CellTag™ 700 Stain

In-Cell Western™ Assay Kits I and II

Developed for:

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

Please refer to your manual to confirm that this protocol is appropriate for the applications compatible with your Odyssey Imager model.

Part Numbers: 926-41091 and 926-41092

Storage:

IRDye® secondary antibody	4 °C
Odyssey Blocking Buffer	4 °C
CellTag 700 Stain	-20 °C

See Sections IV and V for complete storage recommendations

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I. Introduction

The In-Cell Western Kits provide detection reagents for cell-based In-Cell Western Assays. Each kit includes blocking buffer, IRDye® 800CW secondary antibody for detection of a specific protein target in the 800 nm channel, and CellTag 700 Stain to normalize well-to-well variations in cell number. This cost-effective normalization method makes quantification of the target protein more precise.

Detection of two proteins can be achieved using two different primary antibodies, followed by detection using two different secondary antibodies (one labeled with IRDye 800CW and the other with IRDye 680RD) in a multiplex assay. IRDye secondary antibodies for multiplex detection can be purchased at: www.licor.com/catalog. Dilution factors and blocking conditions should be optimized for target and primary antibody combinations.

II. Using CellTag 700 Stain for Cell Number Normalization

CellTag 700 Stain is a near-infrared fluorescent, non-specific cell stain that provides accurate normalization to cell number for In-Cell Western applications. The stain accumulates in both the nucleus and cytoplasm of permeabilized cells, and provides linear fluorescent signal across a wide range of cell types and cell numbers (see Figure 1). CellTag 700 Stain is detected in the 700 nm channel of Odyssey® CLx, Classic, and Sa Imaging Systems. CellTag 700 Stain is applied to the cells during incubation with IRDye 800CW secondary antibody, and enables accurate measurement of target protein levels (see Figure 2) with much higher throughput than Western blotting.

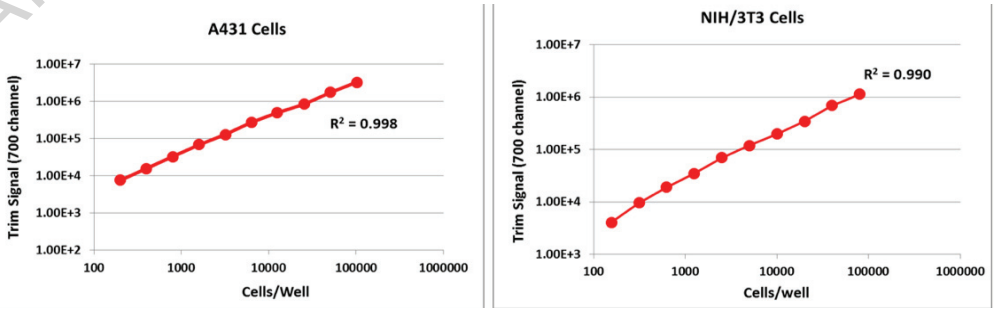

Figure 1. Linear Relationship between Fluorescence and Cell Number. Two-fold serial dilutions of A431 or NIH/3T3 cells were plated in 96-well plate. Cells were fixed, permeabilized, stained with CellTag 700 Stain (0.2 µM), and detected with Odyssey Classic Infrared Imaging System (Resolution: 169 µm; Quality: medium; Focus offset: 4.0 mm; Intensity: 5). The Trim Signals were used to generate the graphs. Linear fluorescent signal was obtained across a very wide range of cell numbers (~ 200 – 100,000 cells).

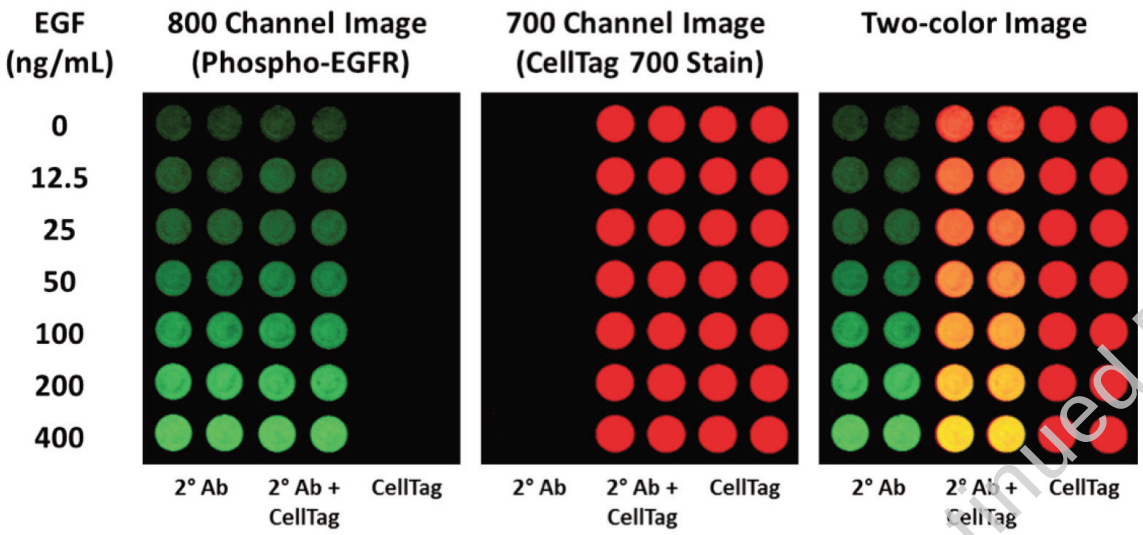

Figure 2. In-Cell Western Assay with CellTag 700 Stain in EGF-stimulated A431 Cells. EGF-stimulated A431cells were fixed and permeabilized. Phosphorylated EGFR was measured using rabbit anti-phospho-EGFR primary antibody followed by detection with IRDye® 800CW Goat anti-Rabbit IgG (LI-COR P/N 926-32211). CellTag 700 Stain (LI-COR, P/N 926-41090) was used for normalization to cell number. The data demonstrate that phosphorylated levels of EGFR increase with treatment of EGF. The plate was scanned on Odyssey® Classic Infrared Imaging System. (Resolution: 169 µm; Quality: medium; Focus offset: 4.0 mm; Intensity: 5 for both channels).

III. In-Cell Western Protocol

Kit Components (sufficient for 20 x 96-well plates)

- IRDye 800CW secondary antibody, 0.5 mg (lyophilized) (LI-COR, P/N 926-32210 or 926-32211)
- Odyssey Blocking Buffer, 1 x 500 mL (LI-COR, P/N 927-40000)
- CellTag 700 Stain, 2 x 10 nmole (LI-COR, P/N 926-41090)

Additional Reagents (required but not included)

- Primary antibody
- 1X PBS
- Tissue culture reagents (serum DMEM, trypsin, etc.)
- Clear or black 96-well microplate (see IX. Experimental Considerations)
- 37% formaldehyde
- 20% Tween® 20
- 10% Triton® X-100

IV. Reconstitution of Antibody

1. Protect from light. Store IRDye 800CW secondary antibody at 4 °C prior to reconstitution.

2. Reconstitute contents of antibody vial with 0.5 mL sterile distilled water. Mix gently by inverting, and allow to rehydrate for at least 30 minutes before use. Centrifuge product if solution is not completely transparent after standing at room temperature.

3. Dilute only immediately prior to use. Reconstituted antibody is stable for 3 months at 4 °C when stored undiluted as directed.

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V. Reconstitution of CellTag 700 Stain

1. Protect from light. Store CellTag 700 Stain at -20 °C prior to reconstitution. Use the stain within 6 months.

2. Reconstitute contents of vial with 0.1 mL 1X PBS for a final concentration of 0.1 mM. Mix thoroughly by vortexing, and allow to rehydrate for at least 30 minutes before use. Store the reconstituted stain at 4 °C.

VI. Cell Preparation and Fixation

1. Treat cells as desired with drug, stimulant, etc. Detailed In-Cell Western (ICW) protocols for certain cell lines and target proteins may be downloaded at: http://biosupport.licor.com. See In-Cell Western Assay Cell Fixation/Permeabilization document at the ICW Assay Application page (http://biosupport.licor.com/docs/ICW_fix_and_perm.pdf)

2. Remove media manually or by aspiration. Immediately fix cells with Fixing Solution (3.7% formaldehyde in 1X PBS) for 20 minutes at room temperature (RT).

   a. Prepare fresh Fixing Solution as follows:

1X PBS	45 mL
37% Formaldehyde	5 mL
3.7% Formaldehyde	50 mL

   b. Using a multi-channel pipettor, add 150 µL of fresh, room temperature Fixing Solution to each well. Add carefully by pipetting down the sides of the wells to avoid detaching the cells from the well bottom.

   c. Allow incubation on the bench top for 20 minutes at RT with no shaking.
   NOTE: If optimal fixation conditions for immunofluorescent staining of your cell line and/or target protein are already known, these conditions may be more appropriate than the fixation protocol described here and would be an excellent starting point for In-Cell Western assay development. Most fixatives and fixation protocols for immunofluorescent staining may be adapted to the In-Cell Western format.

3. To permeabilize, wash five times with 1X PBS containing 0.1% Triton X-100 for 5 minutes per wash.

   a. Prepare Triton Washing Solution as follows:

1X PBS	495 mL
10% Triton X-100	5 mL
1X PBS + 0.1% Triton X-100	500 mL

   b. Remove Fixing Solution to an appropriate waste container (contains formaldehyde).

   c. Using a multi-channel pipettor, add 200 µL of room-temperature Triton Washing Solution to each well. Make sure to carefully add the solution down the sides of the wells to avoid detaching the cells.

   d. Allow wash to shake on a plate shaker for 5 minutes.

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e. Repeat washing steps 4 more times, removing wash manually each time. Do not allow cells/wells to become dry during washing. Immediately add the next wash after each manual disposal.
NOTE: If an alternative permeabilization method (for example, ice-cold methanol) is known to work well for immunofluorescent staining of your protein target, you may prefer to use that permeabilization method rather than the Triton method described here.

VII. Cell Staining

1. Using a multi-channel pipettor, block cells by adding 150 µL of Odyssey® Blocking Buffer to each well. Add the solution carefully by pipetting down the sides of the wells to avoid detaching the cells.
NOTES: No single blocking reagent will be optimal for every antigen-antibody pair. Some primary antibodies may exhibit greatly reduced signal or non-specific binding in different blocking solutions. If you have difficulty detecting your target protein, changing the blocking solution may dramatically improve performance. If you have used the primary antibody successfully for immunofluorescent staining, consider trying the same blocking buffer for In-Cell Western detection.

Odyssey Blocking Buffer often yields higher and more consistent sensitivity and performance than other blockers. Nonfat dry milk or casein dissolved in PBS, or commercial blocking buffers, can also be used for blocking and antibody dilution. When using anti-goat antibodies, milk-based reagents may be contaminated with endogenous IgG, biotin, or phosphoepitopes that can interfere with detection.

2. Allow blocking for 1.5 hours at room temperature with moderate shaking on a plate shaker.

3. Dilute desired primary antibody in Odyssey Blocking Buffer or other appropriate blocker. As a general guideline, 1:50 to 1:200 dilutions are recommended, depending on the primary antibody. If the antibody supplier provides dilution guidelines for immunofluorescent staining, start with that recommended range.
NOTE: If using CellTag 700 Stain for normalization, only one primary antibody will be used. Alternatively, you may choose to normalize with a second primary antibody in your assay. The second primary antibody MUST be from a different host, and an appropriate IRDye® 680RD secondary antibody (not provided in the kit) will be required for detection.

   a. It is important to include control wells that DO NOT contain primary antibody. These wells will be treated with secondary antibody only, and should be used to correct for background staining in the data analysis.

   b. Remove blocking buffer from step 1.

   c. Add 50 µL of Odyssey Blocking Buffer to the control wells and 50 µL of the desired primary antibody in Odyssey Blocking Buffer to the rest of the wells.

4. Incubate with primary antibody for 2.5 hours at room temperature or overnight at 4 °C with gentle shaking.

5. Wash the plate five times with 1X PBS + 0.1% Tween 20 for 5 minutes at RT with gentle shaking, using a generous amount of buffer.

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a. Prepare Tween® Washing Solution as follows:

1X PBS	995 mL
20% Tween 20	5 mL
1X PBS with 0.1% Tween 20	1000 mL

b. Using a multi-channel pipettor, add 200 µL of Tween Washing Solution. Make sure to carefully add the solution down the sides of the wells to avoid detaching the cells from the well bottom.

c. Allow wash to shake on a plate shaker for 5 minutes.

d. Repeat washing steps 4 more times.

6. Dilute the fluorescently-labeled secondary antibody in Odyssey® Blocking Buffer or other appropriate blocker. The recommended dilution range is 1:200 to 1:1,200, with a suggested starting dilution of 1:800. The optimal dilution for your assay should be determined empirically. To lower background, add Tween 20 at a final concentration of 0.2% to the diluted antibody. Avoid prolonged exposure of the antibody vials to light.

   a. Secondary antibody staining and normalization staining are carried out simultaneously. To stain for normalization, add CellTag 700 Stain to the diluted secondary antibody solution and apply this mixture to the cells. Suggested concentration for CellTag 700 Stain is 0.2 µM (1:500 dilution).

   b. For control wells (used to calculate background), do not add CellTag 700 Stain. Add only diluted secondary antibody to these wells.

7. Add 50 µL of secondary antibody solution without CellTag 700 Stain into each of the control wells and 50 µL of secondary antibody solution with CellTag 700 Stain into remaining wells. Incubate for 1 hour at room temperature with gentle shaking. Protect plate from light during incubation.

8. Wash the plate five times with 1X PBS + 0.1% Tween 20 for 5 minutes at room temperature with gentle shaking, using a generous amount of buffer.

   a. Using a multi-channel pipettor, add 200 µL of Tween Washing Solution. Make sure to carefully add the solution down the sides of the wells to avoid detaching the cells from the well bottom.

   b. Allow wash to shake on a plate shaker for 5 minutes.

   c. Repeat washing steps 4 more times. Protect plate from light during washing.

VIII. Imaging

1. After final wash, remove wash solution completely from wells. Turn the plate upside down and tap or blot gently on paper towels to remove traces of wash buffer. For best results, scan plate immediately; plates may also be stored at 4 °C for up to several weeks (protected from light).

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2. Before plate scanning, clean the bottom plate surface and the scanning bed (if applicable) with moist lint-free paper.

3. Scan plate with detection in both 700 and 800 nm channels using an Odyssey® or Aerius System, as described below:
   NOTE: All settings may require adjustment for optimal data quality (see Section IX).

Instrument	Resolution*	Scan Quality*	Intensity Setting (700/800)	Scan Time Medium Quality
Odyssey Classic	169 µM	medium-lowest	5 / 5	7 min
Odyssey CLx	169 µM	medium-lowest	5 / 5	7 min
	169 µM	medium-lowest	AutoMode	16 min
Odyssey Sa	200 µM	medium-lowest	7 / 7	3 min
Aerius	200 µM	medium-lowest	7 / 7	3 min

**Higher resolution or scan quality may be used, but scan time will increase.*

IX. Experimental Considerations

Establish the specificity of the primary antibody by screening lysates of cells treated in the same manner as the In-Cell Western samples, using Western blotting and detection with an Odyssey or Aerius instrument. If significant non-specific binding is present, choose alternative primary antibodies to avoid non-specific signals that may affect In-Cell Western assay results.

Proper selection of microplates can significantly affect results, as each plate has its own characteristics, including well depth, plate autofluorescence, and well-to-well signal crossover. Use the following general considerations for microplate selection.

- In-Cell Western analyses use detection at the well surface with no liquid present. This results in minimal well-to-well signal spread, allowing the use of either clear plates or black-sided plates with clear well bottoms. Do not use plates with white walls, since autofluorescence from the white surface will create significant noise.
- Protect plates from light before imaging to ensure highest sensitivity. When storing plates after imaging, the plates should remain protected from light at 4 °C.
- In-Cell Western assays require sterile plates for tissue culture growth. The following plates are recommended by LI-COR Biosciences:

96-well format	Nunc® Plates (P/N 161093, 165305)
96-well format	Falcon™ Plates (P/N 353075, 353948)
384-well format	Nunc Plates (P/N 164688, 164730)
384-well format	Falcon Plates (P/N 353961, 353962)

- **Focus Offset Optimization** – If plates other than those recommended above are used, the focus offset can be determined empirically by scanning a plate containing experimental and control samples using the following focus offset settings.

Instrument	Focus Offset Determination (mm)
Odyssey Classic & Odyssey CLx	0.5, 1.0, 2.0, 3.0 & 4.0
Odyssey Sa & Aerius	1.7, 2.0, 3.0 & 4.0

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Use the same intensity settings for each scan. After reviewing the scans, use the focus offset with the highest signal-to-noise for experiments. The actual minimum and maximum focus offset will vary with each instrument. Alternatively, consult the plate manufacturer for the recommended measured distance from the skirt to the bottom of the plate.

- All Odyssey® and Aerius imaging systems (excluding Odyssey Fc) require microplates that have a maximum 4.0 mm distance from the base of the microplate to the target detection area of the plate (actual maximum focus offset varies with each Odyssey Sa or Aerius instrument, and is found by choosing Settings > System Administration, then clicking Scanner Information). When using plates specified previously for In-Cell Western assays, the recommended focus offset is 3.0 mm.

- **Intensity Setting Optimization –**

Instrument	Initial Intensity Setting (700/800 nm)	Intensity Settings Weak Signal (700/800 nm)	Intensity Settings Saturated Signal (700/800 nm)
Odyssey Classic	5 / 5	7.5 / 7.5	2.5 / 2.5
Odyssey CLx	5 / 5	7.5 / 7.5	2.5 / 2.5
	AutoMode*	-	-
Odyssey Sa	7 / 7	8 / 8	4 / 4
Aerius	7 / 7	8 / 8	4 / 4

**The Odyssey CLx AutoMode function alleviates the need to scan the plate at multiple intensity settings.*

- Protect plates from light before imaging to ensure highest sensitivity. When storing plates after imaging, the plates should remain protected from light at 4 °C.

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