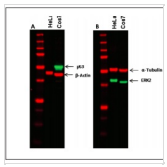

Western Blot Detection

LI-COR Biosciences[1]

[1]LI-COR Biosciences

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LI-COR Biosciences

Working

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ABSTRACT

This protocol is a guide to using IRDye Subclass Specific antibodies for Western blotting. For more detailed descriptions of Western blotting, refer to Western Blot Analysis and In-Cell Western Kits I and II on the LI-COR Biosciences website (www.licor.com).

Developed for: Odyssey® Family of Imagers

EXTERNAL LINK

https://www.licor.com/documents/86xilzixljcaz6rreqy9fx8dwz4rps55

TechNote_IRWesternBlot_ICW_SubclassSpecAb_0311_11784.pdf

PROTOCOL STATUS

Working

We use this protocol in our group and it is working

GUIDELINES

I. Introduction

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; IgG_1, IgG_{2a}, IgG2b, IgG_{2c} and IgG_3. 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 IgG_1 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.

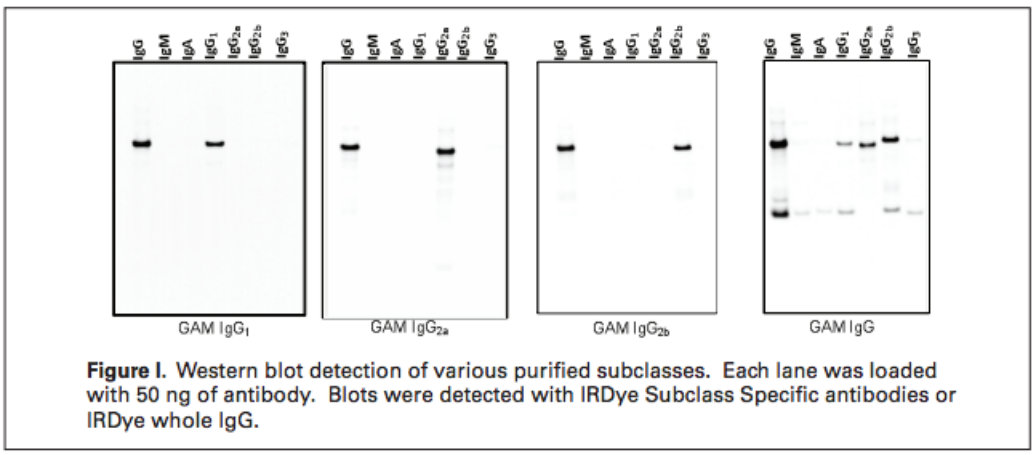

Figure I. Western blot detection of various purified subclasses. Each lane was loaded with 50 ng of antibody. Blots were detected with IRDye Subclass Specific antibodies or IRDye whole IgG.

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 IgG_{2b} (LI-COR P/N 926-42212)
  - Alpha-Tubulin Mouse mAb IgG_1 (LI-COR P/N 926-42213)
- One or two of the following IRDye secondary antibodies

Description	LI-COR Part Number
IRDye 800CW Goat anti-Mouse IgG_1 Specific	926-32350
IRDye 800CW Goat anti-Mouse IgG_{2a} Specific	926-32351
IRDye 800CW Goat anti-Mouse IgG_{2b} Specific	926-32352
IRDye 680LT Goat anti-Mouse IgG_1 Specific	926-68050
IRDye 680LT Goat anti-Mouse IgG_{2a} Specific	926-68051
IRDye 680LT Goat anti-Mouse IgG_{2b} Specific	926-68052

III. Western Blot Detection

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

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 IgG_1, IgG_{2a} or IgG_{2b}, IRDye Subclass Specific secondary antibodies must be used. The same subclasses cannot be combined in a two-color Western blot (for example, two IgG_1 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.

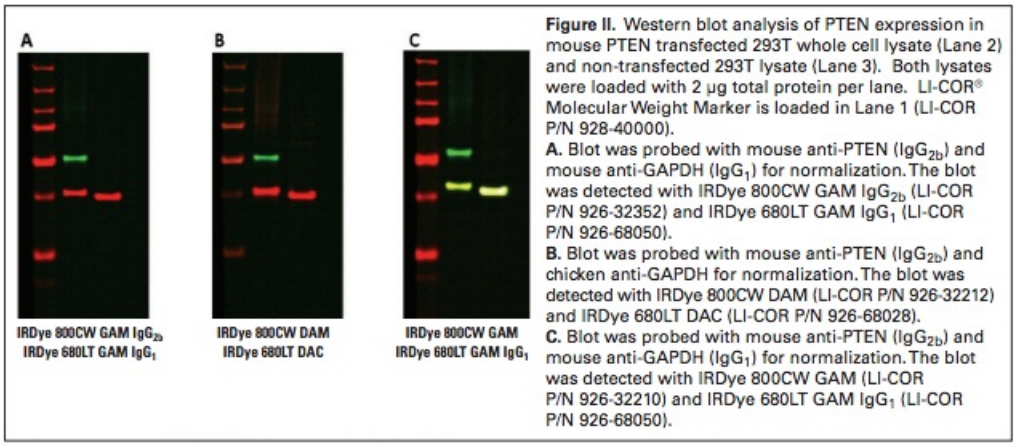

Figure II. Western blot analysis of PTEN expression in mouse PTEN transfected 293T whole cell lysate (Lane 2) and non-transfected 293T lysate (Lane 3). Both lysates were loaded with 2 µg total protein per lane. LI-COR® Molecular Weight Marker is loaded in Lane 1 (LI-COR P/N 928-40000).
A. Blot was probed with mouse anti-PTEN (IgG_{2b}) and mouse anti-GAPDH (IgG_1) for normalization. The blot was detected with IRDye 800CW GAM IgG_{2b} (LI-COR P/N 926-32352) and IRDye 680LT GAM IgG_1 (LI-COR P/N 926-68050).
B. Blot was probed with mouse anti-PTEN (IgG_{2b}) 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 (IgG_{2b}) and mouse anti-GAPDH (IgG_1) for normalization. The blot was detected with IRDye 800CW GAM (LI-COR P/N 926-32210) and IRDye 680LT GAM IgG_1 (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.*

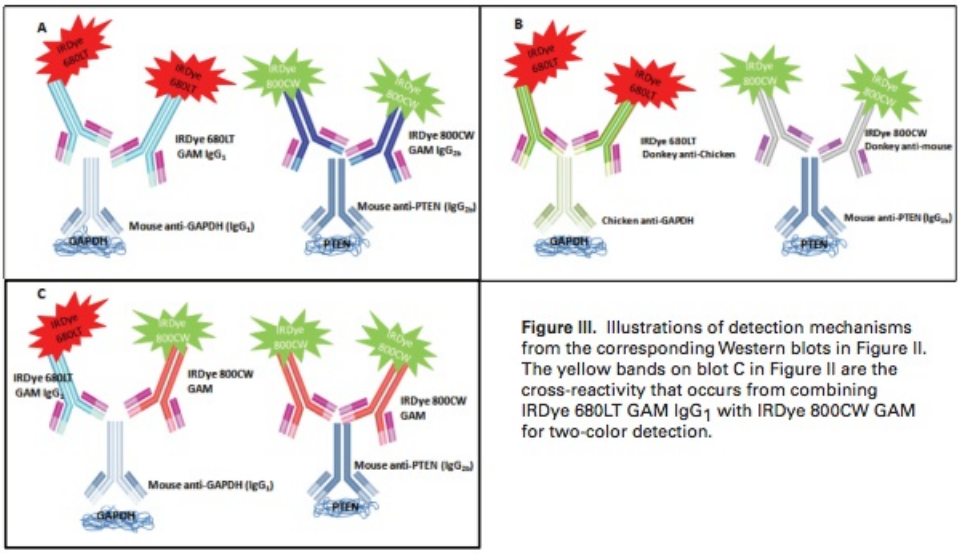

Figure III. Illustrations of detection mechanisms from the corresponding Western blots in Figure II. The yellow bands on blot C in Figure II are the cross-reactivity that occurs from combining IRDye 680LT GAM IgG_1 with IRDye 800CW GAM for two-color detection.

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.

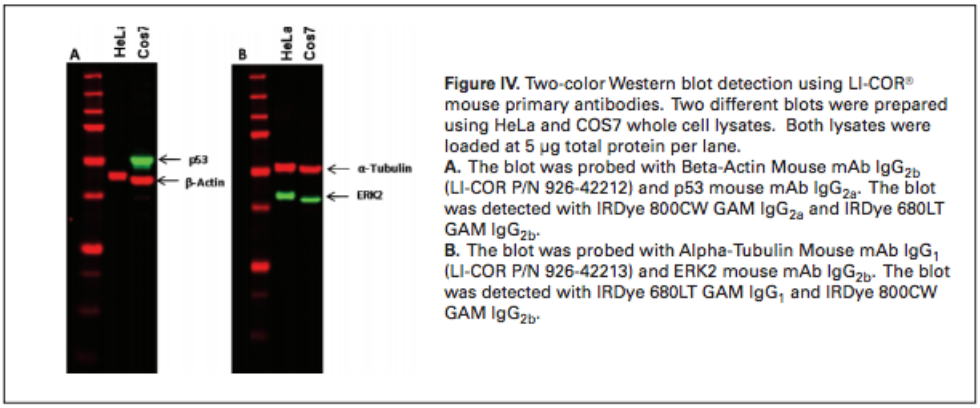

Figure IV. Two-color Western blot detection using LI-COR® mouse primary antibodies. Two different blots were prepared using HeLa and COS7 whole cell lysates. Both lysates were loaded at 5 µg total protein per lane.
A. The blot was probed with Beta-Actin Mouse mAb IgG_{2b} (LI-COR P/N 926-42212) and p53 mouse mAb IgG_{2a}. The blot was detected with IRDye 800CW GAM IgG_{2a} and IRDye 680LT GAM IgG_{2b}.
B. The blot was probed with Alpha-Tubulin Mouse mAb IgG_1 (LI-COR P/N 926-42213) and ERK2 mouse mAb IgG_{2b}. The blot was detected with IRDye 680LT GAM IgG_1 and IRDye 800CW GAM IgG_{2b}.

MATERIALS

NAME	CATALOG #	VENDOR
Beta-Actin Mouse mAb IgG2b	926-42212	LI-COR
Alpha-Tubulin Mouse mAb IgG1	926-42213	LI-COR
IRDye 800CW Secondary Antibodies	926-32350 926-32351 926-32352	LI-COR
IRDye 680LT Secondary Antibodies	926-68050 926-68051 926-68052	LI-COR

SAFETY WARNINGS

See SDS (Safety Data Sheets) for warnings and hazards.

1 Wet the membrane in PBS for several minutes.

NOTE
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/cm^2 is suggested).

01:00:00

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
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.

01:00:00 **minimum incubation at room temp**

NOTE
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.(1/4)

00:05:00

6 Wash membrane for 5 minutes each at room temperature in PBS + 0.1% Tween® 20 with gentle shaking, using a generous amount of buffer.(2/4)

00:05:00

7 Wash membrane for 5 minutes each at room temperature in PBS + 0.1% Tween® 20 with gentle shaking, using a generous amount of buffer.(3/4)

00:05:00

8 Wash membrane for 5 minutes each at room temperature in PBS + 0.1% Tween® 20 with gentle shaking, using a generous amount of buffer.(4/4)

00:05:00

9
- 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

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.

10
- Incubate blot in secondary antibody solution for 30-60 minutes at room temperature with gentle shaking.
- **Protect from light during incubation.**

01:00:00

11 Wash membrane 4 times for 5 minutes each at room temperature in PBS + 0.1% Tween 20, with gentle shaking. **Protect from light** .(1/4)

00:05:00

12 Wash membrane for 5 minutes each at room temperature in PBS + 0.1% Tween 20, with gentle shaking. Protect from light.(2/4)

00:05:00

13 Wash membrane for 5 minutes each at room temperature in PBS + 0.1% Tween 20, with gentle shaking. Protect from light.(3/4)

00:05:00

14 Wash membrane for 5 minutes each at room temperature in PBS + 0.1% Tween 20, with gentle shaking. Protect from light.(4/4)

00:05:00

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