Components for Kit 827-14642 (sufficient for 5 x 96-well plates)

Description	Quantity	Reorder
IRDye® 800CW Goat anti-Mouse Secondary Antibody ***Warning:*** *Contains sodium azide.*	2 x 25 µL (1 mg/mL)	licor.com/antibodies
Intercept® (TBS) Blocking Buffer	125 mL	licor.com/intercept
CellTag™ 700 Stain	10 nmole	licor.com/CellTag
Greiner Bio-One 96-well CELLSTAR® Plates, TC Sterile, Black µClear®, with Lid	8 plates	Reorder LI-COR PN 926-19156 (8 pack) or 926-19157 (32 pack)

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.*

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 800CW Goat anti-Mouse 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 700 Stain

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.

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.

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.

Introduction

This kit provides detection reagents for cell-based In-Cell Western™ Assays. The In-Cell Western enables accurate target quantification with higher throughput than Western blotting. The conventional In-Cell Western protocol, provided in this document, includes the detection of one target protein in the 800 nm channel with IRDye® 800CW and normalization to CellTag™ 700 Stain detected in the 700 nm channel.

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.

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.

Additional Reagents (required but not included)

- Tissue Culture Reagents (serum, media, trypsin, etc.)
- Primary Antibody
- 1X TBS
- 1X PBS
- Intercept® T20 (TBS) Antibody Diluent or equivalent
- 37% Formaldehyde
- 20% Tween® 20
- 10% Triton® X-100

Solutions Used in the Protocol

Reconstitute CellTag 700 Stain

See "Reconstitute CellTag 700 Stain" on the previous page.

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.

   Tip: Generally, cells should be about 80 - 85% confluent at time of fixation.

2. Seed the appropriate number of cells per well into the 96 well plate provided with this kit.

3. Allow cells to settle and adhere to the well bottom for one hour on a flat surface at room temperature.

4. Move the plates to the appropriate incubator until cells reach the desired confluency.

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.

Additional Resources

Single Antibody Titration Experiment

The following plate layout shows a template experiment that can be adapted for the specifics in your research to determine the best antibody dilution to use in your experiment. Plate layouts may also be created and printed from Empiria Studio® Software.

Note: *The section* *Plate Loading Guides* *on the facing page has loading guides designed to fit under a plate to help keep track of which samples should be loaded in which wells.*

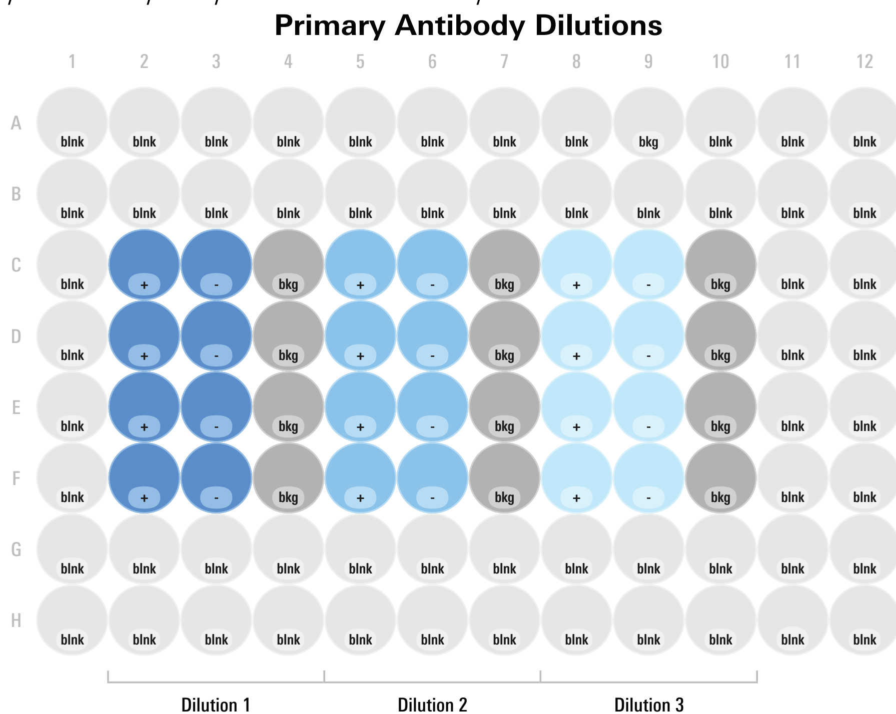


Figure 1. This is the same design used in the **[Preset] Antibody Titration (Single Antibody)** Plate Template provided in Empiria Studio® Software. Read the Plate Layout Legend below or open the Plate Template in Empiria Studio for more information.

Plate Layout Legend

Symbol	Definition
blnk	**Blank** wells contain only TBS or PBS buffer and are used to mitigate the potential impact of plate well edge effects.
bkg	**Background** wells that contain secondary antibody without primary antibody or normalization reagent.
+	**Positive** control wells contain primary and secondary antibody. Depending on your experiment, positive controls could be an over-expressed cell line, a treatment known to stimulate target expression, etc.
-	**Negative** control wells contain primary and secondary antibody. Depending on your experiment, negative controls could be a CRISPR knockdown, a treatment known to inhibit target expression, etc.

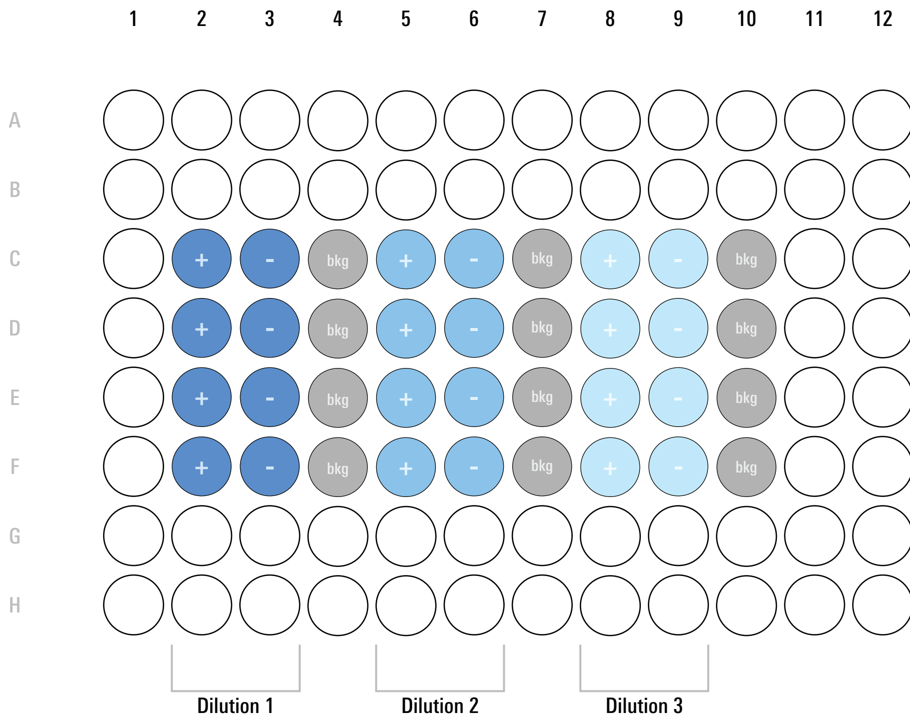
Plate Loading Guides

To use the following plate loading guides, cut out the layout, then place the layout underneath the plate to make it easier to load samples in the correct wells. Plate layouts may also be created and printed from Empiria Studio® Software.

Note: *More Plate Loading guides are available at licor.com/plate-loading-guides.*

Plate Layout for Loading Antibody Dilutions

The following plate layout can be used as a guide for loading antibody dilutions according to the Plate Layout shown in "Single Antibody Titration Experiment" on the previous page.



Symbol	Well Type	Cells	Primary Antibody	Secondary Antibody	CellTag™ 700 Stain
bkg	Background	✔		✔	
+	Positive Control	✔	✔	✔	✔
-	Negative Control	✔	✔	✔	✔

Blank Plate Loading Guides

Label the following blank Loading Guides as needed for your In-Cell Western Assay.

Note: *More Plate Loading guides are available at licor.com/plate-loading-guides.*

	1	2	3	4	5	6	7	8	9	10	11	12
A												
B												
C												
D												
E												
F												
G												
H												

	1	2	3	4	5	6	7	8	9	10	11	12
A												
B												
C												
D												
E												
F												
G												
H												

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