

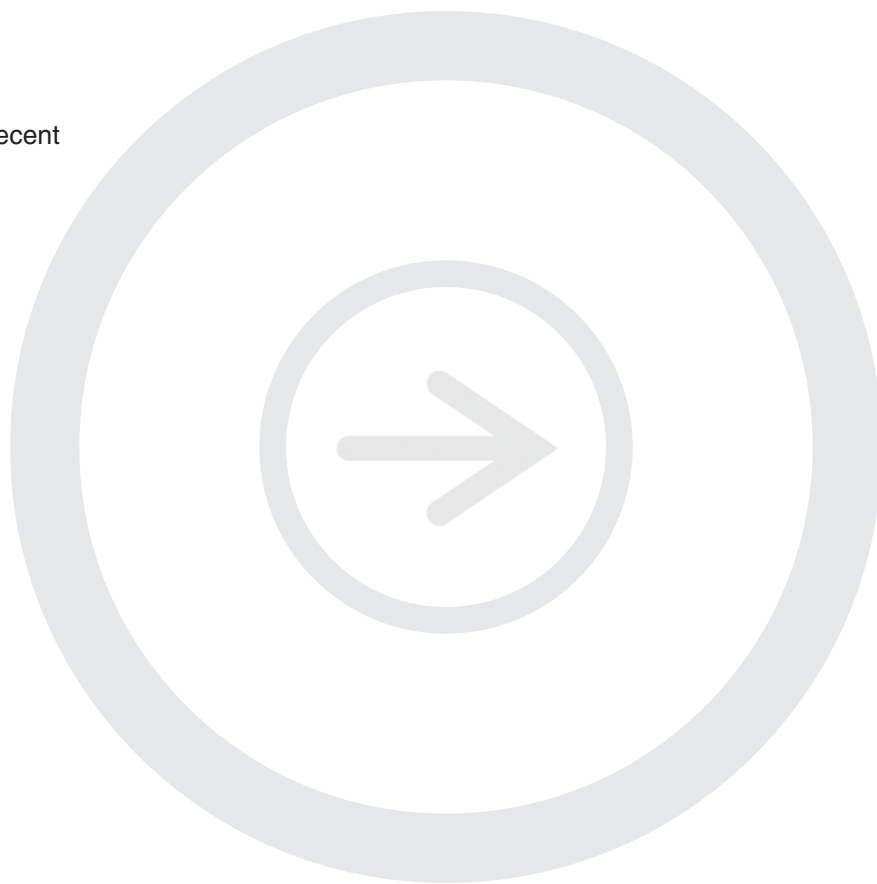
# Odyssey<sup>®</sup>

Infrared Imaging System

## **In-Cell Western Assay**

**Complete Sample Protocol for Measuring IC<sub>50</sub> of Inhibitor U0126 in NIH3T3 Responding to Acidic Fibroblast Growth Factor (aFGF-1)**

Revised January, 2006. The most recent version of this protocol is posted at <http://biosupport.licor.com/support>



***LI-COR***<sup>®</sup>

Biosciences

Doc# 988-08334

# Contents

	Page
I. Required Reagents.....	1
II. Sample Protocol .....	2
III. Experimental Considerations.....	5
IV. Experimental Results.....	6

## I. Required Reagents

### Odyssey® Reagents

- IRDye™ 800CW-labeled goat anti-mouse secondary antibodies (LI-COR, Cat.# 926-32210)\*
- IRDye™ 680-labeled goat anti-rabbit secondary antibodies (LI-COR, Cat.# 926-32221)\*
- Odyssey® Blocking Buffer (Cat.# 927-40000)

### Additional Reagents


- 1X PBS wash buffer
- Tissue culture reagents (serum, D-MEM, trypsin, 1X PBS)
- SIGMAScreen™ Poly-D-Lysine coated 96-well microplate (Sigma®, Cat.# Z38249-3)
- Heparin (CALBIOCHEM®, Cat.# 375097)
- Acidic Fibroblast Growth Factor (Upstate Group Inc., Cat.# 01-116)
- MEK inhibitor U0126 (Promega®, Cat.# V1121)
- Primary antibodies
- 20% Tween®-20
- 37% formaldehyde
- 10% Triton® X-100


**Special Note:** NIH3T3 cells do not adhere strongly to TC treated plates resulting in the need for poly-D-lysine coated plates in this assay. However, even with lysine coated plates the adherence of cells remains relatively weak compared to other cell lines.



 **Be very cautious and delicate with plate handling and pipetting when washing, removing, and adding solutions to avoid detaching the cells.**

\* IRDye™ 800CW-labeled secondary antibodies are also available from Rockland Immunochemicals, Inc. Alexa Fluor® 680-labeled secondary antibodies are available from Invitrogen Corporation.

## II. Sample Protocol

1.	Allow NIH3T3 (ATCC; CRL-1555) cell growth in a T75 flask using standard tissue culture procedures until cells reach near confluency ( $\sim 1.5 \times 10^7$ cells; D-MEM, 10% FBS; Gibco®).						
2.	Remove growth media, wash cells with sterile 1X PBS, and trypsinize cells for displacement.						
3.	Neutralize displaced cells with culture media and clarify by centrifugation (500 x g).						
4.	Remove supernatant and disrupt the cell pellet manually by hand tapping the collection tube. Avoid employment of pipet or vortex during pellet disruption to maintain cell integrity.						
5.	Resuspend cells in 20 ml of complete media and count cells using a hemacytometer.						
6.	Reconstitute cells and dilute in 40 ml of complete media such that 75,000 cells/ml is achieved (2 plates x 96 wells x 200 $\mu$ l/well = $\sim$ 40 ml).						
7.	Manually mix the cell suspension thoroughly.						
8.	Under sterile conditions, dispense 200 $\mu$ l of the cell suspension per well into a SIGMA Screen™ Poly-D-Lysine 96-well microplate (15,000 cells plated per well).						
9.	Incubate cells and monitor cell density until 70% confluency is achieved (it takes about 24 hours).  <b>70% confluency is very important. 90 to 100% confluent cells are certain to detach during washing.</b>						
10.	Warm serum-free media (D-MEM; Gibco) to 37 °C.						
11.	Dissolve U0126 in DMSO to make 10 mM stock. Make two fold serial dilutions of inhibitor using D-MEM. Add 10 $\mu$ l of serial diluted inhibitor into cells so that the final concentration of inhibitor range from 1 to 125 $\mu$ M (see Figure 1, section IV. <i>Experimental Results</i> ). Incubate 1 to 2 hours.						
12.	Remove media and inhibitor from plate wells by aspiration or manual displacement.						
13.	Add either serum free media for resting cells (mock) or serum free media with 100 ng/ml aFGF combined with 10 $\mu$ g/ml heparin for activated cells. Use 100 $\mu$ l of resting/activation media per well.						
14.	Allow incubation at 37 °C for 7.5 minutes.						
15.	Remove activation or stimulation media manually or by aspiration. Immediately fix cells with 4% formaldehyde in 1X PBS for 20 minutes at room temperature. <ul style="list-style-type: none"> <li>a. Prepare fresh <b>Fixing Solution</b> as follows: <table style="margin-left: 40px; border-collapse: collapse;"> <tr> <td style="padding-right: 20px;">1X PBS</td> <td style="text-align: right;">45 ml</td> </tr> <tr> <td>37% Formaldehyde</td> <td style="text-align: right;">5 ml</td> </tr> <tr> <td style="border-top: 1px solid black; padding-top: 2px;">3.7% Formaldehyde</td> <td style="text-align: right; border-top: 1px solid black; padding-top: 2px;">50 ml</td> </tr> </table> </li> <li>b. Using a multi-channel pipettor, add 150 <math>\mu</math>l of fresh <i>Fixing Solution</i> (room temperature solution, RT). <b>Make sure to carefully add the solution down the sides of the wells to avoid detaching the cells from the well bottom.</b></li> <li>c. Allow incubation on bench top for 20 minutes at RT with no shaking.</li> </ul>	1X PBS	45 ml	37% Formaldehyde	5 ml	3.7% Formaldehyde	50 ml
1X PBS	45 ml						
37% Formaldehyde	5 ml						
3.7% Formaldehyde	50 ml						

<p>16.</p>	<p>Wash five times with 1X PBS containing 0.1% Triton X-100 (cell permeabilization) for 5 minutes per wash.</p> <p>a. Prepare <b><i>Triton Washing Solution</i></b> as follows:</p> <table border="0" style="margin-left: 20px;"> <tr> <td style="padding-right: 40px;">1X PBS</td> <td style="text-align: right;">495 ml</td> </tr> <tr> <td>10% Triton X-100</td> <td style="text-align: right;">5 ml</td> </tr> <tr> <td style="border-top: 1px solid black; padding-top: 5px;">1X PBS + 0.1% Triton X-100</td> <td style="border-top: 1px solid black; text-align: right; padding-top: 5px;">500 ml</td> </tr> </table> <p>b. Remove <i>Fixing Solution</i> to an appropriate waste container (contains formaldehyde).</p> <p>c. Using a multi-channel pipettor, add 200 µl of <i>Triton Washing Solution</i> (RT). Make sure to carefully add the solution down the sides of the wells to avoid detaching the cells from the well bottom.</p> <p>d. Allow wash to shake on a rotator for 5 minutes at RT.</p> <p>e. Repeat washing steps 4 more times after removing wash manually.</p> <p> <b>Do not allow cells/wells to become dry during washing. Immediately add the next wash after manual disposal.</b></p>	1X PBS	495 ml	10% Triton X-100	5 ml	1X PBS + 0.1% Triton X-100	500 ml
1X PBS	495 ml						
10% Triton X-100	5 ml						
1X PBS + 0.1% Triton X-100	500 ml						
<p>17.</p>	<p>Using a multi-channel pipettor, block cells/wells by adding 150 µl of LI-COR Odyssey Blocking Buffer to each well. <b>Add the solution carefully by pipetting down the sides of the wells to avoid detaching the cells.</b></p> <p><b>Notes:</b></p> <ul style="list-style-type: none"> <li>• No single blocking reagent will be optimal for every antigen-antibody pair. Some primary antibodies may exhibit greatly reduced signal or different nonspecific banding in different blocking solutions. If you have difficulty detecting your target protein, changing the blocking solution may dramatically improve performance. If the primary antibody has worked well in the past using chemiluminescent detection, try that blocking solution for Aeries detection.</li> <li>• Odyssey Blocking Buffer often yields higher and more consistent sensitivity and performance than other blockers. Nonfat dry milk or casein dissolved in PBS can also be used for blocking and antibody dilution. Milk-based reagents can interfere with detection when using anti-goat antibodies. They also deteriorate rapidly at 4°C, so diluted antibodies cannot be kept and re-used for more than a few days. If using casein, a 0.1% solution in 0.2 X PBS buffer is recommended (Hammersten-grade casein is not required).</li> <li>• Milk-based reagents can interfere with detection when using anti-goat antibodies. They also deteriorate rapidly at 4°C, so diluted antibodies cannot be kept and re-used for more than a few days.</li> <li>• Blocking solutions containing BSA can be used, but in some cases they may cause high membrane background. BSA-containing blockers are not generally recommended and should be used only when the primary antibody requires BSA as blocker.</li> </ul>						
<p>18.</p>	<p>Allow blocking for 90 minutes at RT with moderate shaking on a rotator.</p>						

19.	<p>Add the two primary antibodies to a tube containing Odyssey Blocking Buffer. Combine the solutions defined below for ERK target analysis:</p> <ul style="list-style-type: none"> <li>• Phospho-ERK (Rabbit; 1:100 dilution; Cell Signaling Technology 9101) Total ERK2 (Mouse; 1:75 dilution; Santa Cruz Biotechnology SC-1647)</li> </ul> <ol style="list-style-type: none"> <li>a. Mix the primary antibody solution well before addition to wells.</li> <li>b. Remove blocking buffer from the blocking step and add 50 µl of the desired primary antibody or antibodies in Odyssey Blocking Buffer to cover the bottom of each well.</li> <li>c. <b>Make sure to include control wells without primary antibody to serve as a source for background well intensity.</b> Add 50 µl of Odyssey Blocking Buffer only to control wells.</li> </ol>						
20.	<p>Incubate with primary antibody overnight with gentle shaking at RT.</p>						
21.	<p>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.</p> <ol style="list-style-type: none"> <li>a. Prepare <b>Tween Washing Solution</b> as follows: <table style="margin-left: 40px; border-collapse: collapse;"> <tr> <td style="padding-right: 20px;">1X PBS</td> <td style="text-align: right;">995 ml</td> </tr> <tr> <td>20% Tween-20</td> <td style="text-align: right;">5 ml</td> </tr> <tr> <td style="border-top: 1px solid black; padding-top: 5px;">1X PBS with 0.1% Tween-20</td> <td style="border-top: 1px solid black; text-align: right; padding-top: 5px;">1000 ml</td> </tr> </table> </li> <li>b. Using a multi-channel pipettor, add 200 µl of <i>Tween Washing Solution</i> (RT). <b>Make sure to carefully add the solution down the sides of the wells to avoid detaching the cells from the well bottom.</b></li> <li>c. Allow wash to shake on a rotator for 5 minutes at RT.</li> <li>d. Repeat washing steps 4 more times.</li> </ol>	1X PBS	995 ml	20% Tween-20	5 ml	1X PBS with 0.1% Tween-20	1000 ml
1X PBS	995 ml						
20% Tween-20	5 ml						
1X PBS with 0.1% Tween-20	1000 ml						
22.	<p>Dilute the fluorescently labeled secondary antibody in Odyssey Blocking Buffer as specified below. To lower background, add Tween-20 to the diluted antibody to a final concentration of 0.2%.</p> <p style="margin-left: 40px;">Goat anti-rabbit IRDye™ 680 (1:200 dilution; LI-COR) Goat anti-mouse IRDye™ 800CW (1:800 dilution; LI-COR)</p> <p>Recommended dilution range is 1:200 to 1:1,200.</p> <p> Avoid prolonged exposure of the antibody vials to light.</p>						
23.	<p>Mix the antibody solutions well and add 50 µl of the secondary antibody solution to each well. Incubate for 60 minutes with gentle shaking at RT. Protect plate from light during incubation.</p>						
24.	<p>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.</p> <ol style="list-style-type: none"> <li>a. Using a multi-channel pipettor, add 200 µl of <i>Tween Washing Solution</i> at RT (see step 21). <b>Make sure to carefully add the solution down the sides of the wells to avoid detaching the cells from the well bottom.</b></li> <li>b. Allow wash to shake on a rotator for 5 minutes at RT.</li> <li>c. Repeat washing steps 4 more times after removing wash manually.</li> </ol> <p> Protect plate from light during washing.</p>						

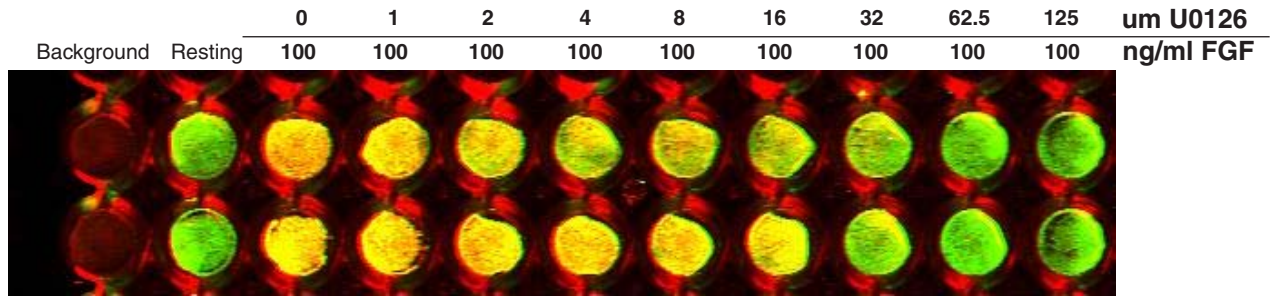
25.	After final wash, remove wash solution completely from wells. Turn the plate upside down and tap or blot gently on paper towels to remove traces of wash buffer. For best results, scan plate immediately; plates may also be stored at 4 °C for up to several weeks (protected from light).
25.	Before plate scanning, clean the bottom plate surface and the Odyssey Imager scanning bed with moist lint free paper to avoid any obstructions during scanning.
26.	Scan the plate with detection in both the 700 and 800 channels using the Odyssey instrument (700 nm detection for IRDye™ 680 antibody and 800 nm detection for IRDye™ 800CW antibody). Use medium scan quality, 169 µm resolution, 3.0 mm focus offset, and an intensity setting of 5 for both 700 and 800 nm channels

### III. Experimental Considerations

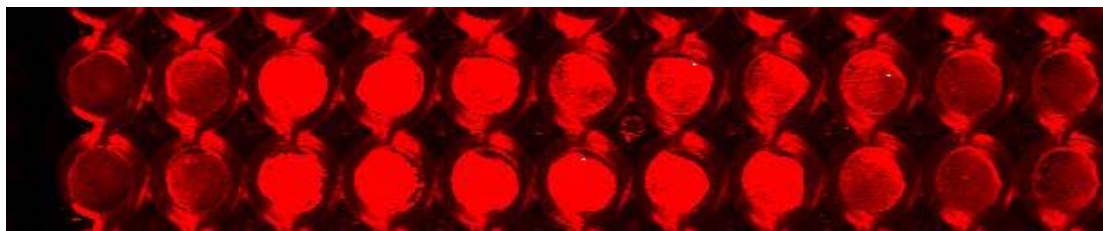
- The Odyssey Imager requires that microplates have a maximum 4.0 mm distance from the Odyssey scanning surface to the target detection area of the plate. The recommended focus offset is 3.0 mm for the SigmaScreen™ microplates specified for this assay.
- If you use plates other than the recommended SigmaScreen™ microplates, the focus offset can be determined 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 as your focus offset for experiments.
- 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 for both 700 and 800 nm channels should 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., 2.5). If your image signal is too low, re-scan using a higher intensity setting (i.e., 7.5).
- Scan settings of medium to lowest quality, with 169 µm resolution, provide satisfactory results with minimal scan time. Higher scan quality or resolution may be used, but scan time will increase.
- Establish the specificity of your primary antibody by screening lysates through Western blotting and detection on the Odyssey instrument. If significant non-specific banding is present, choose alternative primary antibodies. Non-specific binding of primaries will complicate interpretation of In-Cell Western assay results.

# IV. Experimental Results

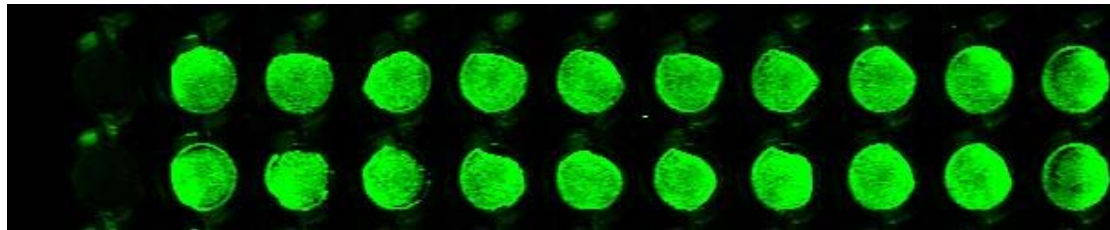
Color images can be seen at <http://biosupport.licor.com/support>.



Two-color display of both 700 and 800 nm channels.



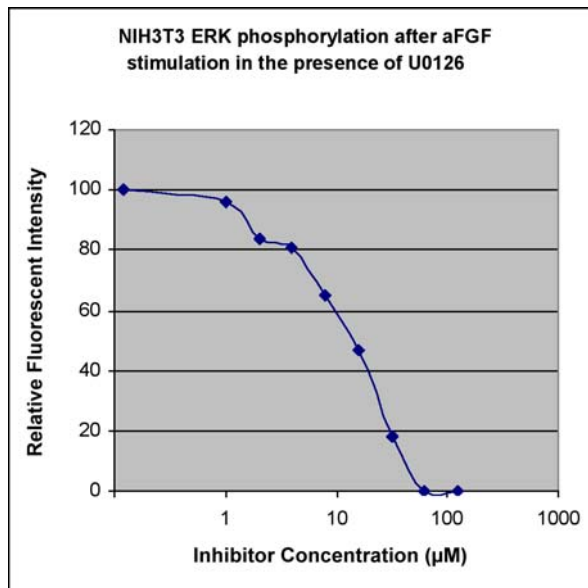
700 nm image (phospho-ERK).



800 nm image (total ERK).

**Figure 1. U0126 inhibition of ERK phosphorylation in NIH3T3 cells stimulated with FGF. The graph demonstrates the inhibitory effect of the MEK inhibitor U0126 as determined through the detection of ERK phosphorylation (Thr202/Tyr204) within an ICW assay. Resulting data were plotted and the IC<sub>50</sub> of U0126 was determined to be ~15µM, correlating well with the IC<sub>50</sub> reported in literature (1) for in vitro and in vivo assays.**

1. Ahn, N.G. *et al* (1999). U0126: An Inhibitor of MKK/ERK Signal Transduction in Mammalian Cells. *Promega Notes* 71, p. 4.



**LI-COR<sup>®</sup>**

Biosciences

4308 Progressive Avenue • P.O. Box 4000 • Lincoln, Nebraska 68504 USA

Technical Support: 800-645-4260

North America: 800-645-4267

International: 402-467-0700 • 402-467-0819

LI-COR GmbH (Germany, Austria, Switzerland, Czech Republic, Hungary, Slovakia): +49 (0) 6172 17 17 771

LI-COR UK Ltd.: +44 (0) 1223 422104

[www.licor.com](http://www.licor.com)

LI-COR is an ISO 9001 registered company. © 2006 LI-COR Inc. LI-COR, Odyssey, and IRDye are trademarks or registered trademarks of LI-COR, inc. Alexa Fluor and Gibco are registered trademarks of Invitrogen Corporation. Tween is a registered trademark of ICI Americas, Inc. Triton is a registered trademark of Union Carbide Chemicals and Plastics Corp. Promega is a registered trademark of Promega Corporation. Sigma and SIGMAscreen are trademarks or registered trademarks of Sigma-Aldrich Inc. CALBIOCHEM is a registered trademark of EMD Biosciences Inc. The Odyssey Infrared Imaging System is covered by U.S. patent (6,495,812), foreign equivalents, and patents pending.