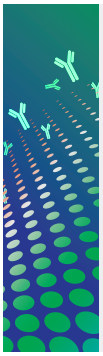


In-Cell Western Application Using the ChemiDoc MP Imaging System

Protocol

Introduction

The In-Cell Western (ICW) Assay, also known as in-cell ELISA (ICE), is both a qualitative and quantitative immunofluorescence assay performed in microplates (6- to 384-well format) that combines the specificity of western blotting with the reproducibility and higher output of an enzyme-linked immunosorbent assay (ELISA). As an immunofluorescence application, ICW allows researchers to use primary antibodies and spectrally distinct conjugated secondary antibodies to detect their protein of interest in fixed and permeabilized cultured cells. Secondary antibodies conjugated with near infrared (NIR) fluorescent dyes are commonly used for ICW multiplex detection and normalization.

While NIR fluorescent dyes can be useful, there is only a small number of commercially available dyes. In this protocol, we replace NIR fluorescent dyes with DyLight Conjugated Secondary Antibodies. After secondary incubation, cells are imaged and quantitated using a western blot imager and analysis software, which use the fluorescent signal of the cells generated in each well to determine the protein expression levels or signaling status. ICW allows for a rapid and accurate measure of relative protein levels in numerous samples without the need for lysing cells, which prevents possible artifacts. With the elimination of sample prep, SDS-PAGE, and membrane transfer steps used in traditional western blotting protocols, ICW requires less hands-on time. This streamlined experimental procedure and data analysis let researchers process numerous samples in parallel with quantitative data output. Though ICW functional assays are not covered in this protocol, it is worth noting that ICW can be used to analyze posttranslational modifications like protein phosphorylation. Here, we describe the basic method for quantitative immunofluorescence ICW assay.

Materials Required

- HEK 293 cells
- 6- to 384-well black/clear or clear/clear flat bottom tissue culture (TC)-treated imaging microplate with lid
- 10x phosphate buffered saline (PBS)
- Paraformaldehyde (PFA) solution, 4% in PBS
- Triton X-100 Detergent
- 1x Tris Buffered Saline (TBS) with 1% Casein (Bio-Rad™ Laboratories, Inc., #1610782)
- Tween 20 Detergent
- Vacuum with hose attachment (or manual aspiration with a pipet)
- Goat Anti-Mouse IgG (H+L) Secondary Antibody, DyLight 680 (Thermo Fisher Scientific Inc., #35518)
- Goat Anti-Rabbit IgG (H+L) Cross-Adsorbed Secondary Antibody, DyLight 800 (Thermo Fisher Scientific Inc., #SA5-10036)
- Optional: hFAB Rhodamine Anti-GAPDH Primary Antibody (Bio-Rad, #12004167)
- ChemiDoc MP Imaging System (Bio-Rad, #12003154)
- Blot/UV/Stain-Free Sample Tray for ChemiDoc MP/ChemiDoc Imaging Systems (Bio-Rad, #12003028)
- Image Lab Software for PC Version 6.1 (Bio-Rad, #SOFT-LIT-170-9690-ILSPC-V-6-1) or Image Lab Software for Mac Version 6.1 (Bio-Rad, #SOFT-LIT-170-9690-ILSMAC-V-6-1)

BIO-RAD

Performing the ICW

The protocol described in this section is for 96-well microtiter plates, but you can also use plate sizes ranging from 6- to 384-wells. Adjust buffer volumes to account for plates with smaller or larger well volumes. We optimized this protocol for adherent HEK 293 cells, and further optimization for different cell lines may be necessary. This protocol was developed and imaged with the ChemiDoc MP System, but is applicable to other Bio-Rad imagers using the appropriate fluorescent channels. It may work on other imaging systems that can detect in the near infrared spectrum.

1. Culture adherent cells in a black-walled, clear flat bottom cell culture plate until they are 80–90% confluent. Treat the cells if needed. For adherent HEK 293 cells, cell density should be 25,000 cells. For the tests described, we used a 96-well black/clear flat bottom TC-treated imaging microplate with a lid, but any 6- to 384-well black/clear or clear/clear flat bottom TC-treated imaging microplate with a lid is acceptable.
2. Fix cells with 150 µl of 4% PFA in PBS for 20 min at room temperature. Add the solution by pipetting down the side of the wells carefully to avoid cell detachment.
3. Permeate cells with 200 µl of 1x PBS + 0.1% Triton X-100. Incubate for 5 min before aspirating. Repeat for a total of four washes.
4. Aspirate using a vacuum with a hose attachment and pipet tip at the end. Tilt the plate at a 45° angle and place the vacuum-attached hose with pipet tip to the bottom of the plate, sliding it along the bottom wall of the well. Minimize moving the tip around the well to decrease how many cells detach.

   Note: Aspiration can be performed manually using a pipet by tilting the plate to a 45° angle and running the pipet tip to the bottom of the plate, sliding along the bottom wall of the well.
5. Add 150 µl of 1x TBS with 1% Casein Blocking Buffer to cells. Incubate for 1.5 hr at room temperature.
6. Dilute primary antibodies according to the vendor's recommended antibody dilution (in this test, we used 1:1,000) in 1x TBS with 1% Casein Blocking Buffer, with up to three targets per well. Add 50 µl per well and incubate for 2 hr at room temperature.

   Multiplexing capabilities:

   a. Bio-Rad ChemiDoc MP System: up to three targets

   b. Licor CLx Imager: up to two targets
7. Wash four times with 200 µl of 1x PBS + 0.1% Tween 20. Wait 5 min per wash before aspirating.
8. Dilute fluorophore-conjugated secondary antibodies as recommended by the vendor in 1x TBS with 1% Casein Blocking Buffer. Add 50 µl per well and incubate for 1 hr in the dark at room temperature.

   Note: For the ChemiDoc MP System, use DyLight 680 and 800, which are comparable to IRDye 700 and 800. If using fewer than three targets, we recommend using Bio-Rad hFAB Rhodamine Housekeeping Protein Fluorescent Primary Antibodies. We used hFAB Rhodamine Anti-GAPDH Primary Antibody in this experiment.
9. Prepare 1x PBS with 0.1% Tween 20 wash solution and add 200 µl to each well. Wait 5 min before aspirating and repeat for a total of four washes. Ensure wells are dry before imaging.
10. Image microplate with the top side down, as shown in Figure 1, on the ChemiDoc MP imaging stage using the Blot/UV/Stain-Free Sample Tray for ChemiDoc MP Systems/ChemiDoc Imaging Systems. (Please consult the vendor's ICW recommendations before imaging on other western blot imaging platforms.)

    Note: The ChemiDoc MP System's camera is mounted to the top of the imager and looks downs onto the stage. Optimal plate imaging necessitates you place the plate so the clear bottom is facing up toward the camera.

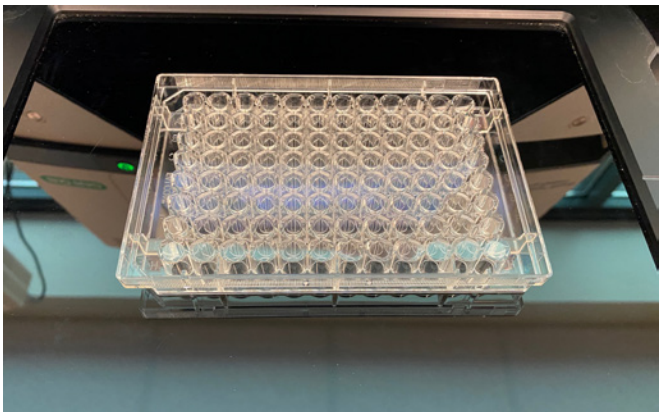
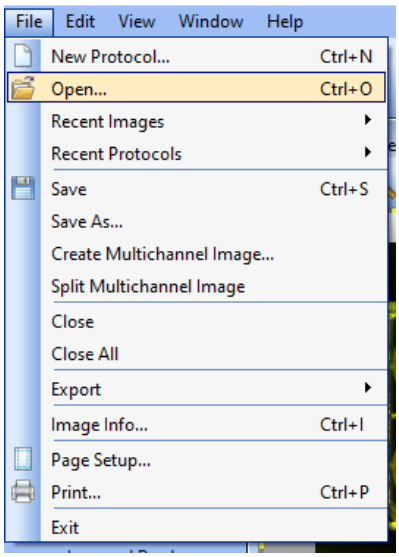


Fig. 1. Example of a 96-well plate being loaded onto the ChemiDoc MP System imaging stage for ICW.

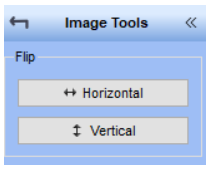
Analyzing the ICW Experiment Using Image Lab Software:

1. Open Image Lab Software.
2. Click **File** for the dropdown menu. Click **Open** and select the Image Lab .mscn or .scn file containing the ICW images.

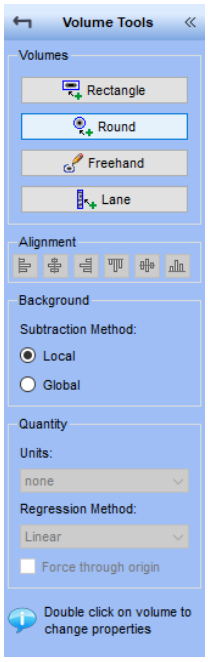


3. In the Analysis Tool Box sidebar, choose **Volume Tools**.

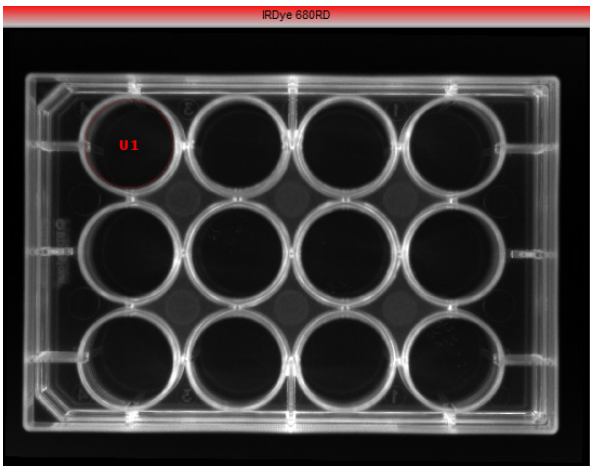
   Note: Before beginning analysis, invert your image by selecting **Image Tools** and choose **Horizontal** flip.



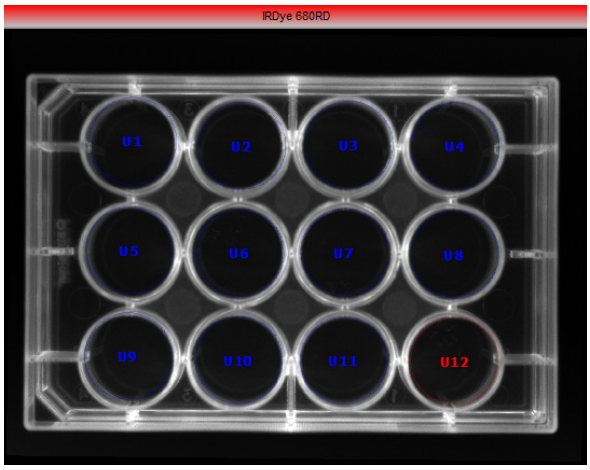
4. In the Volume Tools sidebar, choose **Round**.



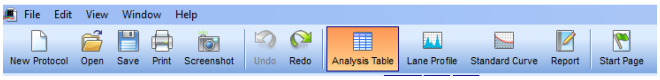
5. Move the cursor to the first well in the plate and click and drag until you get a circle that encompasses the dimensions of the well. This circle will be labeled **U1**.



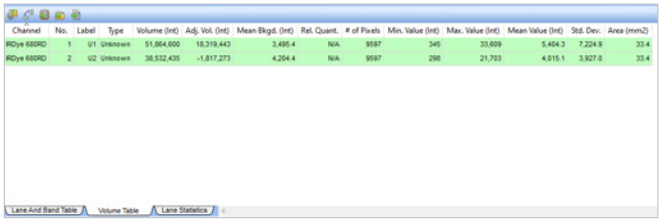
6. Click the **U1** circle and type **Ctrl+C** (for PC) or **Command+C** (for Mac) to copy the circle. Type **Ctrl+V** (for PC) or **Command+V** (for Mac) on the keyboard to paste a new circle and click and drag it to the next well of interest. Repeat until you have positioned the circles to all the wells of interest on the plate. If possible, include one empty well for background subtraction.



7. Click **Analysis Table**.



8. Click the **Volume Table** tab to get the ICW plate analysis.



Example Results

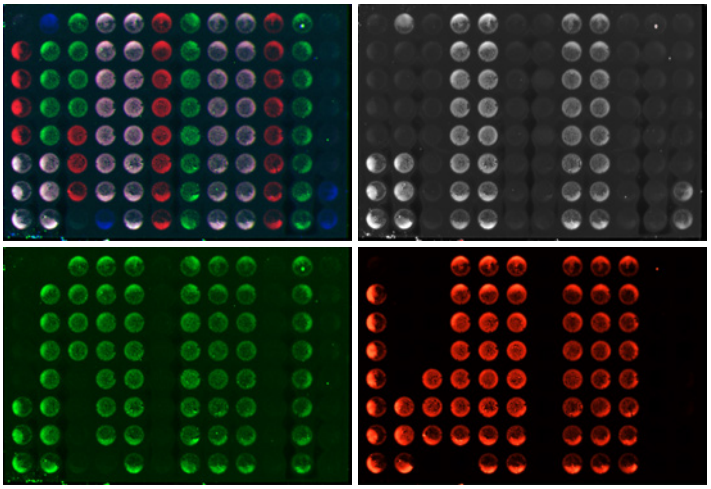


Fig. 2. ICW Assay was used to quantitate HEK 293 cells (25,000–45,000 cell density) in 96-well microplates. GAPDH (rhodamine, gray), mouse anti-actin gamma (DyLight 680, green), and rabbit anti-p53 (DyLight 800, red) images were acquired on a ChemiDoc MP System under autoexposure settings.

Visit bio-rad.com/InCellWestern for more information.

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Russian Federation *00 800 00 24 67 23* ***Singapore*** *65 6415 3188* ***South Africa*** *00 800 00 24 67 23* ***Spain*** *00 800 00 24 67 23* ***Sweden*** *00 800 00 24 67 23*
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