Protocol

In-Cell Western™ Assay Primary Antibody Selection and Validation

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I. Introduction

This document describes important considerations for selecting and validating a primary antibody for the In-Cell Western™ (ICW) Assay.

Antibody selection and validation are critical for development of a high-quality In-Cell Western assay. One major difference between conventional Western blots and the In-Cell Western is the context in which proteins are analyzed. ICW assays are performed on cultured cells that have been fixed to the bottom of a microplate. Fixed antigens have a slightly different conformational structure than those that have been removed from the cell and processed by SDS-PAGE. Consequently, some primary antibodies that perform well for Western blotting may exhibit poor binding characteristics on fixed protein epitopes, resulting in low or absent fluorescent signal during the detection step.

A second major difference with the ICW assay is the lack of protein separation. There is no electrophoresis step to separate individual proteins by their molecular weight; all cellular proteins are attached to the bottom of the microplate, within their cellular context. Since nonspecific antibody interactions are indistinguishable in the microplate well, antibody specificity **must** be confirmed prior to performing the ICW assay.

II. Antibody Selection

When purchasing antibodies from commercial suppliers, try to select those that have the following characteristics:

- Validated for immunofluorescence (IF) applications. Antibodies validated for IF may or may not perform well in the ICW assay, but are a good place to start. Conversely, the fact that an

antibody has not been tested in IF applications does not necessarily mean it will be unsuitable for ICW.

- Affinity purified.
- Pre-adsorbed with other proteins or serum from other species to eliminate any antibody that may cross-react. This is especially important for polyclonal antibodies.
- Monoclonal antibodies are usually preferable due to their specificity and batch-to-batch consistency. However, monoclonal antibodies (mAbs) may be too specific for your assay (e.g. if you are using the same antibody to evaluate samples from different species); also, mAbs are more vulnerable to loss of the single epitope through chemical or other treatments of the antigen during ICW processing.
- Bear in mind that the ICW assay measures "on/off" responses such as changes in protein expression or modification. It will not measure changes in protein translocation.

Specific guidelines for producing custom antibodies are beyond the scope of this document. When producing custom antibodies, the type and quality of immunogen, host animal species, purification method, and pre-adsorption process are preparation aspects that should be carefully considered.

III. Antibody Specificity

Antibody specificity should be evaluated by both Western blot and immunocytochemistry (cell staining). Western blotting can yield useful information about antibody specificity, even though antigen presentation is different than in the cell-based assay. An antibody that generates strong nonspecific bands on a Western blot may not be suitable for ICW experiments, or the blocking and antibody incubation conditions may need further optimization. Western blot analysis is a very reliable way to determine if the desired cellular response has been triggered in your experiment. You may need to optimize cell treatment conditions or timing to capture the cellular response at its peak. For example, some phosphorylation events increase and decrease rapidly, so sampling at the wrong time may cause you to overlook or underestimate the response.

IV. Western Blot Validation

Prepare your samples and perform the Western blot procedure:

- Whole cell lysates should be prepared from a representative cell line or cell type of interest.
- Lysates should be prepared from cells treated under representative conditions; be sure to include lysates from untreated cells as a control for non-specific binding. Use a mild lysis buffer to prepare lysates. Avoid using harsh detergents such as SDS, as these may damage epitopes.

- Example lysis buffer formulation: 50 mM Tris-HCl (pH 7.8), 150 mM NaCl, 1% NP-40, 0.2% Sodium Deoxycholate. Estimate total protein concentration, preferably by BCA, Bradford, or Lowry spectrophotometric assays, and load equal amounts of total protein onto the SDS-PAGE gel for each of the prepared lysate samples.
- Include a high-quality protein molecular weight marker (e.g. LI-COR P/N 928-40000) to verify target size. Follow standard protocols for gel electrophoresis and electrotransfer.
- Follow LI-COR protocols for blocking, primary antibody incubation, secondary antibody incubation, wash steps, and image acquisition. Include a second primary antibody against total protein for use as a loading control. Use LI-COR IRDye® secondary antibodies appropriate to the primary antibody you are evaluating.

Verify size and specificity

- The vertical position of the band(s) of interest should be close to the expected molecular weight of the target protein.
- Only one band should be visible in the lane of interest (Figure 1 and Figure 2). If multiple bands are present – especially strong ones - the primary antibody is likely cross-reactive and not suitable for use in the ICW assay (see Figure 3).
- For activation-state-specific antibodies (e.g. phospho-specific Abs), ensure that basal signal in the negative control lysate is not too high.

Adjust cell treatment conditions and timing as needed.

Examples of primary antibodies with good Western blot specificity are shown in Figure 1 and Figure 2.

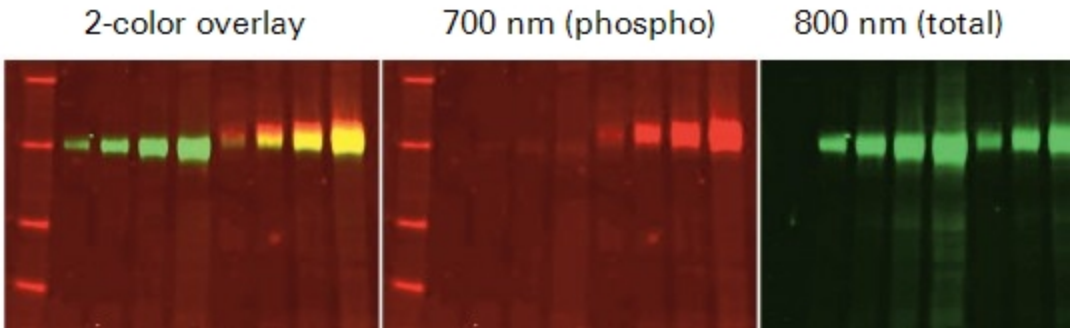

Figure 1. Serial dilution of EGF-treated A431 cell lysate; phospho-EGFR (700 nm) and total EGFR (800 nm) detection. Rabbit anti-phospho-EGFR was obtained from Cell Signaling Technology (2237), and mouse anti-EGFR from Invitrogen (AHR5062).

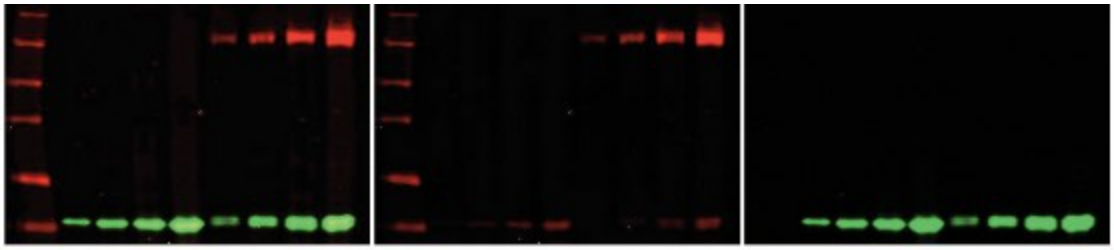

Figure 2. Serial dilution of EGF-treated A431 cell lysate; phospho-EGFR (700 nm) and total ERK (800 nm) detection. Rabbit anti-phospho-EGFR was obtained from Cell Signaling Technology (2237), and mouse anti-ERK from Santa Cruz (sc-1647).

Example of primary antibody with poor Western blot specificity is shown in Figure 3.

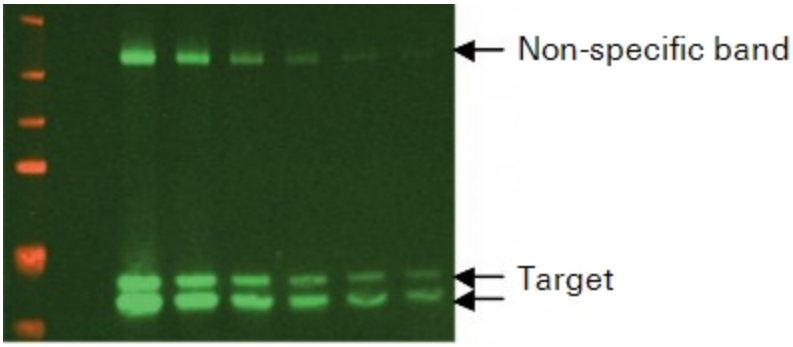

Figure 3. Serial dilution of EGF-treated A431 lysate; phospho-ERK (800 nm) detection. The anti-phospho-ERK primary antibody is recognizing a spurious, strong high molecular weight band. Mouse anti-phospho-ERK was obtained from Santa Cruz (sc-7383).

V. Immunocytochemistry Validation

Antibody specificity should also be evaluated in immunocytochemistry assays to ensure optimal assay performance and interpretation. Signal can be assessed using microplate assays on an Odyssey® Imager, or by near-infrared fluorescence microscopy. General methods for specificity evaluation are described below. The primary antibody supplier may have additional suggestions or information about antibody specificity and optimization.

- Primary antibodies should be titrated to determine the concentration that provides optimal signal and lowest background. The microplate format of the In-Cell Western assay makes it easy to quickly screen a range of antibody concentrations. Careful titration may reduce background staining.
- If target signal is low, consider whether alternate fixation or permeabilization conditions might improve performance.

- For validation of phospho-antibodies used to monitor cell signaling:
    - Treat fixed cells with phosphatase to reduce target phosphorylation. Signal should be reduced in treated cells.

      > **Note:** This test cannot determine if the antibody cross-reacts with another phospho-epitope.

    - Treat cells with a target-specific ligand or inhibitor that modulates only the pathway of interest, to determine if signal responds accordingly; confirm results by Western blotting.
- For validation of primary antibodies in general, create conditions under which the target signal increases or decreases (in each case, confirm the change in target signal by Western blotting). Suggestions include:
    - If possible, perform staining of cells that express the target protein, and cells that do not. This is the most conclusive test. If this is not practical, look for cell lines that are high or low expressors of your target protein, or transfect the target protein into a cell line that does not endogenously express it.
    - Expose the cells to conditions, ligands, or inhibitors that affect the abundance of your target.
    - Run antibody isotype controls (with Ig concentration and isotype that match your primary antibody) to assess nonspecific binding.
    - Pre-block the primary antibody using a blocking peptide to reduce signal (blocking peptides may be commercially available from the primary antibody supplier).
    - Examine stained cells with a fluorescence microscope, to determine if target signal is localized to the proper cell compartment.

LI-COR Biosciences

4647 Superior Street
Lincoln, NE 68504
Phone: +1-402-467-0700
Toll free: 800-645-4267
biosales@licor.com
licor.com/bio

Regional Offices

LI-COR Biosciences GmbH

Siemensstraße 25A
61352 Bad Homburg
Germany
Phone: +49 (0) 6172 17 17 771
bio-eu@licor.com

LI-COR Biosciences UK Ltd.

St. John's Innovation Centre
Cowley Road • Cambridge
CB4 0WS • United Kingdom
Phone: +44 (0) 1223 422104
bio-eu@licor.com

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