CellTag™ 700 Stain Kits I and II Pack Insert

Components (sufficient for 20 x 96-well plates)

For part numbers 926-42091 and 926-42092.

- IRDye® 800CW Secondary Antibody (PN 926-32210 or 926-32211), 0.5 mg (lyophilized)

  Sodium azide is used as a preservative for the IRDye secondary antibody. After reconstituting the antibody according to the provided instructions, the resulting solution will contain 0.01% sodium azide.

  Warning: *Sodium azide is a poisonous and hazardous substance. Handle with care and dispose of properly.*

- Intercept® (PBS) Blocking Buffer, 500 mL
- CellTag 700 Stain, 2 x 10 nmole

Additional Reagents (required but not included)

- Primary Antibody
- 1X PBS
- Tissue Culture Reagents (serum DMEM, trypsin, etc.)
- Black-sided 96-well microplates with clear well bottoms
- 37% Formaldehyde
- 20% Tween® 20
- 10% Triton® X-100

Storage

- IRDye® Secondary Antibody: -20 °C
- Intercept Blocking Buffer: 4 °C

CellTag 700 Stain Storage

Protect from light.

- Upon receipt, immediately store at -20 °C. This product is stable for one year when stored as recommended.
- After reconstitution, store at 4 °C. This product can be stored for 6 months as recommended.

Introduction

The In-Cell Western Kits provide detection reagents for cell-based In-Cell Western Assays. The cost-effective CellTag™ 700 Stain normalization method makes quantification of the target protein more precise.

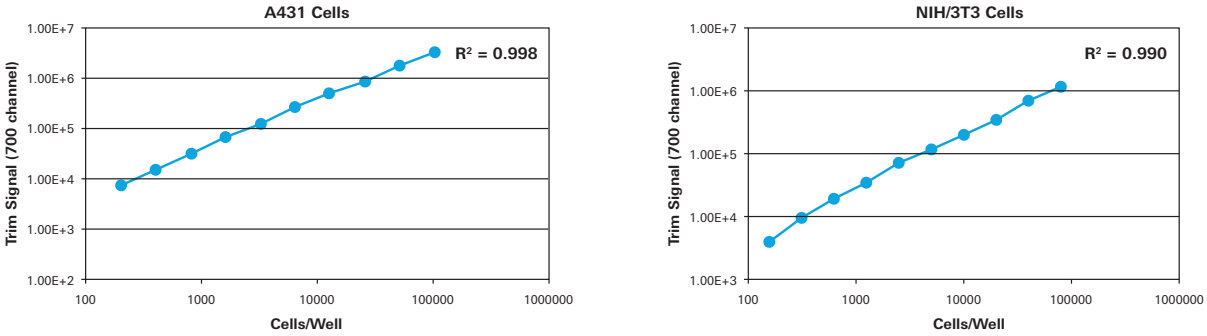
Detection of two proteins can be achieved using two different primary antibodies, followed by detection using two different secondary antibodies (one labeled with IRDye® 800CW and the other with IRDye 680RD) in a multiplex assay. IRDye secondary antibodies for multiplex detection can be

purchased at: licor.com/bio. Dilution factors and blocking conditions should be optimized for each target and antibody combination.

Using CellTag 700 Stain for Cell Number Normalization

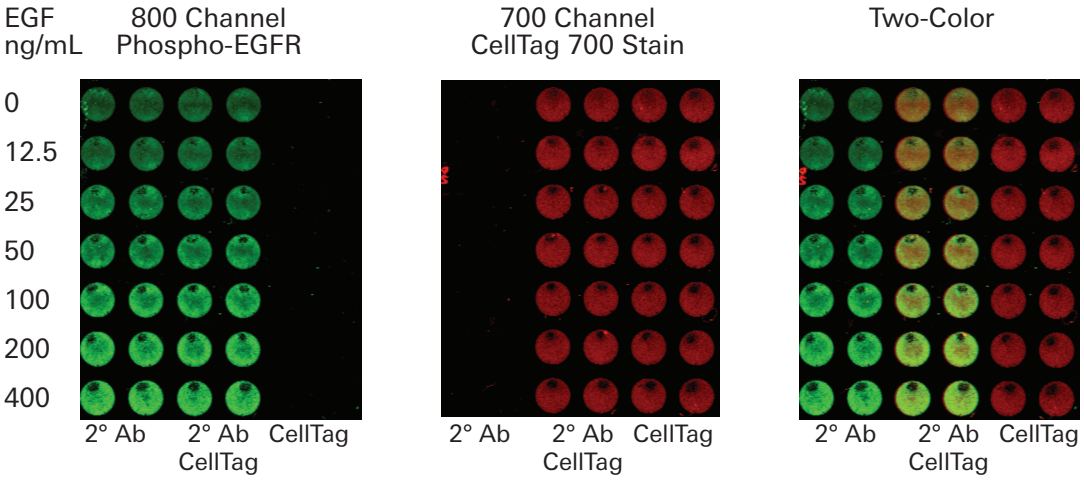
CellTag 700 Stain is a near-infrared fluorescent, non-specific cell stain that provides accurate normalization to cell number for In-Cell Western applications. The stain accumulates in both the nucleus and cytoplasm of permeabilized cells, and provides linear fluorescent signal across a wide range of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

Linear Relationship between Fluorescence and Cell Number



Two-fold serial dilutions of A431 or NIH/3T3 cells were plated in 96-well plates. Cells were fixed, permeabilized, stained with CellTag 700 Stain (0.2 µM), and detected with Odyssey Classic Infrared Imaging System. The Trim Signals were used to generate the graphs.

Example Data



In-Cell Western Assay with CellTag 700 Stain in EGF-stimulated A431 cells. EGF-stimulated A431 cells were fixed, permeabilized, and blocked with Intercept Blocking Buffer (LI-COR, P/N: 927-70000). 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 an Odyssey® CLx Imaging System. (Resolution: 169 µm; Quality: lowest, Focus offset: 3.5 mm; Intensity: Auto for both channels).

Reconstitute Secondary Antibody

Only dilute the antibody immediately before you are ready to use it. Protect from light.

Sodium azide is used as a preservative for the IRDye secondary antibody. After reconstituting the antibody according to the provided instructions, the resulting solution will contain 0.01% sodium azide.

Warning: *Sodium azide is a poisonous and hazardous substance. Handle with care and dispose of properly.*

1. Combine the contents of each vial of secondary antibody with 0.5 mL of sterile distilled water.
2. Mix gently by inverting.
3. Allow the solution to stand at room temperature for at least 30 minutes before use, protected from light.

Reconstitute CellTag 700 Stain

Protect from light.

1. Combine the contents of the vial with 0.1 mL 1X PBS for a final concentration of 0.1 mM.
2. Mix thoroughly by vortexing.
3. Allow mixture to rehydrate for at least 30 minutes before use.

Protocol: In-Cell Western™ Assay with CellTag 700 Stain

Detailed protocols can be found at licor.com/support.

Step 1. Apply Treatment to Cells

Apply treatment to cells as appropriate with drug, stimulant, etc.

Step 2. Remove Media

Remove media manually or by aspiration.

Note: *Proceed to Step 3 (Fix Cells) immediately after removing media.*

Step 3. Fix Cells

Immediately fix cells with Fixing Solution.

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.*

1. Prepare fresh Fixing Solution.

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

2. Using a multi-channel pipettor, add 150 µL of fresh, room temperature Fixing Solution to each well.

   Carefully add the solution down the sides of the wells to avoid detaching cells.

3. Incubate at room temperature on the bench top for 20 minutes with no shaking.

Step 4. Permeabilize Cells

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.

1. Prepare Triton Washing solution as shown:

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

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

3. Using a multi-channel pipettor, add 200 µL of room temperature Triton Washing Solution to each well.

   Carefully add the solution down the sides of the wells to avoid detaching cells.

4. Allow plate to shake on a plate shaker for 5 minutes.

5. Repeat washing steps for a total of 4 washes.

   Note: *Do not allow wells to become dry during washing. Immediately add the next wash after each manual disposal.*

Step 5. Block Cells

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.

1. Using a multi-channel pipettor, add 150 µL of Intercept Blocking Buffer to each well.

   Add the solution carefully by pipetting down the sides of the wells to avoid detaching the cells.

2. Allow blocking for 1 hour at room temperature with moderate shaking on a plate shaker.

Step 6. Dilute Primary Antibody

Dilute primary antibody in Intercept Blocking Buffer.

- Depending on the primary antibody, dilutions may range from 1:50 to 1:200.
- If the primary antibody vendor provides dilution guidelines for immunofluorescent staining, start with that recommended range.

Step 7. Incubate with Primary Antibody

1. Remove blocking buffer.
2. Add 50 µL of Intercept Blocking Buffer to the control wells and 50 µL of the diluted primary antibody to the rest of the wells.
3. Incubate with primary antibody for 1 - 2.5 hours (depending on antibody) at room temperature or overnight at 4 °C with gentle shaking.

Step 8. Wash Plate

1. Prepare Tween® 20 Washing Solution as follows:

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

2. Using a multi-channel pipettor, add 200 µL of Tween 20 Washing solution.
   Carefully add the solution down the sides of the wells to avoid detaching cells.
3. Allow plate to shake on a plate shaker for 5 minutes.
4. Repeat washing steps for a total of 4 washes.

Step 9. Dilute Secondary Antibody

Dilute the fluorescently-labeled secondary antibody in Intercept Blocking Buffer. The optimal dilution for your assay should be determined empirically.

Note: *Avoid prolonged exposure of the antibody vials to light.*

- The recommended dilution range is 1:200 to 1:1,200 with a suggested starting dilution of 1:800.
- To lower background, add Tween 20 at a final concentration of 0.2% to the diluted antibody.

Step 10. Incubate with Secondary Antibody

Secondary antibody staining and normalization staining are carried out simultaneously.

For control wells (used to calculate background), add **only** diluted secondary antibody **without** CellTag 700 Stain.

1. Add CellTag 700 Stain to the diluted secondary antibody solution.
   The suggested concentration for CellTag 700 Stain is 0.2 µM (1:500 dilution).
2. To the control wells, add 50 µL of secondary antibody solution without CellTag 700 Stain.
3. To the remaining wells, add 50 µL of secondary antibody solution with CellTag 700 Stain.

4. Incubate for 1 hour at room temperature with gentle shaking.
   Note: *Protect from light during incubation.*

Step 11. Wash Plate

Protect plate from light during washing.

1. Using a multi-channel pipettor, add 200 µL of Tween 20 Washing solution.
   Carefully add the solution down the sides of the wells to avoid detaching cells.
2. Allow plate to shake on a plate shaker for 5 minutes.
3. Repeat washing steps for a total of 4 washes.

Step 12. Remove Wash Solution

After the 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.

Scan

For best results, scan the plate immediately. Plates may be stored at 4 °C for up to several weeks (protected from light).

1. Clean the bottom plate surface and the scanning bed (if applicable) with moist, lint-free paper.
2. Scan the plate with the 700 and 800 nm channel using an Odyssey® Imager.

Suggested Scan Settings

All settings may require adjustment for optimal data quality. Higher resolutions or scan qualities can be used, but the scan time will increase.

Instrument	Resolution	Scan Quality	Intensity Setting (700 nm)	Intensity Setting (800 nm)
Odyssey Classic	169 µM	lowest	5	5
Odyssey CLx	169 µM	lowest	Auto Mode	Auto Mode
Odyssey Sa	200 µM	lowest	7	7
Aerius™ Imager	200 µM	lowest	7	7

Experimental Design Considerations

Consider the following points carefully when planning your In-Cell Western™ Assay.

Establish Primary Antibody Specificity

Antibody specificity and selectivity are highly dependent on assay context and can be difficult to predict. It is good practice to select an antibody that has been well characterized by the vendor for the intended application. However, even if the antibody has been tested in a specific application by the vendor or elsewhere, you should still experimentally verify antibody performance in your intended assays and experimental contexts.

Select Plates

Proper selection of microwell plates can significantly affect In-Cell Western results. Use **black-sided plates** with clear well bottoms. In-Cell Western analyses are detected at the well surface bottom with no liquid present. Do **not** use plates with white walls, because autofluorescence from the white surface will create significant noise. Sterile plates are required for tissue culture growth.

Recommended Microwell Plates

LI-COR recommends the following plates for the In-Cell Western™ Assay. These plates are sterile, include a lid, and have a clear, polystyrene bottom with black walls.

- For adherent cells, LI-COR recommends a 96-well plate with a clear, flat bottom and black wells, such as the Greiner Bio-One CELLSTAR® Black µClear® Microplate, LI-COR PN 926-19156 (8 pack) or 926-19157 (32 pack).
- For suspension cells, LI-COR recommends growing cells in a 96-well U-bottom plate and transferring cells to Greiner Bio-One CELLSTAR® Black µClear® Microplate, LI-COR PN 926-19156 (8 pack) or 926-19157 (32 pack), for imaging.

Focus Offset

The recommended focus offset for the recommended plates is 3.0 mm.

Determine Focus Offset

If you are using plates other than those recommended, the focus offset can be determined empirically by scanning a plate containing experimental samples and control samples.

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 to determine the distance from the skirt to the bottom of the plate to help you begin the focus offset optimization process.

Intensity Setting Optimization

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

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

Plate Storage

Protect plates from light before imaging to ensure highest sensitivity.

When storing plates after imaging, the plates should be sealed with plate tape or paraffin wrap and remain protected from light at 4 °C.

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