



# In-Cell Western™

QUANTITATIVE CELL-BASED ASSAYS

ACCURATE QUANTIFICATION

MULTIPLEX DETECTION

HIGH SENSITIVITY

DIRECT DETECTION

IMMUNOFLUORESCENT-BASED ASSAY

MULTIPLE TARGETS

**LI-COR**®  
Biosciences



# In-Cell Western™

QUANTITATIVE CELL-BASED ASSAYS

## Accurate Quantification

Wide linear dynamic range

## Multiplex Detection

Normalization increases quantification accuracy

## High Sensitivity

Equal to or better than chemiluminescence

## Direct Detection

Analyzes multiple targets in 96- or 384-well plates.

## Immunofluorescent-Based Assay

Fast, microplate-based assay. Lysate preparation, gel loading, electrophoresis, and membrane transfer are eliminated.

## Multiple Targets

Ideal for screening cell treatments or drug candidates for effects on target proteins.

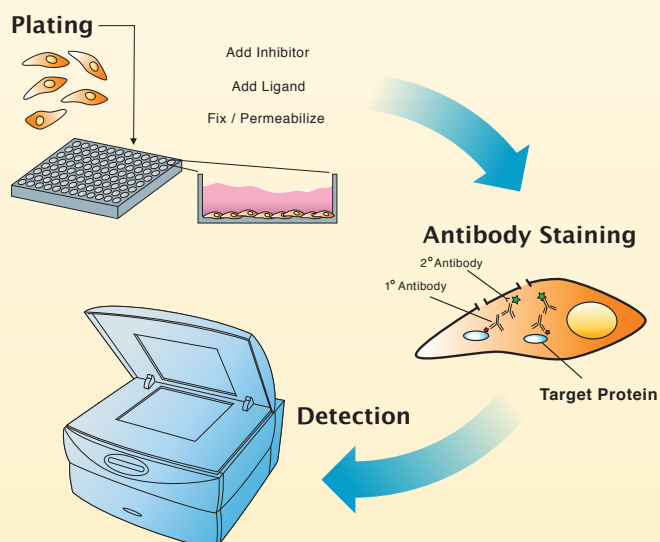
## Quantitative Cell-Based Assays For Ratiometric Protein Analysis

The In-Cell Western Assay is an immunocytochemical assay performed in microplate format. Target-specific primary antibodies and infrared dye-labeled secondary antibodies are used to detect target proteins in fixed cells, and fluorescent signal from each well is quantified. Accuracy is enhanced and data are more meaningful because proteins are detected in their cellular context.

### Application Overview

The unique advantages of infrared fluorescence allow In-Cell Western Assays to provide extremely sensitive and quantitative analysis of cellular signaling pathways in cultured cells in a higher throughput manner. Use of infrared fluorescence reduces interference from cell, plate, and drug compound autofluorescence when compared to standard methods.

## In-Cell Western Assay Workflow



In-Cell Western Assays simultaneously detect two targets at 700 and 800 nm using two spectrally distinct dyes. Separate lasers and fluorescence detectors are used for each dye and offer a wide linear detection range.

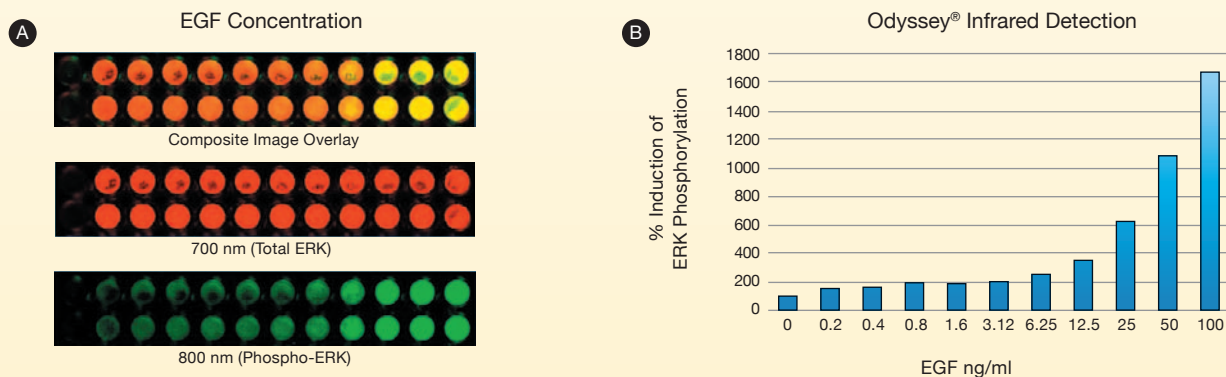
With two detection channels you can probe two separate targets or increase quantification accuracy by using the second channel for normalization. Quantification accuracy is maximized by normalization because adjustments can be made for differences in cell number from well to well. Two-color normalization also helps prevent false negatives and provides more accurate evaluation of cell treatment or drug candidate effects.

In-Cell Western Assays offer broad application to the analysis of protein signaling pathways, reliable protein quantification, and cell-based determinations of  $IC_{50}$  concentrations for lead optimization. In-Cell Western Assays are also a powerful tool in the study of the effects of drug components on multiple points within one or more signaling pathways.

Other traditional protein assay methods such as Western blotting are cumbersome and labor intensive. High content screening methods employ very expensive complex instrumentation and consumable costs. In-Cell Western Assays offer a practical alternative for medium to high throughput analysis.

### Phosphorylation of ERK in Response to Pathway Stimulation

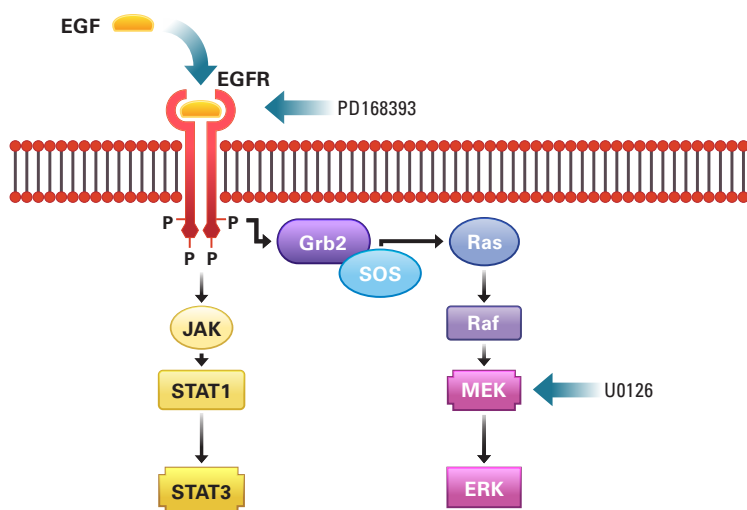
A431 cells were stimulated with serial dilutions of EGF to optimize activation of ERK1/2<sup>1</sup>



**A.** Detection of ERK phosphorylation. These images show a portion of a 96-well plate. Duplicate rows of microplate wells are shown. The top panel is a composite image showing the fluorescence in both the 700 nm and 800 nm detection channels. The middle panel shows detection of total ERK protein regardless of phosphorylation status. The bottom panel shows detection of increasing amounts of phospho-ERK as a function of increasing EGF stimulation.

**B.** Quantification of fluorescence. Phospho-ERK signal has been normalized using the total ERK signal from each well, to correct for well-to-well variation in cell number. This experiment shows a greater than 16-fold increase in ERK phosphorylation compared to the resting state.

## Pathway Analysis with the In-Cell Western™ Assay



### EGFR Signaling

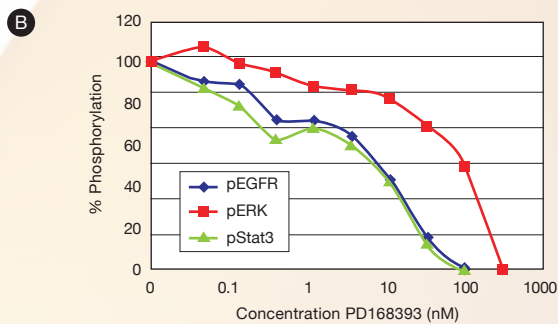
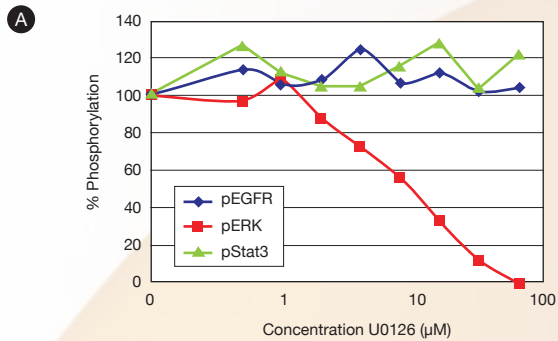
Signaling by the Ras/Raf/MEK/ERK pathway was activated in A431 cells by stimulation of the epidermal growth factor receptor (EGFR) with EGF. Cells were also treated with drug compounds (blue arrows) that inhibit the signaling cascade at two different points. PD168393 is a known EGFR inhibitor and should affect not only EGFR auto-phosphorylation but also downstream targets such as STAT3 and ERK1/2. In contrast, U0126 is an inhibitor of MEK1/2. This drug should inhibit signaling by MEK1/2 and cause a decrease in ERK1/2 phosphorylation, without affecting phosphorylation of EGFR or STAT3.

## In-Cell Western Assay and RNAi

The In-Cell Western Assay can be used in a functional siRNA screen measuring the effects of knockdowns in cultured cells.

Hoffmann et al.<sup>2</sup> used In-Cell Western Assay screening to assess the effects of knockdowns on mTORC1-dependent phosphorylation of ribosomal protein S6 (rpS6).

- HeLa cells were transfected with siRNA and screened for phosphorylation of rpS6 at Ser235/236.
- NHS ester cell labeling was used for cell number normalization.
- A pilot small molecule screen was performed with a library of ~2500 compounds.
- The Dharmacon Human Druggable Genome siRNA library gave 7,317 genes from the human genome that are likely targets for pharmacological inhibition.
- Knockdown of components required for both the growth factor and amino acid branches of the mTORC1 signaling network caused reduction in phospho-rpS6.
- Known genes involved in growth factor-mediated inputs leading to rpS6 phosphorylation (IRS2, IGF1R, PI3-kinase, PDK1, and S6K2) scored as hits in the screen, validating the approach.
- In addition to known pathway components, a number of uncharacterized genes scored as strong hits.
- In-Cell Western RNAi screening was found to be faster and less expensive than high content microscopy (IF), and gave similar or better statistical reproducibility.

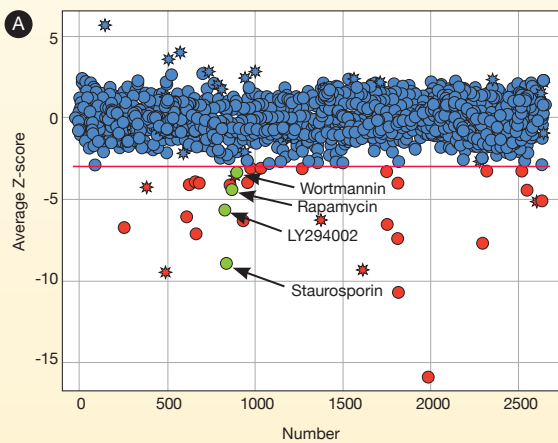


## Effects of pathway inhibitors on EGFR, STAT3, and ERK phosphorylation<sup>1</sup>

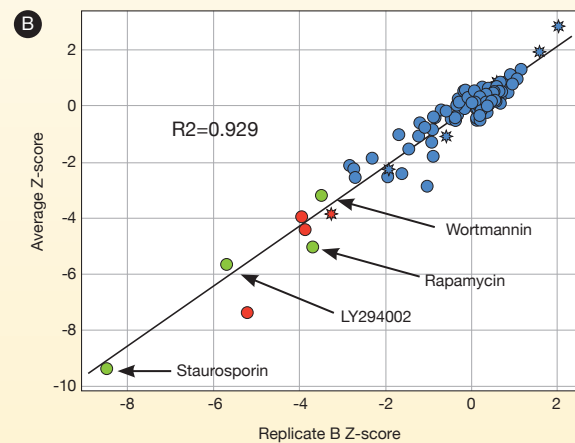
A431 cells were cultured and treated with serial dilutions of drug, then stimulated with EGF. IC<sub>50</sub> curves for each drug were determined in a single microwell plate using duplicate samples.

**A.** The MEK inhibitor U0126 displayed the expected specificity and caused a dramatic decrease in ERK phosphorylation, but did not affect phosphorylation of EGFR or STAT3.

**B.** The EGFR inhibitor PD168393 decreased the phosphorylation observed for all three target proteins in the pathway – the receptor as well as its downstream effectors. However, the concentration of drug required to achieve 50% inhibition of phosphorylation (IC<sub>50</sub>) was almost 10-fold higher for ERK (~100 nM) than for EGFR and STAT3 (~11.1 nM). This may be due to EGFR-independent signaling pathways that also signal through ERK and were not inhibited by the drug tested.



**A.** Results of pilot small molecule screen performed with a library of ~2500 known bioactive compounds. Compounds with average Z-score < -2 are considered hits and are shown in red. Known inhibitors of mTORC1 signaling found in the library are shown in green. Star-shaped symbols represent compounds with > 4-fold reduction in cell number.



**B.** Plate to plate reproducibility is shown for a representative plate from the small molecule library.

# Reproducibility of the In-Cell Western™ Assay<sup>1</sup>

In-Cell Western Assays use a two-color ratiometric approach for precise, quantitative analysis of cell signaling with good reproducibility. IC<sub>50</sub> values were determined for four experimental compounds that inhibit PDGF-induced phosphorylation of Akt in NIH 3T3 cells. At each drug concentration, the integrated fluorescence intensity for phospho-Akt staining was determined. For each data point, eight replicates were performed; the mean and standard deviation are shown. Each experiment was repeated five times (plates 1-5) and IC<sub>50</sub> values for each plate are shown. Each compound gave IC<sub>50</sub> values that were indistinguishable across the five replicates.

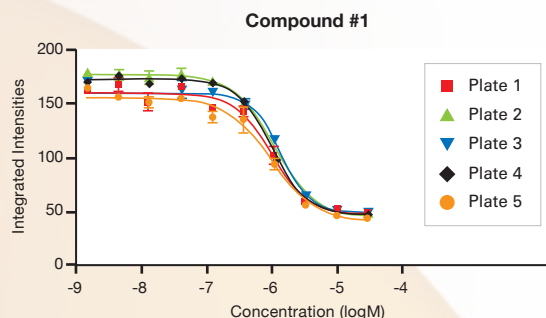


Plate	IC <sub>50</sub>
Plate 1	9.93E-07
Plate 2	1.09E-06
Plate 3	1.39E-06
Plate 4	1.00E-06
Plate 5	9.65E-07

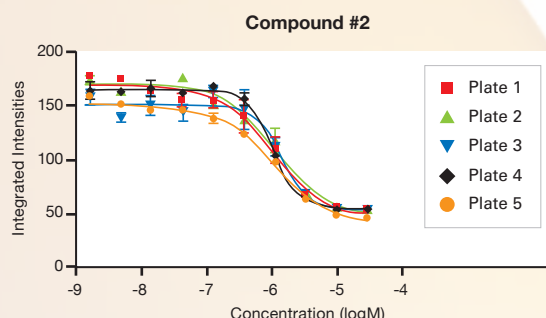


Plate	IC <sub>50</sub>
Plate 1	9.86E-07
Plate 2	1.10E-06
Plate 3	1.40E-06
Plate 4	1.02E-06
Plate 5	1.04E-06

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

- Sensitive, quantitative analysis of cellular signaling pathways
- “Snapshot” of signal transduction or protein expression of cell population in each well
- Enhanced throughput
- Increased precision
- Easily screen cell treatments or drug candidates for their effects on target proteins



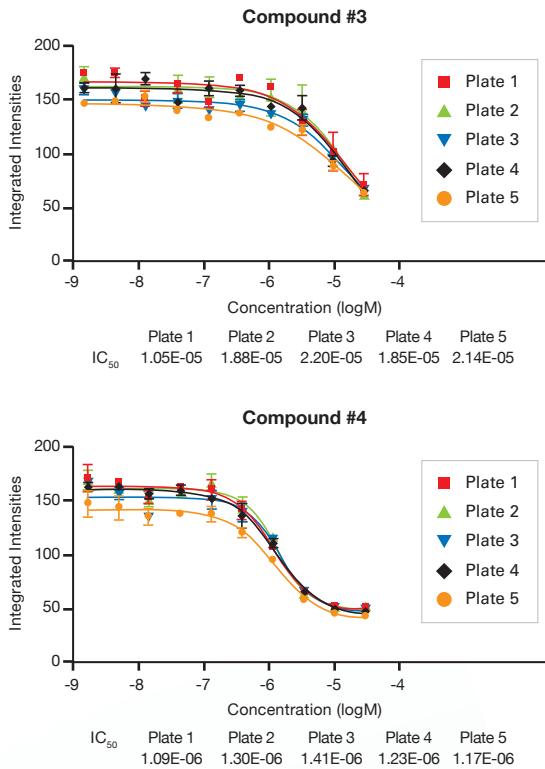
1. Chen, H., J. Kovar, S. Sissons, K. Cox, W. Matter, F. Chadwell, P. Luan, C.J. Vlahos, A. Schutz-Geschwender, and D.M. Olive. A Cell Based Immunocytochemical Assay for Monitoring Kinase Signaling Pathways and Drug Efficacy. *Analytical Biochemistry* 338:136-42 (2005).

2. Hoffmann et al. A functional siRNA screen for novel regulators of mTORC1 signaling. Poster presentation, ASCB Annual Meeting (2008).



## Related Products

In addition to the In-Cell Western™ Assay kits and IRDye conjugated secondary antibodies, LI-COR® Biosciences is committed to providing high-quality products optimized for use in the ICW assay and LI-COR imaging systems.



## Products and Applications Guide

Check out the Products and Applications Guide for a list of instruments, reagents and accessories designed for your infrared imaging needs.

Available online at:  
[www.licor.com/bio](http://www.licor.com/bio)



### Description

P/N

#### In-Cell Western Assay Kits:

In-Cell Western Assay Kit I Goat $\alpha$ -mouse IRDye 800CW Ab	926-31070
In-Cell Western Assay Kit II Goat $\alpha$ -rabbit IRDye 800CW Ab	926-31072

#### Secondary Antibodies:

Goat $\alpha$ -mouse IRDye 800CW, 0.5 mg	926-32210
Goat $\alpha$ -rabbit IRDye 800CW, 0.5 mg	926-32211
Donkey $\alpha$ -mouse IRDye 800CW, 0.5 mg	926-32212
Donkey $\alpha$ -rabbit IRDye 800CW, 0.5 mg	926-32213
Donkey $\alpha$ -goat IRDye 800CW, 0.5 mg	926-32214
Donkey $\alpha$ -chicken IRDye 800CW, 0.5 mg	926-32218
Goat $\alpha$ -rat IRDye 800CW, 0.5 mg	926-32219
Goat $\alpha$ -mouse IRDye 680LT, 0.5 mg	926-68020
Goat $\alpha$ -rabbit IRDye 680LT, 0.5 mg	926-68021
Donkey $\alpha$ -mouse IRDye 680LT, 0.5 mg	926-68022
Donkey $\alpha$ -rabbit IRDye 680LT, 0.5 mg	926-68023
Donkey $\alpha$ -goat IRDye 680LT, 0.5 mg	926-68024
Donkey $\alpha$ -chicken IRDye 680LT, 0.5 mg	926-68028
Goat $\alpha$ -rat IRDye 680LT, 0.5 mg	926-68029
Donkey $\alpha$ -guinea pig IRDye 680LT, 0.5 mg	926-68030
Goat $\alpha$ -mouse IRDye 680, 0.5 mg	926-32220
Goat $\alpha$ -rabbit IRDye 680, 0.5 mg	926-32221
Donkey $\alpha$ -mouse IRDye 680, 0.5 mg	926-32222
Donkey $\alpha$ -rabbit IRDye 680, 0.5 mg	926-32223
Donkey $\alpha$ -goat IRDye 680, 0.5 mg	926-32224
Donkey $\alpha$ -chicken IRDye 680, 0.5 mg	926-32228
Goat $\alpha$ -rat IRDye 680, 0.5 mg	926-32229
Donkey $\alpha$ -guinea pig IRDye 800CW, 0.5 mg	926-32411
Donkey $\alpha$ -guinea pig IRDye 680, 0.5 mg	926-32421
Goat $\alpha$ -mouse IRDye 700DX, 0.5 mg	926-32510
Goat $\alpha$ -rabbit IRDye 700DX, 0.5 mg	926-32511

#### Primary Antibodies:

$\alpha$ -Actin Rabbit mAb, 100 $\mu$ L	926-32230
$\alpha$ -Tubulin Rabbit Ab, 100 $\mu$ L	926-42211

#### Blocking Buffers:

Odyssey® Blocking Buffer, 500 mL	927-40000
Odyssey Blocking Buffer, 3 Pack	927-40003
Odyssey Blocking Buffer, 10 Pack	927-40010
Odyssey Blocking Buffer, 125 mL	927-40100
Milk Blocking Buffer 10X, 100 mL	927-40400
Milk Blocking Buffer 10X, 20 mL	927-40500
Casein Blocking Buffer, 500 mL	927-40200
Casein Blocking Buffer, 100 mL	927-40300
Blocking Buffer Sample Pack	927-40050

#### Stain:

Sapphire700™ Stain, 100 $\mu$ L	928-40022
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#### Accessories:

Odyssey ICW Software Module	9201-550
6-Multiwell-Plate Alignment Guide	926-72200

## Locations WorldWide

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The LI-COR board of directors would like to take this opportunity to return thanks to God for His merciful providence in allowing LI-COR to develop and commercialize products, through the collective effort of dedicated employees, that enable the examination of the wonders of His works.

“Trust in the LORD with all your heart and do not lean on your own understanding. In all your ways acknowledge Him, and He will make your paths straight.”

—Proverbs 3:5,6

