



ROCKLAND[™]
antibodies & assays

In-cell western assays (ICW) also known as In-cell ELISA (ICE) allow researchers a simple and rapid assay method for detection of biomarkers and signaling proteins in whole cells using antibodies. This protocol is designed for the Odyssey[®] infrared imaging system by LICOR[®]. Other similar imaging systems will yield similar results.

In-Cell Western Protocol

Method for the detection of cellular biomarker proteins using fluorochoime conjugated antibodies.

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<http://www.rockland-inc.com/>

In-Cell Western Protocol

This method contains: (A) a list of reagents required to perform ICW; (B) a general protocol applicable to most In-cell western (ICW) applications; (C) a protocol specifically for ICW detection of tubulin protein as a positive control; and (D) recommended secondary antibodies for optimal results.

A. Reagents

Reagent	Note	Rockland p/n
10 X PBS	Phosphate Buffer Saline. ^(a)	MB-008 <i>or equivalent</i>
10X PBS Fish Gel	PBS Fish Gel Concentrate 10X. ^(b)	MB-066-0100
Blocking Buffer/ Antibody Diluent	Prepare 1X PBS Fish gel.	
Formaldehyde (16% w/v)	Use fresh, store opened vials at 4°C in dark, dilute to 3.7% in PBS for use.	KHF001
Permeabilization buffer	Prepare 80 mL 1X PBS + 0.1% Triton X-100 per plate.	Triton must be supplied by the user.
Wash buffer	Prepare 20 mL 1XPBS + 0.1% tween-20 per plate per wash.	Tween-20 must be supplied by the user.
Primary Antibody	Project specific.	See available primary antibodies
Secondary Antibody	See Part D for recommended IR compatible secondary antibodies.	
Active cell culture	Prepare actively growing cells of interest (see protocol below).	
96-well culture plate	Clear or black walled plate may be used. A black walled plate should be used for critical data collection.	
(a) Use sterile deionized water or equivalent for dilution of stock solutions		
(b) BSA or Non-fat dried milk are not recommended, but can be used in some experiments		

B. General Protocol for ICW

The protocol described below is for 96-well microtiter plates. Please adjust buffer volumes to account for plates with small or larger well volumes. 384-well plates may be used if desired. This protocol is known to work for the LICOR® Odyssey® Classic, Odyssey® CLx, and Odyssey® Sa Imaging Systems. This protocol may work on other imaging systems that can detect in the near IR spectrum.

1. Cell preparation

Grow cells in 96-well or 384-well plates until confluent by standard procedures. Serum-starve cells if necessary and proceed to stimulate or monitor cells.

2. Fixation

Proceed to fix cells with a 3.7% formaldehyde solution in PBS for 20 minutes at room temperature. Add 150 µL of formaldehyde solution. Incubate on bench top for 20 minutes at ambient temperature, do not agitate.

3. Permeabilization

Discard fixative solution. Blot inverted plate by tapping onto clean paper towel. Gently add 200 µL of permeabilization buffer to each well with multichannel pipette; gently rotate plate for 5 minutes at ambient. Repeat the wash step 5 total times.

4. Blocking

Block cells by adding 150 µL of blocking buffer for 1.5 hour at room temperature, with gentle shaking (blocking times up to overnight are acceptable).

*Alternative blocking reagents:

Rabbit Serum Non-Sterile (Rockland p/n [D209-00-0050](#))

Mouse Serum Non-Sterile (Rockland p/n [D208-00-0050](#))

5. Primary antibody

Prepare primary antibody by diluting in Antibody Dilution Buffer. A suggested starting dilution range is 1:50 – 1:500. Generally increase the amount of antibody tenfold relative to what is recommended for a conventional western blot. If performing a double-labeling experiment, combine both primary antibodies as a cocktail to the desired concentration using antibody dilution buffer.

Tap out the blocking solution into the sink, blot onto a paper towel. Add diluted primary antibody (total volume 50 µL/well). Cover plate and incubate for 2 hours at room temperature with gentle shaking then, if greater sensitivity is desired, continue incubation overnight at 4°C with plate stationary.

After primary antibody incubation, rinse plate four times with wash buffer for 5 minutes each wash.

*Note In-Cell western allows for simultaneous detection of two primary antibodies. Primary antibodies must be from different host sources, i.e. mouse, rabbit, or goat. The secondary antibodies must discriminate and not cross react between the hosts of the primary antibody.

Alternatively the primary antibodies must be directly conjugated to a 700 channel and an 800 channel fluorochrome respectively. See appendix B for suitable secondary antibodies).

6. Secondary conjugated antibody

Dilute the detection antibody in dilution buffer or other appropriate blocker. A starting dilution of 1:1000 will work for most basic applications, although dilutions from 1:200 to 1:2000 are commonly used. In some cases the dilution may require optimization. Fluorochrome conjugated antibodies should be shielded from exposure to prolonged periods of direct light.

Add fluorochrome-conjugated secondary antibody* diluted in Antibody Dilution Buffer (total volume 50 μ L/well) and incubate for one hour at room temperature in the dark with gentle shaking.

*NOTE: For double-labeling, prepare a cocktail of fluorochrome-conjugated anti-mouse and anti-rabbit secondary antibodies in Antibody Dilution Buffer. Each should have a different fluorochrome dye conjugate where one dye works in the 700 channel and the second dye works in the 800 channel. For example 611-130-002 Rabbit IgG (H&L) Secondary Antibody IRDye700DX® could be paired with 610-431-020 Mouse IgG (H&L) Antibody IRDye800CW®.

After incubation, rinse plate four times with wash buffer for 5 minutes each and blot dry when finished.

7. Data collection

Scan the dry plate on LICOR® Odyssey™ or equivalent imaging system according to manufacturer's directions.

If using the Odyssey imager, set the device for the appropriate detection channels. The intensity settings should be set for moderate sensitivity. Set both 700 and 800 nm channels to 5. However if the image shows saturation, repeat the scan with lower intensity setting (i.e., 3 or 1.5). If the signal is very low, the intensity setting may be set higher such as 7 or 8.

Resolution settings can be set to medium to obtain a clean result with an acceptable scanning time.

Note that fixed and stained plates can be stored for 1-3 weeks for future use. To preserve the experiment for a later scan, protect the plate from light by wrapping in a foil pouch (or equivalent). Store the plate at 4 °C prior to use.

C. Protocol for Tubulin Detection as a Positive Control

The following example is a positive control experiment to demonstrate the detection of tubulin. In this experiment Capan-2 pancreatic cancer cells are grown and seeded into a 96-well ELISA plate and probed for alpha tubulin. The experiment is simple and fast.

Capan-2 cells are grown to sub-confluence in a T150 flask. The cells are trypsinized and then seeded into a 96-well tissue culture plate (COSTAR p/n 3598 or equivalent). Black walled plates may be used to reduce side-scatter.

The recommended seeding density is 0.2×10^6 cells per mL. Allow the cells to grow to confluence (for Capan-2 cells approximately 48 hours). Since alpha tubulin is cytosolic, the cells are fixed and permeabilized. Permeabilization with Triton-X100 allows for greater antibody penetration into the cell interior. As a negative control cells in a portion of the plate were not permeabilized. Note that permeabilization is not necessary for antibodies that target extracellular protein domains.

Control experiment to stain for alpha tubulin:

- 1) Remove media from wells and gently wash the cells two times with 1X PBS by adding 200 μ L/well for 5 minutes each at room temperature with gentle shaking.
- 2) Add 150 μ L/well 3.7% formaldehyde in 1X PBS. Incubate 20 minutes at room temperature WITHOUT shaking (i.e. stationary).
- 3) Remove fixing solution by gentle aspiration using multichannel pipet.
- 4) Wash and permeabilize cells with 200 μ L/well of 1X PBS+0.1% Triton X-100 four times for 5 minutes each at room temperature with gentle shaking.
- 5) Gently flick out contents of wells between each wash. Periodically check to confirm cells have not detached from the plate.
- 6) Block by adding 150 μ L/well of 1XPBS fish gel solution (Rockland p/n MB-066-0100).
- 7) Incubate blocking step for 1.5 hours at room temperature with gentle shaking.
- 8) Wash by adding 200 μ L /well 1XPBS+0.1% Tween-20 four times for 5 minutes each at room temperature with gentle shaking.
- 9) Add 50 μ L /well mouse anti-alpha tubulin primary antibody (Rockland p/n 200-301-880) diluted to 2.5 μ g/mL (1:400) in blocking buffer.

- 10) Incubate primary antibody for 2 hours at room temperature with gentle shaking then move to 4°C for overnight incubation WITHOUT shaking (i.e.stationary).
- 11) Wash by adding 200 µL/well 1XPBS+0.1% Tween-20 four times for 5 minutes each at room temperature with gentle shaking.
- 12) Add 50 µL/well anti-mouse IgG IRDye 800 (Rockland p/n [610-732-124](#)) diluted 1:1000 in blocking buffer. Cover plate with aluminum foil to protect the IRDye from exposure to light.
- 13) Incubate with secondary antibody for 1 hour at room temperature with gentle shaking.
- 14) Wash by adding 200 µL/well 1XPBS+0.1% Tween-20 four times for 5 minutes each at room temperature with gentle shaking.
- 15) After the last wash gently pipet out any residual liquid and blot plate dry.
- 16) Scan plate.

Permeabilized	+	+	+	+	+	+	-	-	-	-	-	-
anti-Ms-IR800	-	+	+	+	+	+	-	+	+	+	+	+
anti alpha tubulin	+	-	+	+	+	+	+	-	+	+	+	+

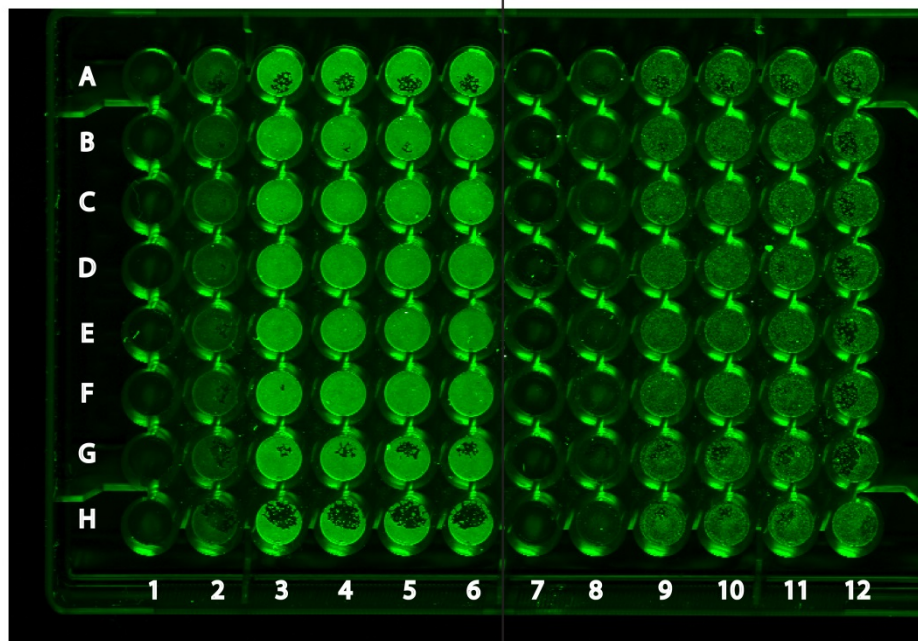


Figure 1: In-cell western data showing detection of tubulin in permeabilized Capan-2 cells. A 96-well tissue culture plate was seeded with Capan-2 cells, and after treatment with mouse anti-tubulin and IRDye® 800 conjugated anti-mouse secondary, alpha tubulin is detected. Note that detection of tubulin is demonstrated only in the permeabilized cells (columns 3- 6). The dark regions in columns H3-6 is thought to be disruption of the cells by the pipette tips. The legend at top indicated the specific conditions used for each column of cells.

D. Recommended Secondary Antibodies

Fluorochrome conjugated secondary antibodies:

Anti-Rabbit secondary antibodies:

[611-130-002](#) Rabbit IgG (H&L) Secondary Antibody IRDye700DX® Conjugated

[611-144-002](#) Rabbit IgG (H&L) Antibody DyLight™ 680 Conjugated

[611-131-002](#) Rabbit IgG (H&L) Secondary Antibody IRDye800CW® Conjugated

[611-145-002](#) Rabbit IgG (H&L) Antibody DyLight™ 800 Conjugated

Anti-Mouse secondary antibodies:

[610-430-002](#) Mouse IgG (H&L) Antibody IRDye700DX® Conjugated

[610-144-002](#) Mouse IgG (H&L) Antibody DyLight™ 680 Conjugated

[610-431-020](#) Mouse IgG (H&L) Antibody IRDye800CW® Conjugated Pre-Adsorbed

[610-145-002](#) Mouse IgG (H&L) Antibody DyLight™ 800 Conjugated Anti-Rabbit IgG

[610-732-124](#) Anti-MOUSE IgG (H&L) (DONKEY) Antibody IRDye® 800 Conjugated (Min X Bv Ch Gt GP Ham Hs Hu Rb Rt & Sh Serum Proteins)