Sapphire700™ Stain Pack Insert

Specifications

For part number 928-40022

- Shelf Life: 1 year from date of receipt
- Storage: Room Temperature

Required Reagents

- IRDye 800CW Secondary Antibody (LI-COR, various PNs)
- Blocking buffer
- Sapphire700 Stain (LI-COR, PN 928-40022)
- DRAQ5 Stain (Biostatus, PN DR50200)
- Primary antibody
- 1X PBS
- Tissue culture reagents (serum, DMEM, trypsin, etc.)
- 96-well multiwell plate (see Experimental Design Considerations on page 6)
- 37% formaldehyde
- 20% Tween® 20
- 10% Triton® X-100s

Introduction

In-Cell Western assays commonly use primary and secondary antibodies for normalization in the 700 nm channel. For example, if phospho-ERK is the target of interest, an antibody against total ERK can be used to normalize for variations in ERK protein levels. An alternative, more cost-effective approach involves the use of two fluorescent cell stains in the 700 nm channel for normalization. Sapphire700 Stain and DRAQ5 Stain are both cell staining agents that can be used in combination for accurate normalization of well-to-well variation in cell density. The combination of these two stains expands the detection range over that provided by a single stain alone.

Using Sapphire700 Stain and DRAQ5 Stain for Cell Number Normalization

Simultaneous staining of cells with Sapphire700 Stain and DRAQ5 Stain expands the linear range, allowing more accurate normalization of cell number across both low- and high-cell densities. Staining is performed at the time that IRDye 800CW secondary antibody is applied to the cells.

Sapphire700 Stain

Sapphire700 Stain is a non-specific cell stain that accumulates in both the nucleus and cytoplasm of fixed or dead cells but not live cells. When used to stain serial dilutions of A431 cells in 96-well plates, Sapphire700 Stain displays linearity of fluorescent signal for higher cell densities, from ~50,000 to ~250,000 cells/well (see Example Data on the next page).

DRAQ5 Stain

DRAQ5 Stain is a cell-permeable DNA-interactive agent which can be used for stoichiometric staining of dsDNA in live or fixed cells. For more information about DRAQ5 Stain, please visit the Biostatus Limited web site. When serial dilutions of A431 human epithelial carcinoma cells are plated in 96-well plates, DRAQ5 Stain alone demonstrates linearity of fluorescent signal for lower cell densities, from ~3,000 to ~50,000 cells/well (see Example Data below).

Example Data

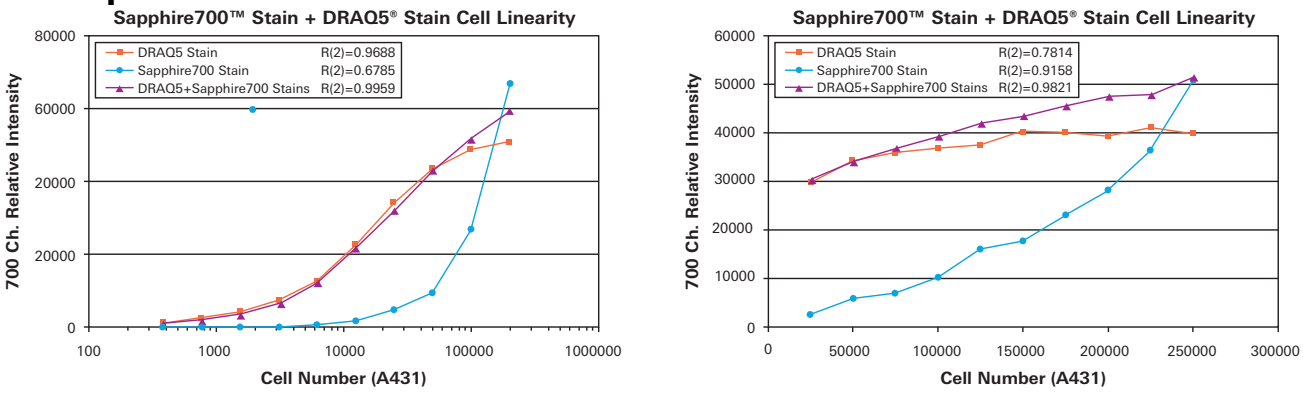

Figure 1. Sapphire700 Stain and DRAQ5 Stain as normalizing agents for In-Cell Western Assays. Dilutions of A431 cells were plated on flat-bottomed, 96-well plates, then fixed and permeabilized. Cells were stained with DRAQ5 Stain alone, Sapphire700 Stain alone, or both stains combined. The left graph shows two-fold cell dilutions over a wide range of cell densities (0 - 200,000 cells/well). The right graph shows a closer examination of linearity of signal over the range of 25,000 - 250,000 cells/well, in dilution increments of 25,000 cells.

Protocol: In-Cell Western™ Assay with Sapphire700 Stain and DRAQ5 Stain

Dilutions of Sapphire700™ Stain and DRAQ5 Stain are mixed with the diluted secondary antibody. Following incubation, washes and imaging are carried out according to the standard In-Cell Western™ protocol.

LI-COR also offers a one-step solution for In-Cell Western assay normalization using CellTag™ 700 Stain (LI-COR PN 926-41090) or CellTag 520 (LI-COR PN 926-41094). CellTag displays linearity across the same range of cell densities as Sapphire700 Stain and DRAQ5 Stains combined and enables accurate normalization of target protein levels when combined with an IRDye® 800CW secondary antibody. Visit licor.com/celltag700 for more detailed information regarding CellTag.

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 method (for example, ice-cold methanol) is known to work well for immunofluorescent staining of your protein target, you may prefer to use that method rather than the 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

1. Using a multi-channel pipettor, add 150 µL of 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 1.5 hours at room temperature with moderate shaking on a plate shaker.

Step 6. Dilute Primary Antibody

Dilute primary antibody in 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 blocking buffer to the control wells and 50 µL of the diluted primary antibody to the rest of the wells.

   Note: *It is important to include control wells that do not contain primary antibody. These wells will only be treated with secondary antibody and should be used to correct for background staining in the data analysis.*
3. Incubate with primary antibody for 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 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. To stain for normalization, add both Sapphire700 Stain and DRAQ5 Stain to the diluted secondary antibody solution and apply this mixture to the cells. Suggested dilutions for normalization stains:

Sapphire700 Stain	1:1,000
DRAQ5 Stain from Biostatus (5 mM)	1:10,000

1. For control wells (used to calculate background), add **only** diluted secondary antibody **without** Sapphire700 Stain and DRAQ5 Stain.
2. To the control wells, add 50 µL of Secondary Antibody Solution without Sapphire700 Stain and DRAQ5 Stain.
3. To the remaining wells, add 50 µL of Secondary Antibody Solution with Sapphire700 Stain and DRAQ5 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.

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.

Experimental Design Considerations

The following are a few points to consider carefully when planning your In-Cell Western™ Assay.

More Info: *For more information about developing an In-Cell Western Assay, read the In-Cell Western Assay Handbook (licor.com/icw-handbook).*

Select Plates

Proper selection of microwell plates can significantly affect In-Cell Western results.

Recommended Plates

LI-COR Biosciences recommends the following plates.

- 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.

Plate Color

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

Sterile Plates

In-Cell Western Assays require sterile plates for tissue culture growth.

Blocking Buffer

No single blocking reagent will be optimal for every antigen-antibody pair. Some primary antibodies may exhibit greatly reduced signal or different non-specific binding in different blocking solutions.

Be sure to keep your buffer system consistent throughout the protocol for blocking, antibody dilutions, and washes.

Limitation of Liability and Limited Use Label License

For Research Use Only. Not intended for human therapeutic or diagnostic use.

By using this product, you agree to the Limitation of Liability and the Limited Use Label License available online at licor.com/packinserts under the **Pack Inserts** heading (the "License"). The License is incorporated herein by this reference, and the License may be updated from time to time.

LI-COR

LI-COR Biosciences
4647 Superior Street
Lincoln, NE 68504
Phone: +1-402-467-0700
Toll free: 800-645-4267
biosales@licor.com
licor.com/bio

Regional Offices

LI-COR Biosciences GmbH
Siemensstraße 25A
61352 Bad Homburg
Germany
Phone: +49 (0) 6172 17 17 771
bio-eu@licor.com

LI-COR Biosciences UK Ltd.
St. John's Innovation Centre
Cowley Road • Cambridge
CB4 0WS • United Kingdom
Phone: +44 (0) 1223 422104
bio-eu@licor.com

Doc # 988-16471
2022-09-15