



IN-CELL WESTERN™ ASSAY

CELL FIXATION/PERMEABILIZATION

I. Introduction

To enable analysis of cellular proteins for the In-Cell Western (ICW) assay, cells must be immobilized onto a solid microplate surface, and antigens must be made accessible to their antibodies. In general, there are two classes of reagents which accomplish this task: cross-linking reagents and organic solvents.

Cross-linking reagents (usually aldehydes) form a network of intermolecular bridges by linking antigens through free reactive groups (mostly amino groups). Cross-linking in this manner helps to preserve the cell structure; however, a second step is usually required to permeabilize the cell membrane, and cross-linking reagents may denature some proteins. Organic solvents, such as alcohol or acetone, are a good alternative for some proteins. Organic solvents dehydrate the cell, which precipitates cellular proteins and causes the cells to adhere to the plastic surface of the microplate, but may also denature or damage some proteins and severely reduce their antigenicity.

Permeabilization is typically required to provide access to the antigen and subcellular localization should be considered. For example, methanol is often the best choice for nuclear targets, but does not work well for cytoskeletal targets. For detection of a cell surface protein, permeabilization may not be required.

No single fixation/permeabilization method will perform the best for every cell line or protein, so optimal conditions should be determined empirically. When choosing a fixation and permeabilization method for the In-Cell Western assay, your past experience with that antigen may be helpful. If there are established conditions for this antigen for immunofluorescent microscopy or other cell-based assays, these are an excellent starting point. If not, this document describes general formaldehyde and methanol fixation methods that could be evaluated. Primary antibody manufacturers can often recommend fixation and permeabilization conditions for immunofluorescent staining of particular antigens.

II. Recommended Methods

Method 1: Formaldehyde/Triton X-100

The following is the most common procedure used for cell fixation and permeabilization in the ICW assay. In this method, formaldehyde is used as the cross-linking reagent, and Triton[®] X-100 is used for permeabilization. These conditions are a good place to begin optimization if you have little or no experience with your system.

1. Just prior to use, prepare *Fixing Solution*:
 - a. Prepare 20 mL per plate.
 - b. Mix 2 mL 37% Formaldehyde and 18 mL 1X PBS (final formaldehyde concentration = 3.7%)

CAUTION: Formaldehyde is hazardous and should be handled and discarded according to the manufacturer's MSDS.

2. Carefully remove growth medium from the microplate by manual aspiration.
3. Immediately fix cells by adding 150 μ L *Fixing Solution* to each well. **Be sure to carefully add the solution down the sides of the wells to avoid detaching the cells from the well bottom.**
4. Cover the plate and allow incubation on the bench top for 20 min at ambient temperature with no shaking.

- During the fixing step, prepare *Permeabilization Solution* by mixing 5 mL 10% Triton X-100 and 495 mL 1X PBS (final Triton X-100 concentration = 0.1%).
- Remove Fixing Solution from the microplate by manual aspiration to an appropriate hazardous waste container (refer to manufacturer's MSDS).
- Add 200 μ L of Permeabilization Solution to each well; shake on a rotator for 5 minutes at ambient temperature (slow to moderate shaking).
- Repeat permeabilization wash step for a total of 4 washes. **Be sure to add and remove the *Permeabilization Solution* carefully by manual aspiration to avoid detaching the cells; also, allow the *Permeabilization Solution* to incubate for the full 5 minutes with each wash to ensure complete cell permeabilization.**
- After removing the last permeabilization wash by manual aspiration, proceed immediately to blocking and remaining ICW incubation steps.

Method 2: Methanol

The second method uses methanol for both fixation and permeabilization of cells. Methanol fixation/permeabilization may be a better option than formaldehyde in some cases (for example, when using some phospho-Stat3 antibodies or antibodies against some major structural proteins or nuclear matrix proteins).

- Cool 100% methanol to -20 °C.

NOTE: Methanol is flammable and should only be placed in a freezer that is appropriately rated for flammable liquids.

- Carefully remove growth medium from the microplate by manual aspiration.
- Immediately fix and permeabilize cells by adding 50 μ L cold methanol to each well. **Be sure to carefully add the methanol down the sides of the wells to avoid detaching the cells from the well bottom.**
- Allow the plate to incubate at ambient temperature for 10 minutes with gentle shaking.
- Carefully remove the methanol by manual aspiration into an appropriate waste container.
- Gently rinse wells three times with 100 μ L of 1X PBS. **Be sure to add and remove the PBS carefully by manual aspiration to avoid detaching the cells.**
- After removing the last 1X PBS rinse, proceed immediately to blocking and remaining ICW incubation steps.

Method Comparison Example

The graph in Figure 1 shows the Z'-factor results when comparing permeabilization Method 1 and Method 2 for detection of Stat3 phosphorylation in EGF-treated and untreated A431 cells. In this case, methanol cell permeabilization produced consistently more desirable results than detergent permeabilization (Z'-factor values > 0.5 are desirable). For a detailed discussion, see Document # 979-10610, "Using the Z'-Factor Coefficient to Monitor Quality of Near-Infrared Fluorescent Cell-Based Assays".

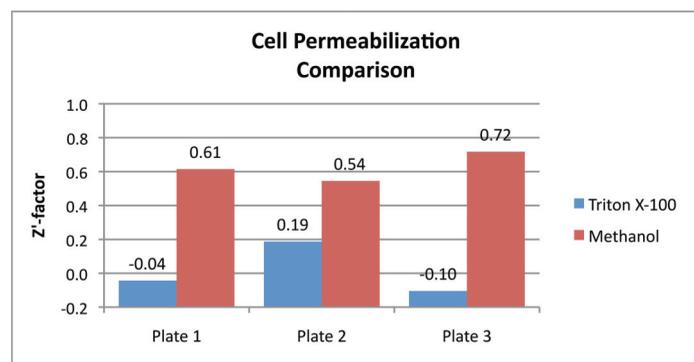


Figure 1. Z'-factor analysis of two independent ICW experiments for detection of phospho Stat3 in EGF-treated and untreated A-431 cells. In the first experiment, cells in three separate plates were fixed with formaldehyde and permeabilized with Triton X-100 (Method 1; blue bars). In the second experiment, cells in three separate plates were fixed and permeabilized using methanol (Method 2; red bars).

III. Alternative Methods

The following reagents/methods may be used as possible alternatives to the aforementioned fixation/permeabilization methods. These methods have not been specifically tested for the ICW assay, and therefore are not supported by LI-COR; however, they are relatively common to other immunocytochemical assays, and may be viable options when optimizing your assay. The steps in these methods are not rigid; optimization of the various methods is recommended. As a general rule, aldehydes must be followed by a permeabilization step, but alcohols and organic solvents do not require a separate permeabilization step; however, this is not always true. In each case, proceed with ICW blocking and incubation steps after the fixation/permeabilization procedure.

1. Acetone
 - a. Fix cells with ice-cold (-20 °C) acetone for 10 min.
 - b. Rinse with 1X PBS.
2. Formaldehyde-Methanol
 - a. Fix cells with 3.7% formaldehyde for 20 min.
 - b. Incubate cells with cold 100% methanol for 10 min.
 - c. Rinse with 1X PBS.
3. Ethanol
 - a. Fix cells with refrigerated (4 °C) 95% ethanol + 5% glacial acetic acid for 10 min.
 - b. Rinse with 1X PBS.
4. Methanol-Acetone
 - a. Fix cells with refrigerated (4 °C) methanol for 10 min at -20 °C.
 - b. Remove methanol and add refrigerated (4 °C) acetone for 1 min.
 - c. Rinse with 1X PBS.
5. Paraformaldehyde
 - a. Fix cells in 4% paraformaldehyde for 20 min.
 - b. Rinse with 1X PBS.
 - c. Wash 4 x 5 min with 0.1% Triton X-100 (diluted in 1X PBS).
6. Paraformaldehyde-Methanol
 - a. Fix cells in 4% paraformaldehyde for 20 min.
 - b. Rinse with 1X PBS.
 - c. Add refrigerated (4 °C) methanol for 5 min.
 - d. Rinse with 1X PBS.
7. Inclusion of glutaraldehyde, at a concentration between 0.05 – 0.1%, along with formaldehyde or paraformaldehyde may enhance the cross-linking action and thereby provide stronger cell fixation.
8. Milder detergents, such as NP40 or saponin, may be used as alternatives to Triton X-100 for cell permeabilization.

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