Technical Note

Detection of post-immunoprecipitation proteins by Western blot using the Quick Western Kit–IRDye® 680RD

Developed for:

Aerius, Odyssey® Classic, Odyssey CLx and Odyssey Sa Imaging Systems

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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I. INTRODUCTION

Immunoprecipitation (IP) is a valuable tool that utilizes antibody:antigen interactions to isolate a specific target protein from thousands of proteins in a complex solution. The specificity of the reaction increases the concentration of dilute proteins as high as 10,000-fold; a property that renders the technique useful in examination of rare proteins. With careful choice of antibodies, a co-IP can also be used to demonstrate protein interaction partners*.

The typical workflow for an IP can be divided into four parts:

1. Incubation of the primary antibody with the sample (e.g., a cell lysate);

2. Capture of the antibody:antigen complex using a solid support such as Protein A agarose beads;

3. Separation of the proteins by SDS-PAGE;

4. Detection of the target protein by Western blot.

The technique is time-consuming and often requires optimization of antibody and lysate concentrations as well as the inclusion of appropriate positive and negative controls. Ultimately, success relies on the choice of antibodies, not only for the IP step, but also for Western blot detection of post-IP proteins. A common solution to avoid detection of the heavy and light chains from the IP step in the Western blot is to use two separate antibodies to the same target, but from different host species. An example would be using a mouse capture antibody for IP, and a rabbit antibody for post-IP Western blot detection. The LI-COR® Quick Western Kit-IRDye 680RD eliminates the need for two separate antibodies in the IP workflow. The proprietary Detection Reagent does not recognize denatured heavy or light chains from monoclonal antibodies; therefore, the same monoclonal antibody can be used for the IP step AND post-IP Western blot detection. In addition, the Quick Western Protocol saves 90 minutes of assay time compared to a traditional two-step Western blot protocol*.

**NOTE: The data, procedure, and troubleshooting outlined in this Technical Note are provided as guidelines. A complete discussion of the IP technique and the Western blot technique are beyond the scope of this material.*

The following data simulate post-IP Western blot detection utilizing traditional IRDye secondary antibody detection (panel A) compared with Quick Western Kit-IRDye 680RD detection (panel B). The traditional detection method recognizes the denatured heavy and/or light chains of the IP antibodies. The Quick Western Kit detection method does not recognize any denatured heavy and light chains from the IP antibodies evaluated. This lack of recognition is the foundation for single antibody IP and post-IP Western blot detection.

NOTE: The Detection Reagent will recognize denatured heavy or light chains of certain polyclonal antibodies. It will not recognize denatured heavy or light chains of monoclonal (mouse or rabbit) antibodies.

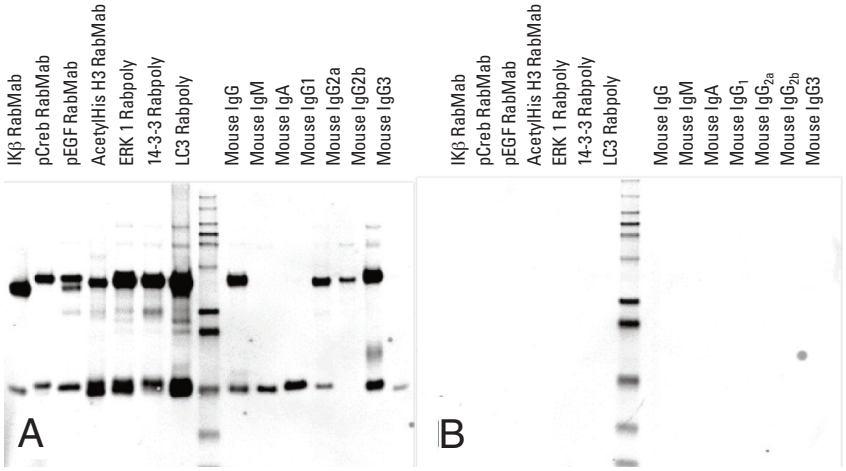


Figure 1. 100 ng of each antibody was denatured in Protein Sample Loading Buffer, heated for 5 minutes at 100°C, cooled, and then loaded in duplicate on 10% Bis-Tris gels. The proteins were transferred to nitrocellulose and detected with either IRDye 680RD Goat anti-Rabbit (Panel A) or Quick Western Kit- IRDye 680RD (Panel B).

II. REQUIRED REAGENTS

- Quick Western Kit-IRDye 680RD (LI-COR, P/N 926-68100)
- A431 Cell Lysate in 50 mM Tris-HCl pH7.8, 150 mM NaCl, 1% NP-40, 0.2% Sodium Deoxycholate, 1X Halt Protease Inhibitor
- Protein A Beads (Thermo Fisher, P/N 20333)
- Anti-ERK2 Mouse Antibody (Santa Cruz Biotechnology, P/N SC1647)
- 1X PBS
- Tween® 20
- 4X Protein Sample Loading Buffer (LI-COR, P/N 928-40004). Dilute to 2X with water.
- 26.5-gauge needle
- 3 mL syringe
- 20X MES Buffer (Invitrogen P/N NP0002)

- NuPAGE® Antioxidant (Invitrogen P/N NP0005)
- NuPAGE 10% Bis-Tris gels (Invitrogen P/N NP0303BOX)
- Odyssey® Protein Molecular Weight Marker (LI-COR® P/N 928-40000)
- Tris-Glycine Buffer (LI-COR P/N 928-40010)
- Nitrocellulose (LI-COR P/N 926-31092)
- Odyssey Blocking Buffer (LI-COR P/N 927-40000)
- IRDye 680RD Goat anti-Mouse (LI-COR P/N 926-68070)

III. SAMPLE PROTOCOL: IMMUNOPRECIPITATION AND DETECTION OF ERK2

NOTE: This is intended as a general guideline for an IP and Western blot procedure. Different targets will require optimization of antibody concentrations, lysate concentrations, and incubation times. In addition, solid supports other than Protein A agarose beads may be substituted.

IMPORTANT: It is critical that the antibody chosen is recommended for both immunoprecipitation AND Western blot by the primary antibody vendor. This information can be found in the primary antibody pack insert or on the vendor's website.

WARNING: Mouse IgG_1 subclass antibodies may not work in this procedure, or may require additional optimization.

Sample Preparation (Day 1)

1. Starting with a total of 820 µg of A431 cell lysate, transfer 200 µg into each of 4 separate 1.5 mL microcentrifuge tubes and place the four tubes on ice.

2. Add 5 µL ERK2 antibody to 2 of the tubes and label as "IP".

3. Add 5 µL 1X PBS to the remaining 2 tubes and label as "negative control". Mix all tubes well.

4. Move all 4 tubes to 4°C overnight with gentle shaking.

5. Transfer the remaining 20 µg (10 µL) aliquot of A431 lysate to a 1.5 mL microcentrifuge tube. Add an equal volume of 2X diluted Protein Sample Loading Buffer and store at -20°C until use. Label as "positive control".

Bead Preparation (Day 2)

6. Add 150 µL of Protein A bead (50:50) slurry to a 1.5 mL microcentrifuge tube and centrifuge 1 minute at highest speed in a microcentrifuge. Remove supernatant with a 26.5-gauge needle and 3 mL syringe. Discard supernatant.

7. Add 1 mL of 1X PBS, making sure the pellet is disrupted, then centrifuge at high speed as in step 6.

8. Repeat step 7 two more times.

9. Resuspend final pellet in 150 µL 1X PBS.

Pull Down of Bead:Protein Complex

10. Add 30 µL of bead slurry (prepared in step 9) to each tube (from step 4) and incubate 2 hours at room temperature with rocking on a Nutator™ mixer.
11. Centrifuge on highest speed for 1 minute to pellet the beads. Immediately remove the supernatant with a 26.5-gauge needle and 3 mL syringe.
12. Wash the beads 3 times with 1 mL of 1X PBS each, pelleting the beads and removing the supernatant after each wash with the needle and syringe. Discard supernatant.
13. Wash the beads 1 time with 1 mL of 1X PBS + 0.1% Tween® 20, pellet, and carefully remove ALL liquid from the tube. Discard supernatant.
14. Resuspend the beads in 10 µL of 2X diluted Orange Protein Sample Loading Buffer and heat 5 minutes at 100°C.
15. Cool tubes immediately on ice.

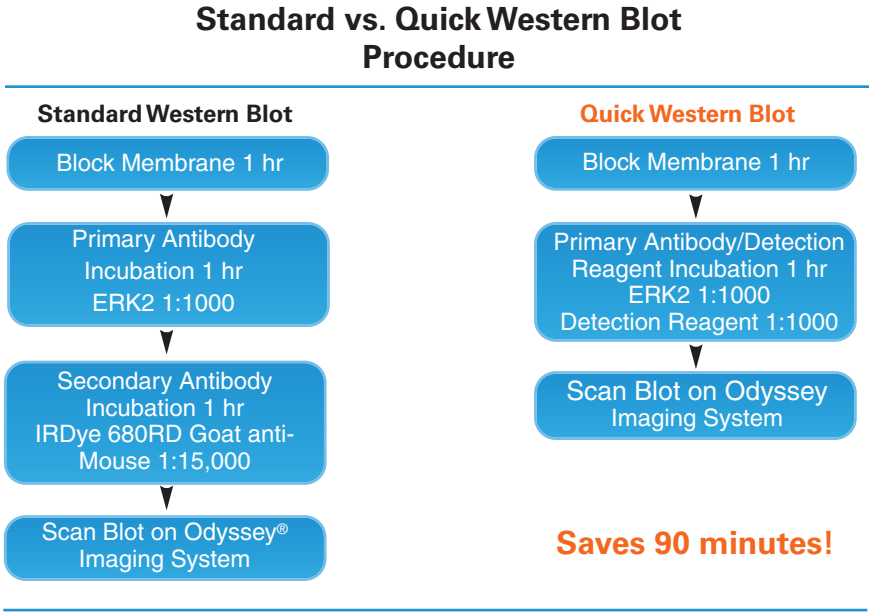
Electrophoresis and Transfer

16. Assemble 10% Bis-Tris gel in electrophoresis box. Add 1X MES-SDS running buffer to entire chamber and 500 µL of antioxidant to inner chamber.
17. Centrifuge beads on highest speed for 1 minute, then load the 10 µL of supernatant on a gel.

NOTE: Duplicate sets of samples should be separated by pre-stained MW Markers and include the positive controls prepared on Day 1 (See Figure 2).

18. Run gel according to manufacturer's recommendations.
19. Transfer the proteins to nitrocellulose using the tank transfer method and 1X Tris-Glycine + 20% methanol buffer for 65 minutes at 100V constant voltage.

Western Blot Workflow:



NOTE: With all Western blotting detection methods, optimal performance will be achieved if blots are allowed to dry prior to detection. Make sure all forceps and incubation boxes have been rinsed with methanol to remove any potential contamination.

20. Place the membrane in a clean incubation box.

21. Rinse briefly with 1X PBS. Decant.

22. Block the membrane in Odyssey® Blocking Buffer for 1 hour at room temperature with shaking.

23. Cut the membrane in half (using clean scissors) and move each half to separate, clean incubation boxes.

NOTE: Perform both detection methods simultaneously

Standard Western Blot Detection

24. Incubate one blot following the LI-COR® standard Western blot protocol using the manufacturer's recommended amount of ERK2 antibody (1:1000) diluted in Odyssey Blocking Buffer plus 0.2% Tween® 20 for detection. For example, add 15 µL of ERK2 antibody to 15 mL Odyssey Blocking Buffer plus Tween 20.

25. Incubate one hour at room temperature.

26. Decant antibody, rinse briefly one time with 1X PBS + 0.1% Tween 20, then add an excess of wash buffer and incubate 5 minutes with shaking.
    a. Decant the wash and repeat for a total of 4 times.

27. Add the IRDye 680RD goat anti-mouse secondary antibody at a 1:15,000 dilution to Odyssey Blocking Buffer + 0.2% Tween 20 and incubate 1 hour at room temperature with shaking. For example, add 1 µL of secondary antibody to 15 mL of Odyssey Blocking Buffer plus 0.2% Tween 20.

28. After 1 hour incubation of the secondary antibody, decant antibody, rinse briefly one time with 1X PBS + 0.1% Tween 20, then add an excess of wash buffer and incubate 5 minutes with shaking.
    a. Decant the wash and repeat for a total of 4 times.

Quick Western Blot Detection

NOTE: Please refer to Quick Western Kit-IRDye 680RD pack insert for more detailed instructions on reconstitution and storage of Detection Reagent.

29. Incubate the second half of the membrane following the Quick Western Blot protocol using ERK2 diluted 1:1000 mixed with 1 µL per mL of the Detection Reagent in Odyssey® Blocking Buffer plus 0.2% Tween® 20. For example, add 15 µL of ERK2 antibody plus 15 µL of Detection Reagent to 15 mL Odyssey Blocking Buffer plus 0.2% Tween 20. Blot should be incubated at room temperature on a shaker using your standard primary antibody incubation time; in this case, one hour.

30. Decant the antibody/Detection Reagent and rinse briefly one time with 1X PBS + 0.1% Tween 20, then add an excess of wash buffer and incubate 5 minutes with shaking.
    a. Decant the wash and repeat for a total of 4 times.

Imaging

31. Scan on Odyssey CLx set to auto, or Odyssey Classic at an intensity of 5 for both channels.

IV. EXPERIMENTAL RESULTS

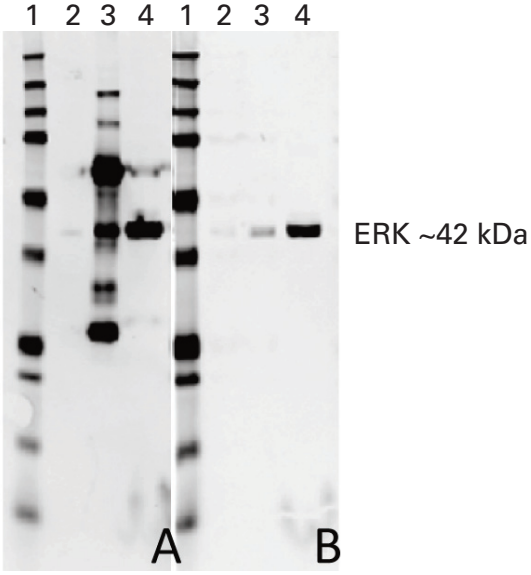


Figure 2. Immunoprecipitaton of ERK2 using the same ERK2 mouse monoclonal antibody for IP and post-IP detection by Western blot. Membranes were scanned on the Odyssey Classic Infrared Imaging System in both channels at intensity of 5, 169 µm resolution, focus offset of 0 and scan quality set to medium-lowest. In Panel A, IRDye 680RD Goat anti-Mouse was used for detection; in Panel B, Quick Western Kit-IRDye 680RD was used for detection. Lane 1, MW markers; Lane 2, IP with PBS (negative control); Lane 3, IP with ERK; Lane 4, lysate only (positive control).

V. EXPERIMENTAL CONSIDERATIONS

- Negative and positive controls are extremely important for correct interpretation of the data and should ALWAYS be included in an immunoprecipitation experiment.

- Careful consideration should be given in the choice of solid support. Be sure that your selection provides the best binding affinity to the type of antibody used. Protein A-sepharose, agarose, magnetic beads, etc., is best for mouse IgG_{2a}, IgG_{2b}, or rabbit IgG. Protein G has a higher affinity for mouse IgG_1. Consult the manufacturer's guide if you are uncertain of the best support for your experiment.

- Mouse or rabbit monoclonal antibodies are recommended for use with this kit (for IP and detection of post-IP proteins by Western blot). The detection reagent recognizes denatured heavy and/or light chains of certain polyclonal antibodies, which may interfere with post-immunoprecipitation detection by Western blot.

- If there are cross-reacting bands in the lysate + PBS (negative control) as well as your lysate + antibody lanes, pre-clearing the lysates is an option to reduce non-specific contaminants and remove proteins with high affinity to Protein A or G.
  - Wash an amount of your solid support equal to 30 µL of 50:50 slurry per sample a minimum of 3 times in 1 mL 1X PBS.
  - Resuspend beads in the starting volume and add 30 µL to each tube of lysate (step 1), including no antibody control tubes (step 3), and incubate 1 hour at room temperature.
  - Centrifuge on highest speed for 1 minute in a microcentrifuge. Transfer the supernatant to a clean tube using a 26.5-gauge needle and syringe.
  - Start the protocol at Step 2.

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