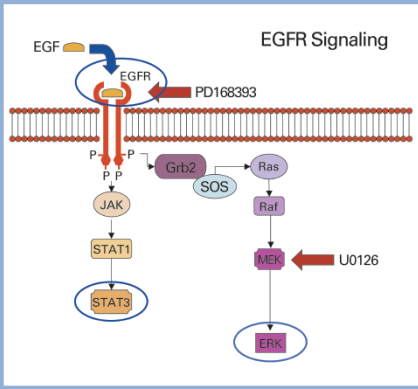
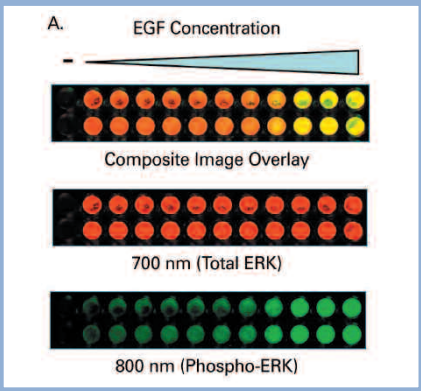
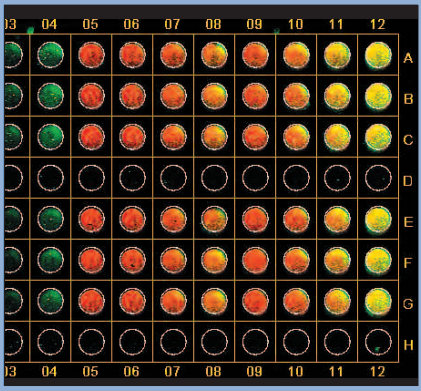


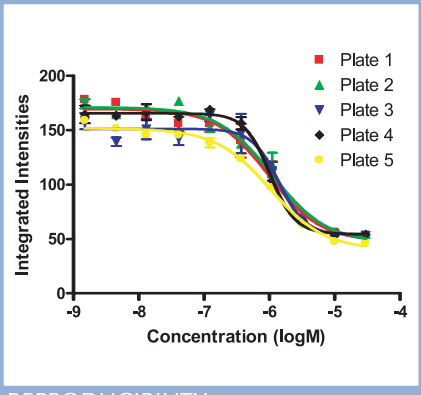
PATHWAY ANALYSIS



ADHERENT CELLS



SUSPENSION CELLS



REPRODUCIBILITY

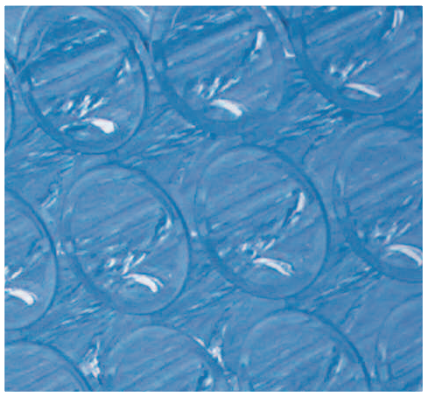
APPLICATION OVERVIEW

IN-CELL WESTERN ASSAY

LI-COR®
Biosciences

Quantitative Cell-Based Assays

For Ratiometric Protein Analysis



Application Summary:
The In-Cell Western is an immunocytochemical assay performed in microplate format. Target-specific primary antibodies and infrared-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 Westerns 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 Westerns 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 against a second target or DNA stain. 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 Assay Workflow

Grow Adherent/Suspension Cells in Microplate

↓

Fix and Permeablize Cells, Block

↓

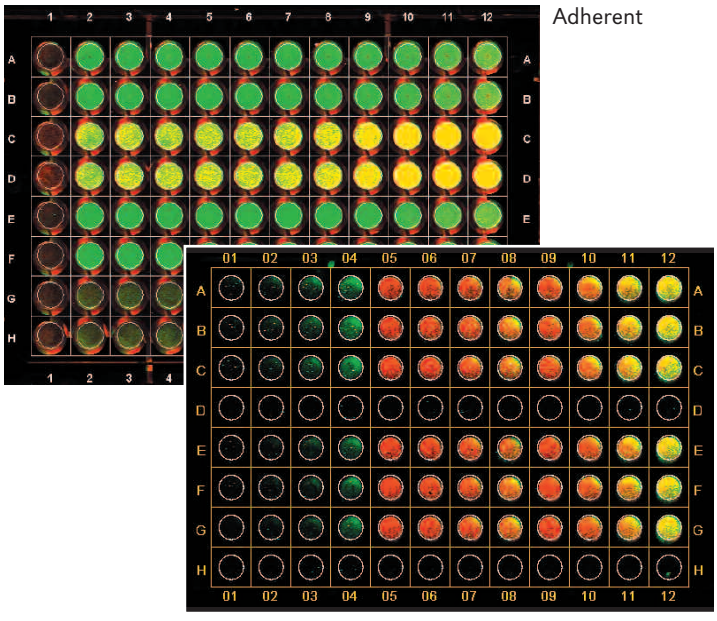
Primary Antibody Incubation

↓

Secondary Antibody Incubation (DNA Staining, if desired)

↓

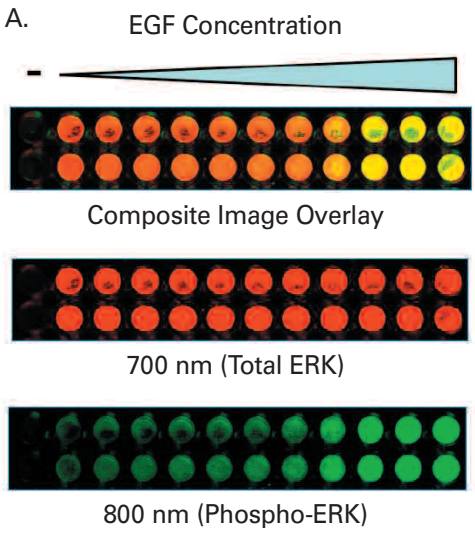
Image and Quantify

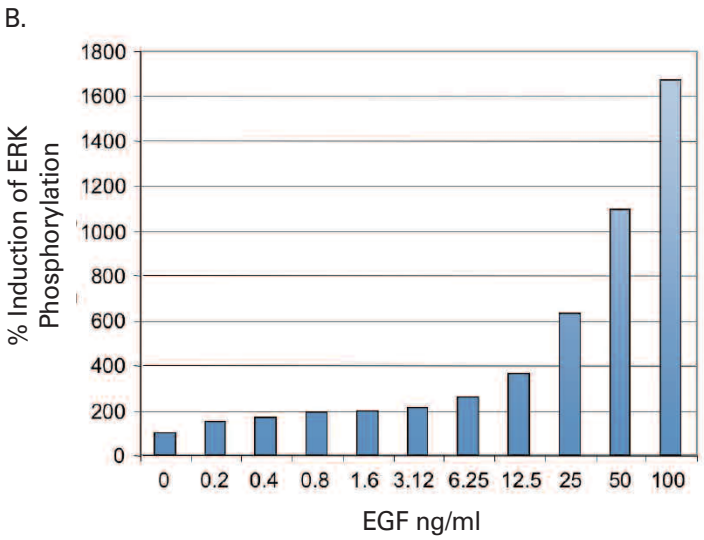


Adherent

Suspension

Phosphorylation of ERK in Response to Pathway Stimulation





A431 cells were stimulated with serial dilutions of EGF to optimize activation of ERK1/2*.

A) Detection of ERK phosphorylation. These images show a portion of a 96-well plate. The top panel is a composite image showing the fluorescence in both the 700 nm and 800 nm detection channels. Duplicate rows of microplate wells are shown. 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.

In-Cell Westerns 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 Westerns 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. In-Cell Westerns offer a practical alternative for medium to high throughput analysis.

Advantages of the In-Cell Western

- Simultaneous, two-target detection enables precise quantification and accurate measurement of abundance or phosphorylation of one target by normalization against another target or DNA stain.
- Direct detection of proteins in their cellular context eliminates variabilities and artifacts caused by cell lysis. In-cell detection can provide more relevant results than enzyme assays with purified proteins.
- Near-infrared probes yield high sensitivity for measuring small changes in protein amount or modification.
- Analyzes multiple targets in 96- or 384-well plates.
- Fast, microplate-based assay – lysate preparation, gel loading, electrophoresis, and membrane transfer are eliminated.
- Ideal for screening cell treatments or drug candidates for effects on target proteins.

In-Cell Westerns use a two-color ratiometric approach for precise, quantitative analysis of cell signaling with good reproducibility.

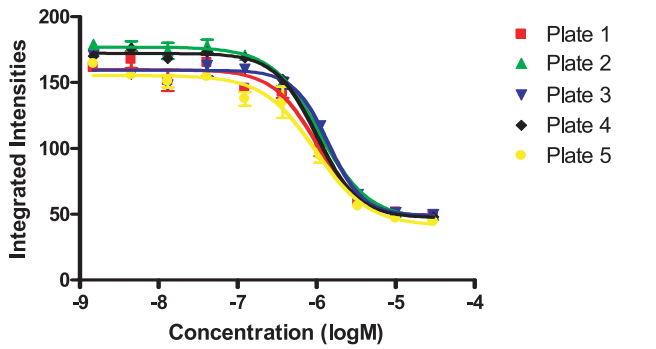


	Plate 1	Plate 2	Plate 3	Plate 4	Plate 5
IC_{50}	9.93E-07	1.09E-06	1.39E-06	1.00E-06	9.65E-07

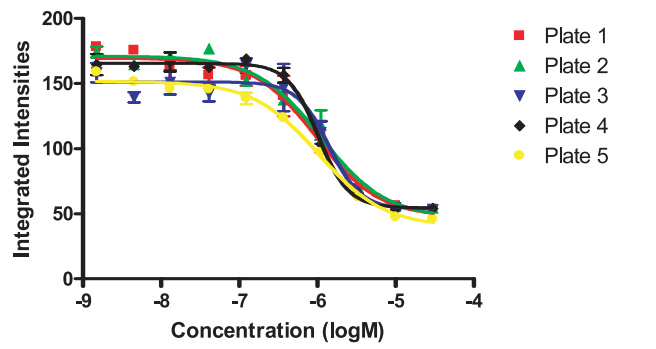


	Plate 1	Plate 2	Plate 3	Plate 4	Plate 5
IC_{50}	9.86E-07	1.10E-06	1.40E-06	1.02E-06	1.04E-06

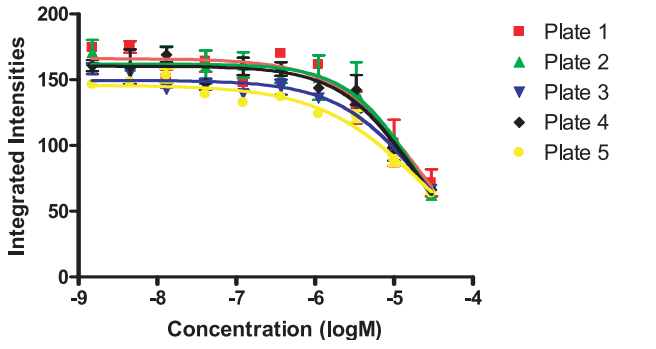


	Plate 1	Plate 2	Plate 3	Plate 4	Plate 5
IC_{50}	1.05E-05	1.88E-05	2.20E-05	1.85E-05	2.14E-05

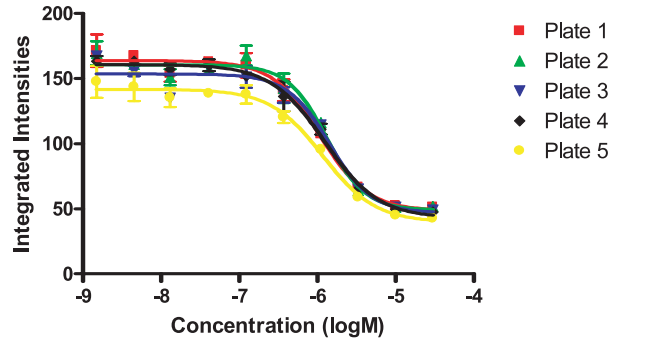
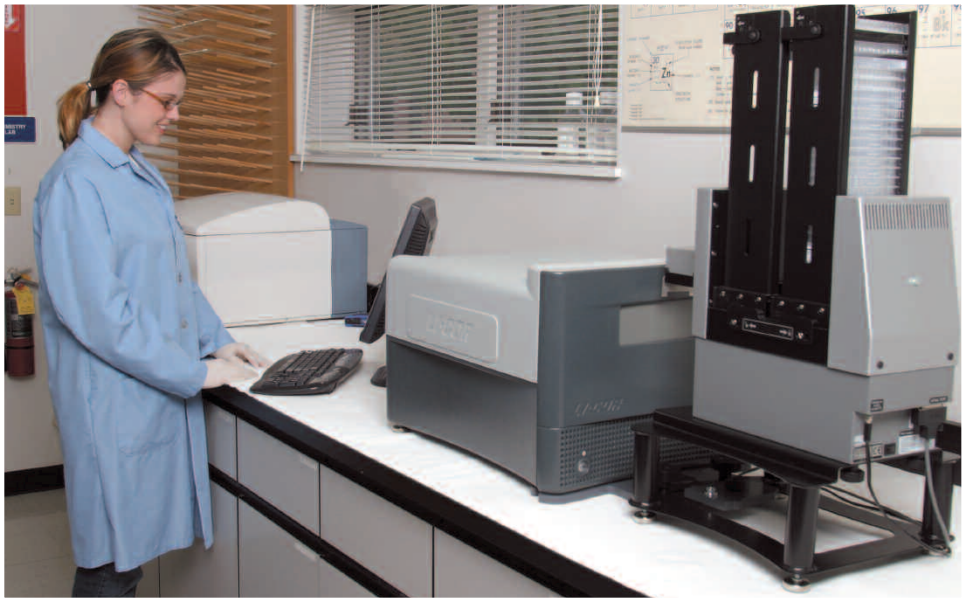


	Plate 1	Plate 2	Plate 3	Plate 4	Plate 5
IC_{50}	1.09E-06	1.30E-06	1.41E-06	1.23E-06	1.17E-06

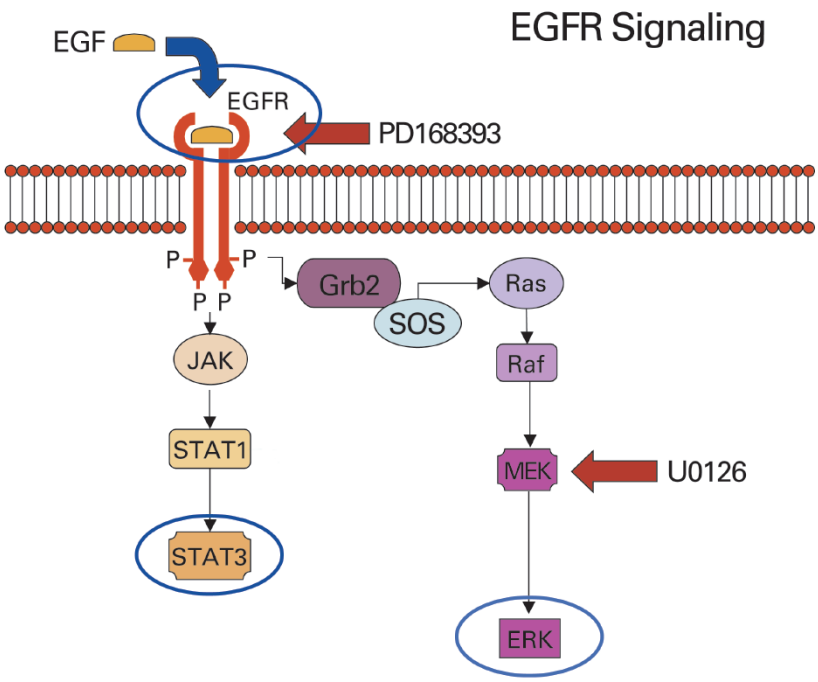
Reproducibility of the ICW assay*. IC_{50} values were determined for four experimental compounds that inhibit PDGF-induced phosphorylation of Akt in NIH 3T3 cells. At each drug concentration, the integrated fluorescence intensity for phospho-Akt staining was determined. For each data point, eight replicates were performed; the mean and standard deviation are shown. Each experiment was repeated five times (plates 1-5) and IC_{50} values for each plate are shown. Each compound gave IC_{50} values that were indistinguishable across the five replicates.



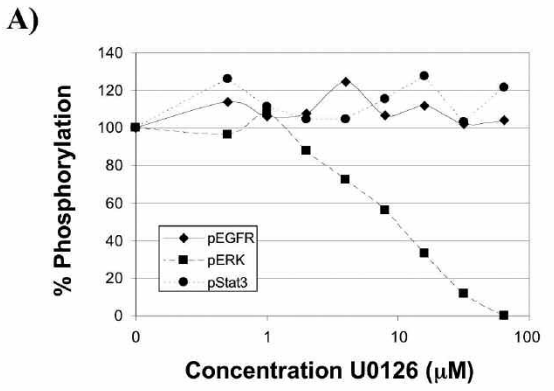
The Aerius® Automated Infrared Imaging System with Bio-Tek® Bio-Stack™ Automated Microplate Stacking System handles up to 30 microplates per run. Optional COM software is available for integration with robotic systems.

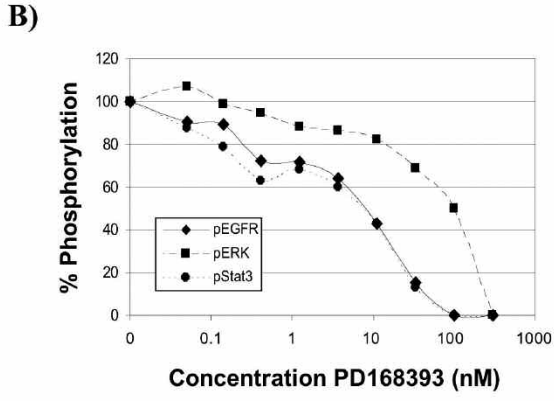
Aerius automates plate stacking, spot finding, analysis and data export for In-Cell Western assays.

Pathway Analysis with the In-Cell Western Assay



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 (red 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.





Effects of pathway inhibitors on EGFR, Stat3, and ERK phosphorylation*. A431 cells were cultured and treated with serial dilutions of drug, then stimulated with EGF. IC_{50} 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_{50}) 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.

*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).

LI-COR Infrared Imaging Systems

Direct infrared detection is available on two different systems from LI-COR Biosciences — The Odyssey® Infrared Imaging System and the Aerius® Automated Infrared Imaging System. Both systems are designed for In-Cell Westerns and other membrane and plate-based assays. Researchers are continually exploring the benefits of LI-COR Systems for a variety of additional applications including *in vivo* imaging, tissue imaging, and On-Cell Westerns.

Odyssey and Aerius simultaneously detect two targets at 700 and 800 nm using two spectrally distinct dyes. Separate lasers and fluorescence detectors are used for each dye. Two-dye detection allows evaluation of two different targets of interest, or one dye can be used for data normalization.

Odyssey Features:

- Membrane, microplate, or gel scanning
- Scan up to six microplates at a time
- In-Cell Western software for response calculations and reporting

Aerius Features:

- Automatic scanning, spot finding, and intensity measurements
- Percent response calculations for In-Cell Westerns
- Scan up to 30 plates per run with optional plate stacker
- Internal bar code reader

Learn More About In-Cell Westerns:

The LI-COR website provides updates on new developments in infrared imaging and research results using the Odyssey and Aerius systems. To learn more, go to **www.licor.com/aerius.**

LI-COR® Biosciences

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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

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