

In-Cell Western™ Assay

Kits I and II

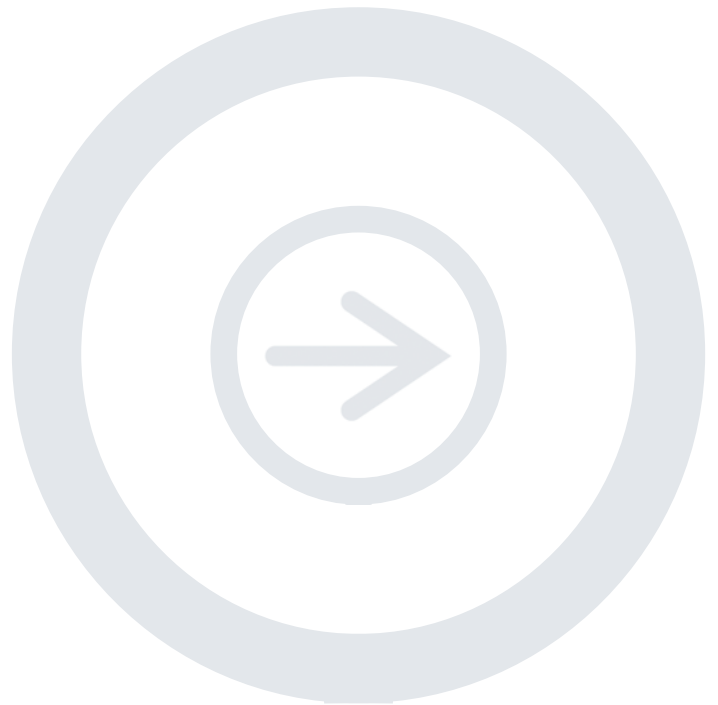
Developed for:

**Aerius, and Odyssey® Classic,
Odyssey CLx, and
Odyssey Sa
Imaging Systems**

*Please refer to your manual to confirm
that this protocol is appropriate for the
applications compatible with your
Odyssey Imager model.*

Part Numbers: 926-31070 and 926-31072

Storage: 4°C



Published November 2006. Revised
October 2011. The most recent version
of this protocol is posted at
<http://biosupport.licor.com/support>

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I. INTRODUCTION

The In-Cell Western Kits provide detection reagents for cell-based In-Cell Western Assays. Each kit includes blocking buffer, IRDye® 800CW secondary antibody for detection of a specific protein target in the 800 nm channel, and two fluorescent cell stains that are used in combination in the 700 nm channel to normalize well-to-well variations in cell number. This approach allows the target of interest to be detected accurately and cost-effectively.

If the application detects two protein targets with two different primary antibodies, use two different secondary antibodies (one labeled with IRDye 800CW and the other with either IRDye 680LT or IRDye 680RD) in a multiplex assay. IRDye secondary antibodies for multiplex detection can be purchased at: www.licor.com/catalog Dilution factors and blocking conditions should be optimized for target and primary antibody combinations.

II. USING DRAQ5® AND SAPPHIRE700™ CELL STAINS FOR CELL NUMBER NORMALIZATION

The cell stains included in this kit are designed to be used in combination to provide accurate normalization over a broad range of cell densities. DRAQ5 Cell Stain is a cell-permeable, DNA-interactive agent that can be used for stoichiometric staining of DNA in live or fixed cells. DRAQ5 Stain is part of this kit, but is sold separately by Biostatus Limited (<http://www.biostatus.com/product/draq5/>). When serial dilutions of A431 human epithelial carcinoma cells are plated in 96-well plates, DRAQ5 Stain demonstrates linearity of fluorescent signal for lower cell densities, up to ~50,000 cells/well (Figure 1A).

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 (Figure 1B).

Simultaneous staining of cells with DRAQ5 and Sapphire700 Stains expands the linear range, allowing more accurate normalization of cell number across both low and high cell densities (Figure 1). Sapphire700 Stain can be purchased separately from LI-COR Biosciences (P/N 928-40022).

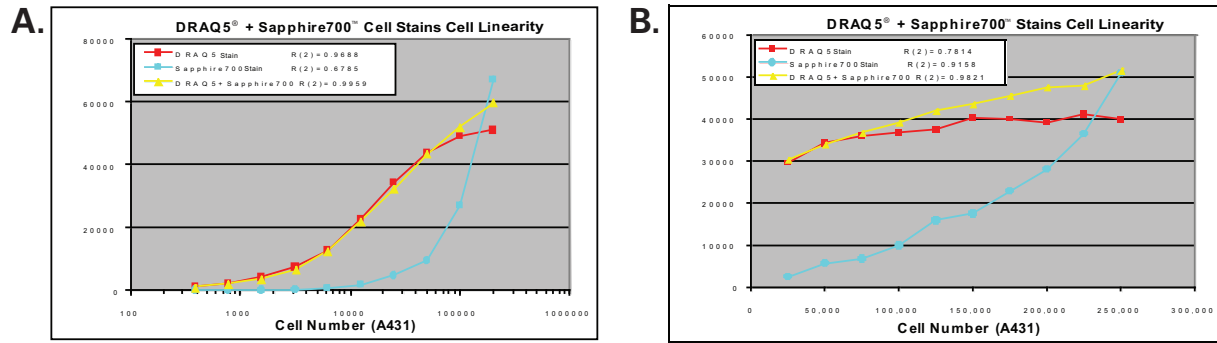


Figure 1. DRAQ5[®] and Sapphire700[™] Cell Stains Cell Linearity. Dilutions of A431 cells were plated on clear, flat-bottom 96-well plates, then fixed and permeabilized. Cells were stained with DRAQ5 Stain alone, Sapphire700 Stain alone, or both stains combined. A) Two-fold dilutions of cells, over a wide range of cell densities (0 - 200,000 cells/well). B) Closer examination of linearity of signal over the range of 25,000 - 250,000 cells/well, in dilution increments of 25,000 cells.

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 (or against a housekeeping protein) can be used to normalize for variations in cell number. Staining with DRAQ5 and Sapphire700 Cell Stains eliminates the need for these additional primary and secondary antibodies, and yields the same quantitative measurement of ERK phosphorylation (Figure 2).

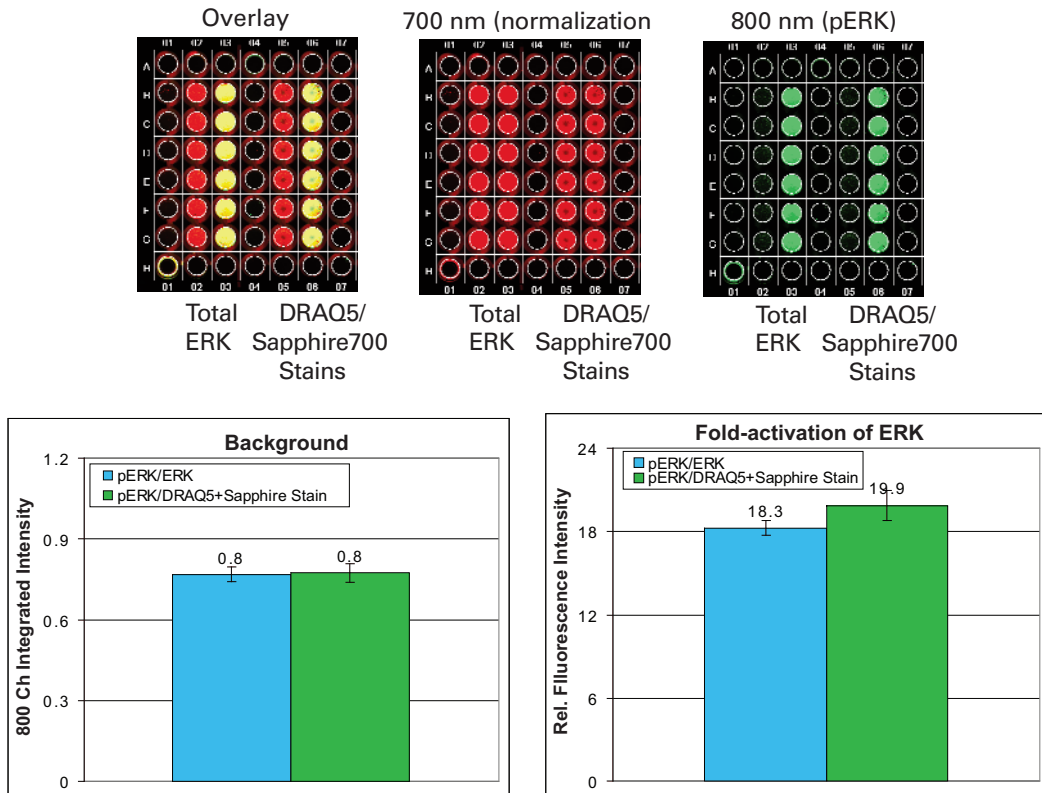


Figure 2. Comparison of normalization methods. ERK activation was induced in A431 cells by stimulation with epidermal growth factor (EGF). Phospho-ERK was detected in the 800 nm channel with anti-phospho-ERK primary antibody and IRDye[®] 800CW secondary antibody. Normalization was performed in two ways: anti-total-ERK primary antibody and IRDye 680 secondary antibody; or DRAQ5 and Sapphire700 Cell Stains. The EGF-induced ERK activation measured by the two methods was indistinguishable.

III. In-Cell Western Demo Kit: Protocol For Use

Kit Components (store kit at 4°C)

- IRDye® 800CW secondary antibody, 0.5 mg (lyophilized)
- Odyssey® Blocking Buffer, 4 x 500 mL (LI-COR®, P/N 927-40000)
- DRAQ5® Stain, 100 µL
- Sapphire700™ Stain, 100 µL (LI-COR, P/N 928-40022)

Additional Reagents (required but not included)

- Primary antibody
- 1X PBS wash buffer (LI-COR, P/N 928-40018, 10X PBS)
- Tissue culture reagents (serum DMEM, trypsin, etc.)
- Clear or black 96-well microplate (see *VIII. Experimental Considerations*)
- 37% formaldehyde
- 20% Tween® 20
- 10% Triton® X-100

IV. Reconstitution of Antibody

1. Protect from light. Store IRDye 800CW secondary antibody at 4°C prior to reconstitution.
2. Reconstitute contents of antibody vial with 0.5 mL sterile distilled water. Mix gently by inverting, and allow to rehydrate for at least 30 minutes before use. Centrifuge product if solution is not completely clear after standing at room temperature.
3. Dilute only immediately prior to use. Reconstituted antibody is stable for 3 months at 4°C when stored undiluted as directed. For extended storage, aliquot and freeze at -20°C or below; avoid repeated freeze-thaw cycles.

V. Cell Preparation and Fixation

1. Treat cells as desired with drug, stimulant, etc. Detailed In-Cell Western protocols for certain cell lines and target proteins may be downloaded at: <http://biosupport.licor.com>
See *In-Cell Western™ Assay Cell Fixation/Permeabilization* document at the ICW Assay Application page (www.licor.com)
2. Remove media manually or by aspiration. Immediately fix cells with Fixing Solution (3.7% formaldehyde in 1X PBS) for 20 minutes at room temperature (RT).

a. Prepare fresh *Fixing Solution* as follows:

1X PBS	45 mL
37% Formaldehyde	5 mL
<hr/>	
3.7% Formaldehyde	50 mL

- b. Using a multi-channel pipettor, add 150 µL of fresh, room temperature *Fixing Solution* to each well. **Add carefully by pipetting down the sides of the wells to avoid detaching the cells from the well bottom.**

- c. Allow incubation on the bench top for 20 minutes at RT with no shaking.

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.

3. To permeabilize, wash five times with 1X PBS containing 0.1% Triton® X-100 for 5 minutes per wash.

- a. Prepare *Triton Washing Solution* as follows:

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

- b. Remove *Fixing Solution* to an appropriate waste container (contains formaldehyde).
 c. Using a multi-channel pipettor, add 200 µL of room-temperature *Triton Washing Solution* to each well. **Make sure to carefully add the solution down the sides of the wells to avoid detaching the cells.**
 d. Allow wash to shake on a plate shaker for 5 minutes.
 e. Repeat washing steps 4 more times, removing wash manually each time. Do not allow cells/wells to become dry during washing. Immediately add the next wash after each manual disposal.

NOTE: 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.

VI. Cell Staining

1. Using a multi-channel pipettor, block cells by adding 150 µL of Odyssey® Blocking Buffer to each well. **Add the solution carefully by pipetting down the sides of the wells to avoid detaching the cells.**

NOTES:

- *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.*
- *Odyssey Blocking Buffer often yields higher and more consistent sensitivity and performance than other blockers. Nonfat dry milk or casein dissolved in PBS, or commercial blocking buffers, can also be used for blocking and antibody dilution. When using anti-goat antibodies, milk-based reagents may be contaminated with endogenous IgG, biotin, or phospho-epitopes that can interfere with detection.*

2. Allow blocking for 1.5 hours at room temperature with moderate shaking on a plate shaker.
3. Dilute desired primary antibody in Odyssey® Blocking Buffer or other appropriate blocker. As a general guideline, 1:50 to 1:200 dilutions are recommended depending on the primary antibody. If the antibody supplier provides dilution guidelines for immunofluorescent staining, start with that recommended range.

NOTE: If using DRAQ5® and Sapphire700™ Cell Stains for normalization, only one primary antibody will be used. Alternatively, you may choose to normalize with a second primary antibody in your assay. The second primary antibody **MUST** be from a different host, and an appropriate IRDye® 680LT or IRDye 680RD secondary antibody (not provided in the kit) will be required for detection.

- a. **It is important to include control wells that DO NOT contain primary antibody. These wells will be treated with secondary antibody only, and should be used to correct for background staining in the data analysis.**
 - b. Remove blocking buffer from step 2.
 - c. Add 50 µL of Odyssey® Blocking Buffer to the control wells and 50 µL of the desired primary antibody in Odyssey Blocking Buffer to the rest of the wells.
4. Incubate with primary antibody for 2.5 hours at room temperature or overnight at 4°C with gentle shaking.
 5. Wash the plate five times with 1X PBS + 0.1% Tween® 20 for 5 minutes at RT with gentle shaking, using a generous amount of buffer.

a. Prepare *Tween Washing Solution* as follows:

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

- b. Using a multi-channel pipettor, add 200 µL of *Tween Washing Solution*. **Make sure to carefully add the solution down the sides of the wells to avoid detaching the cells from the well bottom.**
 - c. Allow wash to shake on a plate shaker for 5 minutes.
 - d. Repeat washing steps 4 more times.
6. Dilute the fluorescently-labeled secondary antibody in Odyssey Blocking Buffer or other appropriate blocker. The recommended dilution range is 1:200 to 1:1,200, with a suggested starting dilution of 1:800. The optimal dilution for your assay should be determined empirically. To lower background, add Tween 20 at a final concentration of 0.2% to the diluted antibody. **Avoid prolonged exposure of the antibody vials to light.**

a. Secondary antibody staining and normalization staining are carried out simultaneously. To stain for normalization, add DRAQ5 Stain and Sapphire700 Stain to the diluted secondary antibody solution and apply this mixture to the cells. Suggested dilutions for normalization stains:

Sapphire700 Stain	1:1000
DRAQ5 Stain from LI-COR® ICW kit (1 mM):	1:2000
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DRAQ5 Stain from Biostatus (5 mM):	1:10,000

- b. For control wells (used to calculate background), do not add DRAQ5 and Sapphire700 Cell Stains. Add only diluted secondary antibody to these wells.
7. Add 50 µL of secondary antibody solution **without** DRAQ5 Stain and Sapphire700 Stain into each of the control wells and 50 µL of secondary antibody solution **with** DRAQ5 Stain and Sapphire700 Stain into rest of wells. Incubate for 1 hour at room temperature with gentle shaking. **Protect plate from light during incubation.**

8. Wash the plate five times with 1X PBS + 0.1% Tween® 20 for 5 minutes at room temperature with gentle shaking, using a generous amount of buffer.
 - a. Using a multi-channel pipettor, add 200 µL of *Tween Washing Solution*. **Make sure to carefully add the solution down the sides of the wells to avoid detaching the cells from the well bottom.**
 - b. Allow wash to shake on a plate shaker for 5 minutes.
 - c. Repeat washing steps 4 more times. **Protect plate from light during washing.**

VII. Imaging

1. After 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. For best results, scan plate immediately; plates may also be stored at 4°C for up to several weeks (protected from light).
2. Before plate scanning, clean the bottom plate surface and the scanning bed (if applicable) with moist lint-free paper.
3. Scan plate with detection in both 700 and 800 nm channels using an Odyssey® or Aerius System described below:

NOTE: All settings may require adjustment for optimal data quality (see Section VIII).

Instrument	Resolution*	Focus Offset	Scan Quality*	Intensity Setting (700/800)	Scan Time Medium Quality
Odyssey Classic	169 µm	3.0	medium-lowest	5 / 5	7 min
Odyssey CLx	169 µm	3.0	medium-lowest	5 / 5	7 min
	169 µm	3.0	medium-lowest	AutoMode	16 min
Odyssey Sa	200 µm	3.0	medium-lowest	7 / 7	3 min
Aerius	200 µm	3.0	medium-lowest	7 / 7	3 min

**Higher resolution or scan quality may be used, but scan time will increase.*

VIII. Experimental Considerations

Establish the specificity of the primary antibody by screening plate-like lysates through Western blotting and detection on an Aerius or Odyssey instrument. If significant non-specific binding is present, choose alternative primary antibodies to avoid results with non-specific signal detection.

Proper selection of microplates can significantly affect results, as each plate has its own characteristics, including well depth, plate autofluorescence, and well-to-well signal crossover. Use the following general considerations for microplate selection.

- In-Cell Western analyses use detection at the well surface with no liquid present. This results in minimal well-to-well signal spread, allowing the use of both clear as well as black-sided plates with clear bottoms. *Do not use plates with white walls, since autofluorescence from the white surface will create significant noise.*
- Protect plates from light before imaging to ensure highest sensitivity. When storing plates after imaging, the plates should remain protected from light at 4°C.
- In-Cell Western assays require sterile plates for tissue culture growth. The following plates are recommended by LI-COR Biosciences:

96-well format	Nunc® (P/N 161093, 165305)
96-well format	Falcon™ (P/N 353075, 353948)
384-well format	Nunc (P/N 164688, 164730)
384-well format	Falcon (P/N 353961, 353962)

- **Focus Offset Optimization** – If plates other than those recommended above are used, the focus offset can be determined empirically by scanning a plate containing experimental and control samples using the following focus offset settings.

Instrument	Focus Offset Determination (mm)
Odyssey® Classic & Odyssey CLx	0.5, 1.0, 2.0, 3.0 & 4.0
Odyssey Sa & Aerius	1.7, 2.0, 3.0 & 4.0

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 for the recommended measured distance from the skirt to the bottom of the plate.

- All Aerius and Odyssey Imaging systems (excluding Odyssey Fc) require microplates that have a maximum 4.0 mm distance from the base of the microplate to the target detection area of the plate (actual maximum focus offset varies with each Aerius and Odyssey Sa instrument and is found by choosing Settings > System Administration, then clicking Scanner Information). When using plates specified above for In-Cell Western assays, the recommended focus offset is 3.0 mm.

- **Intensity Setting Optimization** –

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

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

- Protect plates from light before imaging to ensure highest sensitivity. When storing plates after imaging, the plates should remain protected from light at 4°C.

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