In-Cell Western™ Assay
Kits I and II

Published November, 2006. The most recent version of this protocol is posted at http://biosupport.licor.com/protocols.jsp

Part Numbers: 926-31070 and 926-31072
Storage: 4 °C
The In-Cell Western Kit provides detection reagents for cell-based In-Cell Western™ Assays. The kit includes blocking buffer, fluorescently labeled 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 for well-to-well variations in cell number. This approach allows the target of interest to be detected accurately and cost-effectively.

If your application requires you to discriminate two protein targets with two different primary antibodies, this can be accomplished using secondary antibodies labeled with IRDye 680 and IRDye 800CW in a multiplex assay. Secondary antibodies for multiplex detection can be purchased at www.licor.com.

**Using DRAQ5™ and Sapphire700™ 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™ is a cell permeable DNA-interactive agent that can be used for stoichiometric staining of DNA in live or fixed cells. DRAQ5 is part of this kit, but is otherwise sold separately by Biostatus Limited (visit http://www.biostatus.com/product/draq5/). When serial dilutions of A431 human epithelial carcinoma cells are plated in 96-well plates, DRAQ5 demonstrates linearity of fluorescent signal for lower cell densities, up to ~50,000 cells/well (Figure 1A).

Sapphire700™ 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 displays linearity of fluorescent signal for higher cell densities, from ~50,000 to ~250,000 cells/well (Figure 1B). Simultaneous staining of cells with both DRAQ5 and Sapphire700 expands the linear range, allowing more accurate normalization of cell number across both low and high cell densities (Figure 1A and B). Sapphire700 can be purchased separately from LI-COR (Cat# 928-40022).

![DRAQ5 + Sapphire700 Cell Linearity](image1)

**Figure 1. DRAQ5 and Sapphire700 as normalizing agents for In-Cell Western assays.** Dilutions of A431 cells were plated on clear, flat bottom 96-well plates, then fixed and permeabilized. Cells were stained with DRAQ5 alone, Sapphire700 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 eliminates the need for this additional primary and secondary antibody, and yields the same quantitative measurement of ERK phosphorylation (Figure 2).
In-Cell Western Kit: Protocol for Use

**Kit Components (store kit at 4°C)**
- IRDye 800CW-labeled secondary antibody, 0.5 mg (lyophilized)
- Odyssey Blocking Buffer, 4 x 500 ml (LI-COR, Cat# 927-40000)
- DRAQ5, 100 µl
- Sapphire700, 100 µl (LI-COR, Cat# 928-40022)

**Additional Reagents (required but not included)**
- Primary antibody
- 1X PBS wash buffer (LI-COR, Cat# 928-40018, 10X PBS)
- Tissue culture reagents (serum D-MEM, trypsin, etc.)
- Clear or black 96-well microplate (see V. Hints and Tips for In-Cell Western Assays)
- 37% formaldehyde
- 20% Tween®-20
- 10% Triton® X-100

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**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 staining. The EGF-induced ERK activation measured by the two methods was indistinguishable.
I. 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.

II. 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/protocols.jsp.

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:
   
<table>
<thead>
<tr>
<th>Component</th>
<th>Volume</th>
</tr>
</thead>
<tbody>
<tr>
<td>1X PBS</td>
<td>45 ml</td>
</tr>
<tr>
<td>37% Formaldehyde</td>
<td>5 ml</td>
</tr>
<tr>
<td>3.7% Formaldehyde</td>
<td>50 ml</td>
</tr>
</tbody>
</table>
   
   b. Using a multi-channel pipettor, add 150 µl of fresh, room temperature **Fixing Solution** to each well. Add the **Fixing Solution** 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.
### III. Cell Staining

#### 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:

<table>
<thead>
<tr>
<th>Component</th>
<th>Volume</th>
</tr>
</thead>
<tbody>
<tr>
<td>1X PBS</td>
<td>495 ml</td>
</tr>
<tr>
<td>10% Triton X-100</td>
<td>5 ml</td>
</tr>
<tr>
<td><strong>1X PBS + 0.1% Triton X-100</strong></td>
<td>500 ml</td>
</tr>
</tbody>
</table>

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 rotator 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.

#### 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.**

**Hints and Tips for Blocking:**
- No single blocking reagent will be optimal for every antigen-antibody pair. Some primary antibodies may exhibit greatly reduced signal or nonspecific 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 rotator.
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 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 680 secondary antibody (not provided in the kit) will be required.

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:

<table>
<thead>
<tr>
<th>Component</th>
<th>Volume (ml)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1X PBS</td>
<td>995</td>
</tr>
<tr>
<td>20% Tween-20</td>
<td>5</td>
</tr>
<tr>
<td>1X PBS with 0.1% Tween-20</td>
<td>1000</td>
</tr>
</tbody>
</table>

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 rotator 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 and Sapphire700 to the diluted secondary antibody solution and apply this mixture to the cells. Suggested dilutions for normalization stains:

- Sapphire700: 1:1000
- DRAQ5: 1:2000 for 1 mM solution or 1:10,000 for 5 mM solution

b. For control wells (used to calculate background), do not add DRAQ5 and Sapphire700. Add only diluted secondary antibody to these wells.
IV. Imaging

<table>
<thead>
<tr>
<th>Step</th>
<th>Action</th>
</tr>
</thead>
<tbody>
<tr>
<td>7.</td>
<td>Add 50 µl of secondary antibody solution without DRAQ5 and Sapphire700 into each of the control wells and 50 µl of secondary antibody solution with DRAQ5 and Sapphire700 into rest of wells. Incubate for 1 hour at room temperature with gentle shaking. <strong>Protect plate from light during incubation.</strong></td>
</tr>
</tbody>
</table>
| 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 rotator for 5 minutes.  
   c. Repeat washing steps 4 more times. **Protect plate from light during washing.** |

IV. Imaging

<table>
<thead>
<tr>
<th>Step</th>
<th>Action</th>
</tr>
</thead>
<tbody>
<tr>
<td>1.</td>
<td>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).</td>
</tr>
<tr>
<td>2.</td>
<td>Before plate scanning, clean the bottom plate surface and the Odyssey Imager scanning bed with moist lint-free paper.</td>
</tr>
<tr>
<td>3.</td>
<td>Scan the plate with detection in both the 700 and 800 channels, using the Odyssey or Aerius imagers (700 nm detection for normalization stains, and 800 nm detection for IRDye 800CW antibody). Generally, a scan resolution of 169 µm (Odyssey) or 200 µm (Aerius) is appropriate. An initial intensity setting of 5 is suggested for both 700 and 800 nm channels. Focus offset position is critical to proper plate imaging; for more information about choosing the correct focus offset, see Section V.</td>
</tr>
<tr>
<td>4.</td>
<td>Proceed with data analysis.</td>
</tr>
</tbody>
</table>

V. Hints and Tips for In-Cell Western Assays

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

- Establish the specificity of your primary antibody by Odyssey Western blotting of similar lysates, and/or by immunofluorescent microscopy. To achieve the most consistent results, use the same blocking buffer for validation experiments and In Cell Western assays. If significant non-specific banding is detected on a Western blot, choose alternative primary antibodies. Non-specific binding of primaries will make it difficult to accurately interpret In-Cell Western assay results.

- 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 wells, since the autofluorescence from the white surface will create significant noise.**
• In-Cell Western assays require sterile plates for tissue culture growth. The following plates and focus offset settings are recommended as a starting point. Please be aware that the manufacturers' specifications for culture plates are subject to change.

<table>
<thead>
<tr>
<th>Well Number</th>
<th>Well Bottom</th>
<th>Manufacturer</th>
<th>Part Number</th>
<th>Offset - Odyssey</th>
<th>Offset - Aerius</th>
</tr>
</thead>
<tbody>
<tr>
<td>96</td>
<td>Flat</td>
<td>Nunc™-Nalgene</td>
<td>161093, 165305</td>
<td>3.0 mm</td>
<td>3.0 mm</td>
</tr>
<tr>
<td>96</td>
<td>Flat</td>
<td>BD Falcon™</td>
<td>353075, 353948</td>
<td>3.0 mm</td>
<td>3.0 mm</td>
</tr>
<tr>
<td>96</td>
<td>Round*</td>
<td>BD Falcon</td>
<td>353077</td>
<td>3.0 mm</td>
<td>not recommended</td>
</tr>
<tr>
<td>96</td>
<td>Round*</td>
<td>Nunc-Nalgene</td>
<td>163320</td>
<td>not recommended</td>
<td>3.9 mm</td>
</tr>
<tr>
<td>384</td>
<td>Flat</td>
<td>Nunc-Nalgene</td>
<td>164688, 164730</td>
<td>3.0 mm</td>
<td>3.0 mm</td>
</tr>
<tr>
<td>384</td>
<td>Flat</td>
<td>BD Falcon</td>
<td>353961, 353962</td>
<td>3.0 mm</td>
<td>3.0 mm</td>
</tr>
</tbody>
</table>

* For use with suspension cells. For more information, please refer to the suspension cell protocol available in the Application Protocols Manual or on the LI-COR web site (http://biosupport.licor.com/protocols.jsp).

• The Odyssey and Aerius Imagers require that microplates have a maximum 4.0 mm distance from the scanning surface to the target detection area of the plate. Optimal signal will be achieved when the focus offset position is set as accurately as possible, and the best offset for your experiments may need to be determined empirically (see below).

• Determining the best focus offset is especially important when using plates other than those listed in the table above. Find the optimal focus offset by scanning a plate containing experimental and control samples at 0.5, 1.0, 2.0, 3.0, and 4.0 mm focus offsets. Use the same intensity settings for each scan. After reviewing the collected scans, use the focus offset with the highest signal-to-noise for your experiments. Focus offset can be further fine-tuned in 0.1-0.5 mm increments if desired, and this may result in additional improvement in signal strength.

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

• Intensity settings for both 700 and 800 nm channels should generally be set to 5 for initial scanning. If your image signal is saturated or too high, re-scan using a lower intensity setting (i.e. 2). If your image signal is too low, re-scan using a higher intensity setting (i.e. 7).