically. To lower background, add Tween® 20 at a final concentration of 0.2% to the diluted antibody. Secondary antibody staining is carried out simultaneously. **Avoid prolonged exposure of the antibody vials to light.**

7. Add 50 μL of secondary antibody solution into all wells. Incubate for 1 hour at room temperature, with gentle shaking. **Protect plate from light during incubation.**

8. Wash the plate five times with 1X PBS + 0.1% Tween 20 for 5 minutes at room temperature with gentle shaking, using a generous amount of buffer:
   a. Using a multi-channel pipettor, add 200 μL of Tween Washing Solution. **Make sure to carefully add the solution down the sides of the wells to avoid detaching the cells from the well bottom.**
   b. Allow wash to shake gently on a plate shaker for 5 minutes.
   c. Repeat washing steps 4 more times. **Protect plate from light during washing.**
   The plate is now ready to image.

The same considerations for two-color Western blot detection apply to two-color In-Cell Western™ detection with the following addition:

- Choose primary antibodies that have been recommended for other immunofluorescence techniques such as IF-IC and IHC.
- Establish the specificity of the primary antibody by screening lysates through Western blotting and detection on an Odyssey Imaging system. To achieve the most consistent results, use the same blocking buffer for validation experiments and In-Cell Western assays. If significant non-specific binding is detected on a Western blot, choose alternative primary antibodies. Non-specific binding of primaries will complicate interpretation of In-Cell Western assay results.

Figures V and VI demonstrate In-Cell Western Assay data generated using MitoSciences’ Mito-Biogenesis™ In-Cell ELISA Kit (IR). The kit utilizes IRDye Subclass Specific antibodies for detection.

**Figure V. Inhibition of mitochondrial biogenesis by Chloramphenicol**

The IC₅₀ of a drug’s effect on mitochondrial protein translation was determined using the MitoBiogenesis In-Cell ELISA Kit (IR). In this example, HepG2 cells were seeded at 3000 cells/well and allowed to grow for 3 cell doublings in a drug dilution series. Cells were fixed in a 96-well plate and targets of interest (COX-I and SDH-A) were detected with highly specific, well-characterized monoclonal antibodies supplied in the kit. The plate was scanned using an Odyssey imaging system. Average intensity values for each set of replicates were determined for COX-I and SDH-A and background subtracted (no primary antibody). Relative signal values were determined by normalizing the COX-I average intensity values to the SDH-A average intensity values. Chloramphenicol inhibits mtDNA-encoded COX-I protein synthesis relative to nuclear DNA-encoded SDH-A protein synthesis by 50% at 1.8 μM.
Figure VII compares IRDye whole IgG vs. IRDye Subclass Specific antibody detection by ICW. Extracellular-signal related kinase (ERK) phosphorylation was measured following the LI-COR protocol entitled, *In-Cell Western Assay For Assessing Response of A431 Cells to Stimulation with Epidermal Growth Factor*. This document can be found on the LI-COR website (Doc# 988-11453). All primary antibodies were qualified by Western blot prior to ICW (data not shown).

Figure VI. Odyssey image of In-Cell ELISA
COX-I detection is shown in green (800 channel) and SDH-A detection is shown in red (700 channel). COX-1 protein synthesis decreases with increasing amounts of Chloramphenicol.

**Figure VII. Dose response data**
ERK phosphorylation is graphed relative to ERK2 (blue) or ERK1 (red). The ERK2 data was generated using mouse anti-pERK (IgG2a) and mouse anti-ERK2 (IgG2b) followed by detection with IRDye 800CW GAM IgG2a (LI-COR P/N 926-32351) and IRDye 680LT GAM IgG2b (LI-COR P/N 926-68052). ERK1 data was generated using mouse anti-pERK (IgG2a) and rabbit anti-ERK1 followed by detection with IRDye 800CW GAM (LI-COR P/N 926-32210) and IRDye 680LT GAR.