CellTag™ 700 Stain Pack Insert

Components (926-41090)

CellTag 700 Stain: 2 x 10 nmole (lyophilized)

Specifications

- Recommended concentration: 0.2 µM
- Excitation: 675 nm
- Emission: 697 nm

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 at room temperature before use.

CellTag 700 Stain Storage

Protect from light.

Lyophilized

- Storage temperature: -20 °C
- Storage conditions: Protect from light.
- Shelf life: This product is stable for one year when stored as recommended.

After Reconstitution

- Storage temperature: 4 °C or -20 °C
- Storage conditions: Protect from light.
- Shelf life: This product can be stored for 6 months as recommended.

Description

The In-Cell Western™ Assay is an immunocytochemical assay that uses near-infrared fluorescence to detect and quantify proteins in permeabilized cells. The assay combines the specificity of Western blotting with the reproducibility and throughput of ELISA. Quantitative accuracy can be greatly improved by normalizing to cell number or normalizing to an internal control protein. Normalization to cell number is a fast and inexpensive method that makes quantification more precise by correcting for well-to-well variation.

Normalization Using CellTag™ 700 Stain

The cost-effective CellTag 700 Stain normalization method makes quantification of the target protein more precise.

CellTag 700 Stain is a near-infrared fluorescent, non-specific cell stain that provides accurate normalization to cell number for several multiwell plate assays. 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 an Odyssey® Imager.

CellTag 700 Stain can be used independently (e.g., in a cell proliferation assay) or in combination with immunodetection (e.g., an In-Cell Western), depending on the needs of your research. In an

In-Cell Western™ Assay Assay, CellTag can be applied to cells during incubation with an IRDye® 800CW Secondary Antibody to enable accurate normalization for target protein quantification.

Linear Relationship Between Fluorescence and Cell Number

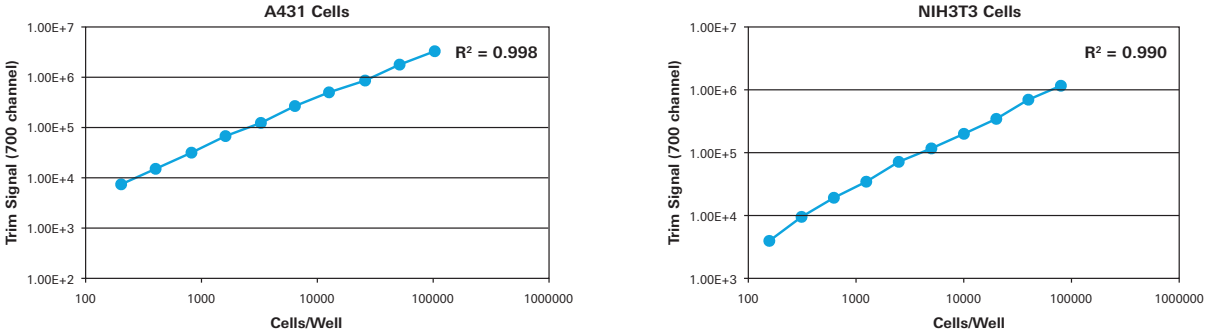

Figure 1. Two-fold serial dilutions of A431 or NIH3T3 cells were plated in 96-well plates. Cells were fixed, permeabilized, stained with CellTag 700 Stain (0.2 µM), and detected with Odyssey® Classic Infrared Imaging System. The Trim Signals were used to generate the graphs.

Example Data

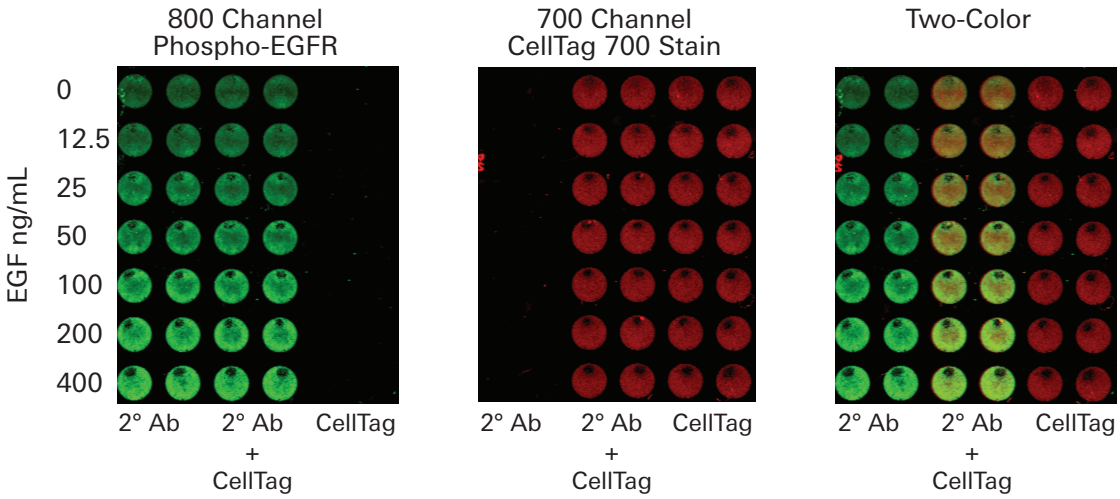

Figure 2. In-Cell Western™ Assay with CellTag™ 700 Stain in EGF-stimulated A431 cells. EGF-stimulated A431 cells were fixed, permeabilized, and blocked with Intercept® Blocking Buffer (LI-COR, P/N: 927-70001). Phosphorylated EGFR was measured using rabbit anti-phospho-EGFR primary antibody followed by 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 EGF treatment. 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).

Other Normalization Strategies

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.

Alternatively, an imager, such as the Odyssey M, which also supports signal capture from visible wavelengths, enables you to detect targets in the 700 nm and 800 nm channels while still normalizing to cell number with CellTag 520 (licor.com/celltag) detected in the 520 nm channel. Additional information about normalization strategies for In-Cell Western Assays can be found at licor.com/support.

Example In-Cell Western™ Assay Protocol

The following is a conventional, representative protocol for an In-Cell Western Assay using adherent cells performed in a 96-well plate.

You may need to optimize this protocol for your particular cell line and target of interest. This protocol can be adapted for most multiwell plate formats from 6 wells to 1536 wells. Guidance for developing an In-Cell Western Assay, detailed protocols, and further information regarding suspension cells can be found at licor.com/support.

Required Materials

- IRDye® 800CW Secondary Antibody
- Intercept® (TBS) Blocking Buffer (licor.com/intercept) or equivalent

  Note: *Be sure to keep your buffer system consistent throughout the protocol for blocking, antibody dilutions, and washes. For example, if you use a TBS-based buffer system, choose Intercept® (TBS) Blocking Buffer. If you use a PBS-based buffer system, choose Intercept® (PBS) Blocking Buffer.*
- Intercept® T20 (TBS) Antibody Diluent or equivalent
- Primary Antibody
- 1X TBS
- 1X PBS
- Tissue culture reagents
- 37% Formaldehyde
- Tween® 20
- Triton® X-100
- Multiwell plates: This protocol can be adapted for most multiwell plates from 6 to 1536 wells.

  Note: *We recommend using black-sided plates to minimize well-to-well crosstalk. White-sided plates are not recommended for this assay due to autofluorescence.*
  - 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.

Solutions Used in the Protocol

Reconstitute CellTag 700 Stain

See "Reconstitute CellTag 700 Stain" on page 1.

Prepare Wash Solution

1. Add 5 mL of 20% Tween® 20 to 995 mL of 1X TBS to make a 0.1% Tween 20 solution.
2. Mix solution well.

Prepare Fresh Fixation Solution

If optimal fixation conditions are known for immunofluorescent staining of your cell line and/or target protein, then use the appropriate conditions. Most fixatives and fixation protocols for immunofluorescent staining may be adapted to the In-Cell Western™ Assay format.

Tip: The LI-COR In-Cell Western protocol uses a 3.7% formaldehyde solution.

1. Add 5 mL of 37% formaldehyde to 45 mL of 1X TBS to make a 3.7% formaldehyde solution.
2. Mix solution well.

Prepare Fresh Permeabilization Solution

If optimal permeabilization conditions are known for immunofluorescent staining of your cell line and target protein then use the appropriate conditions.

1. Add 5 mL of 10% Triton X-100 to 495 mL of 1X TBS to make a 0.1% Triton X-100 solution.
2. Mix solution well.

Prepare Antibody Diluent

Tip: Intercept® T20 (TBS) Antibody Diluent is preformulated with Tween® 20 to the correct concentration.

If you will not be using Intercept® T20 (TBS) Antibody Diluent, you will need to measure and prepare an antibody diluent used in the primary and secondary antibody dilutions. Using the same type of blocking buffer you will use to block the cells, add Tween 20 to a final concentration of 0.2% Tween 20.

Step 1. Seed Cells

Experimental conditions must be optimized for each cell type to determine the appropriate level of confluency required to achieve significant well fluorescence.

1. Count cells to determine cell number.
2. Seed the necessary number of cells per well into a multiwell plate.
3. Allow cells to settle and adhere to the well bottom on a level surface at room temperature for approximately 5 - 20 minutes.
4. Move the plates to the appropriate incubator until cells reach the desired confluency.

   Tip: Generally, cells should be about 80 - 85% confluent at time of fixation.
5. If your experiment does not require a cell treatment, then remove media from the wells and skip to Step 3 "Fix Cells" below.

Step 2. Cell Treatment (Optional)

If your experiment requires a cell treatment, follow the instructions in this section. Otherwise, proceed to Step 3 " Fix Cells".

1. Apply appropriate treatment (e.g., drug, siRNA, virus) for the optimal amount of time to elicit desired cellular response.
2. Remove media containing treatment.
3. Proceed immediately with fixation.

Step 3. Fix Cells

1. Using a multichannel pipette, carefully add 150 µL of fresh, room temperature Fixation Solution down the sides of each well to avoid detaching cells.
2. Incubate for 20 minutes at room temperature without agitation.
3. Remove Fixation Solution and dispose of it in an appropriate waste container.

Step 4. Permeabilize Cells

1. Using a multichannel pipette, carefully add 150 µL of room temperature Permeabilization Solution down the sides of each well to avoid detaching cells.
2. Gently agitate plate for 20 minutes at room temperature.
3. Remove Permeabilization Solution carefully from the wells using the multichannel pipette without removing any cells. Continue immediately with Step 5 "Block Cells".

Step 5. Block Cells

If you have used the primary antibody successfully for immunofluorescent staining, consider trying the same blocking buffer for In-Cell Western™ Assay detection.

1. Using the multichannel pipette, add 150 µL of Intercept® (TBS) Blocking Buffer to each well. Add the solution carefully by pipetting down the sides of the wells to avoid detaching the cells.
2. Block for 1.5 hours at room temperature with gentle agitation.

Step 6. Prepare Primary Antibody Solution

Dilute the primary antibody in antibody diluent.

Tip: 50 µL per well is the recommended volume for 96-well plates.

- Depending on the primary antibody, dilutions typically 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 Primary Antibody

Note: *Secondary Antibody Background control wells measure background attributed only to the secondary antibody. In this step, you will only add diluent (without primary antibody) to the background control wells. Secondary antibody will be added to the background control wells in step 10.*

1. Remove blocking buffer carefully from the wells.
2. Add 50 µL of antibody diluent to the Secondary Antibody Background control wells and 50 µL of diluted primary antibody to the rest of the wells.
3. Depending on vendor recommendations, incubate the plate for 2 hours at room temperature or overnight at 4 °C with gentle agitation.

Step 8. Wash Plate

1. Carefully remove the liquid from the wells, and avoid removing any cells.
2. Using a multichannel pipette, carefully add 150 µL of Wash Solution down the sides of the wells to avoid detaching cells.
3. Allow gentle agitation for 5 minutes at room temperature.
4. Carefully remove liquid from the wells, without removing any cells.
5. Repeat steps 2-4 for a total of 4 washes.

Step 9. Prepare Secondary Antibody and Cell Stain

Dilute the IRDye® fluorescently-labeled secondary antibody in antibody diluent. The optimal dilution for your assays should be determined empirically. The recommended dilution range is 1:200 to 1:1,200, with a suggested starting dilution of 1:800.

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

1. Prepare enough secondary antibody solution for all your wells.

   Tip: 50 µL per well is the recommended volume for 96-well plates.

2. For your Secondary Antibody Background control wells, aliquot enough secondary antibody solution (50 µL per well for a 96-well plate) into another tube and set aside.

   Do **not** add CellTag™ 700 Stain to this aliquot.

3. Add CellTag 700 Stain to the remaining diluted secondary antibody solution.

   The suggested concentration for CellTag 700 Stain is 0.2 µM (1:500 dilution).

Step 10. Incubate Secondary Antibody and Cell Stain

Secondary antibody staining and normalization staining are carried out simultaneously. For Secondary Antibody Background control wells, add only diluted secondary antibody without CellTag 700 Stain.

1. To the Secondary Antibody Background control wells, add 50 µL of secondary antibody solution **without** CellTag 700 Stain stain.

2. To the remaining wells, add 50 µL of secondary antibody solution **with** CellTag 700 Stain.

3. Incubate the plate for 1 hour at room temperature with gentle agitation.

   Note: *Protect plate from light from this step onward.*

Step 11. Wash Plate

1. Carefully remove the liquid from the wells, and avoid removing any cells.

2. Using a multichannel pipette, carefully add 150 µL of Wash Solution down the sides of the wells to avoid detaching cells.

3. Allow plate to gently agitate for 5 minutes at room temperature and protected from light.

4. Carefully remove liquid from the wells, without removing any cells.

5. Repeat steps 2-4 for a total of 4 washes.

Step 12. Measure Signal

1. Ensure all of the final wash solution is removed from the wells using a multichannel pipette.

   Turn the plate upside down on a paper towel, then tap or blot the plate gently on the paper towel. For best results, apply an adhesive plate cover.

2. Clean the scanning bed and the bottom plate surface with lint-free paper and 70% ethanol prior to imaging.

3. Place the Plate Alignment Guide on the glass surface of the imaging bed to align plates for imaging.

4. Scan on an Odyssey® Imager. The suggested scan settings for Greiner Bio-One plates are as shown below. For plates other than Greiner Bio-One, you may need to empirically determine the optimal focus offset.

   For acquiring images in LI-COR® Acquisition Software, follow the Multiwell Plate workflow for In-Cell Western. The following imaging settings are used in this workflow. Other settings can be entered in the Custom workflow.

	Resolution		Focus Offset	
Imager	**96-Well**	**384-Well**	**Default**	**Range**
Odyssey DLx or Odyssey CLx	169 µm	84 µm	4.00 mm	0.00 - 4.00 mm
Odyssey M	100 µm	50 µm	3.8 mm	1.00 - 5.00 mm

   For acquiring images in Image Studio™ Software, use the following settings.

Imager	**Resolution**	**Scan Quality**	**Intensity (700 nm)**	**Intensity (800 nm)**	**Focus Offset**
Odyssey Classic	169 µM	Lowest	5	5	4.0 mm
Odyssey CLx	169 µM	Lowest	Auto	Auto	4.0 mm

5. Analyze data using the Multiwell Plate workflow in Empiria Studio® Software licor.com/empiria

   For analysis using Image Studio, use the details provided at licor.com/is-icw-analysis.

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