Components

For GAM/GAR CellTag 520 ICW Kit III (926-42093) and GAR/GAM CellTag 520 ICW Kit IV (926-42094)

The following quantities are sufficient for 10 x 96-well plates.

Description	Quantity	Reorder
IRDye® 800CW Secondary Antibody (PN 926-32210 or 926-32211) ***Warning:*** *Contains sodium azide.*	0.5 mg (lyophilized)	licor.com/antibodies
IRDye 680RD Secondary Antibody (PN 926-68070 or 926-68071) ***Warning:*** *Contains sodium azide.*	0.5 mg (lyophilized)	
Intercept® (TBS) Blocking Buffer (PN 927-60001)	500 mL	licor.com/intercept
CellTag 520 Stain (PN 926-41094)	2 x 50 nmole	licor.com/CellTag

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.*

Storage

Intercept (TBS) Blocking Buffer

Store at 4 °C. See expiration date on bottle.

IRDye Secondary Antibody

- Storage temperature: 4 °C
- Storage conditions: Protect from light and moisture.
- Shelf life: When stored as recommended, this product is stable for 3 months.

CellTag 520 Stain

Lyophilized

- Storage temperature: -20 °C
- Storage conditions: Protect from light.
- Shelf life: This product is stable for 6 months 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 3 months as recommended.

Plate

After image acquisition, plates may be sealed with plate tape or paraffin wrap and stored at 4 °C for up to several weeks protected from light.

Description

The In-Cell Western™ Assay is an immunocytochemical assay that uses visible and 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.

Using CellTag 520 Stain for Cell Number Normalization

CellTag 520 Stain is a visible fluorescent, non-specific cell stain that provides accurate normalization to cell number for the In-Cell Western Assay and other 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 520 Stain is detected in the 520 nm channel of the Odyssey® M Imager. CellTag 520 Stain can be applied to the cells during incubation with IRDye® secondary antibodies, and enables accurate normalization of multiple target proteins with much higher throughput than Western blotting.

Linear Relationship between Fluorescence and Cell Number

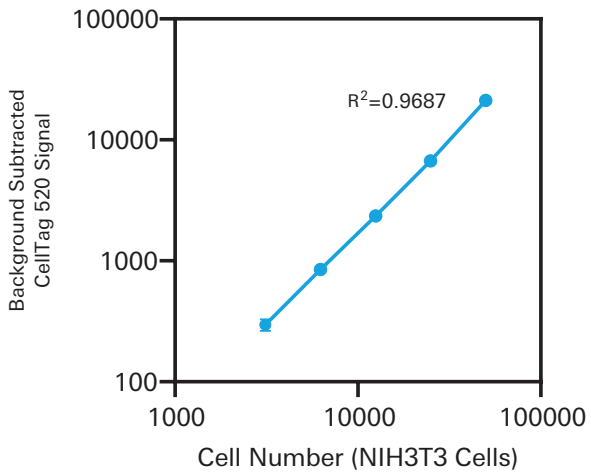

Figure 1. This graph shows the linear relationship between CellTag 520 Stain signal and NIH3T3 cell number. Two-fold serial dilutions of NIH3T3 cells were plated in a 96-well plate. Cells were fixed, permeabilized, stained with CellTag 520 Stain (1.0 µM), and detected with an Odyssey® M Imaging System.

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.

Additional Reagents (required but not included)

- Primary Antibody
- 1X TBS
- 1X PBS
- Intercept® T20 (TBS) Antibody Diluent or equivalent
- Tissue Culture Reagents (serum DMEM, trypsin, etc.)
- 37% Formaldehyde
- 20% Tween® 20
- 10% 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 520 Stain

Protect from light.

1. Reconstitute each vial with 100 µL 1X PBS for a stock concentration of 0.5 mM.
2. Mix thoroughly by vortexing.
3. Allow mixture to rehydrate for at least 30 minutes before use, protected from light.

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: *Contains sodium azide.*

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.

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.

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" on the facing page.

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 Intercept T20 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. Aliquot enough secondary antibody solution (50 µL per well for a 96-well plate) for your Secondary Antibody Background wells into another tube and set aside.

   Do **not** add CellTag™ 520 Stain to this aliquot.
3. Add CellTag 520 Stain to the remaining diluted secondary antibody solution.

   The suggested concentration for CellTag 520 Stain is 1 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 520 Stain.

1. To the Secondary Antibody Background control wells, add 50 µL of secondary antibody solution **without** CellTag 520 Stain stain.
2. To the remaining wells, add 50 µL of secondary antibody solution **with** CellTag 520 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 pipet.
   Turn the plate upside down on a paper towel, then tap or blot the plate gently on the paper towel.
2. For best results, apply an adhesive plate cover.
3. Clean the scanning bed and the bottom plate surface with lint-free paper and 70% ethanol prior to imaging.
4. Place the Plate Alignment Guide on the glass surface of the imaging bed to align plates for imaging.
5. Follow the Multiwell Plate workflow provided in LI-COR® Acquisition Software for In-Cell Western. Use the channels below when selecting detection channels.

Reagent	Channel
CellTag™ 520 Stain	520 nm
IRDye® 680RD Secondary Antibody	700 nm
IRDye 800CW Secondary Antibody	800 nm

6. Scan on an Odyssey® M Imager.

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