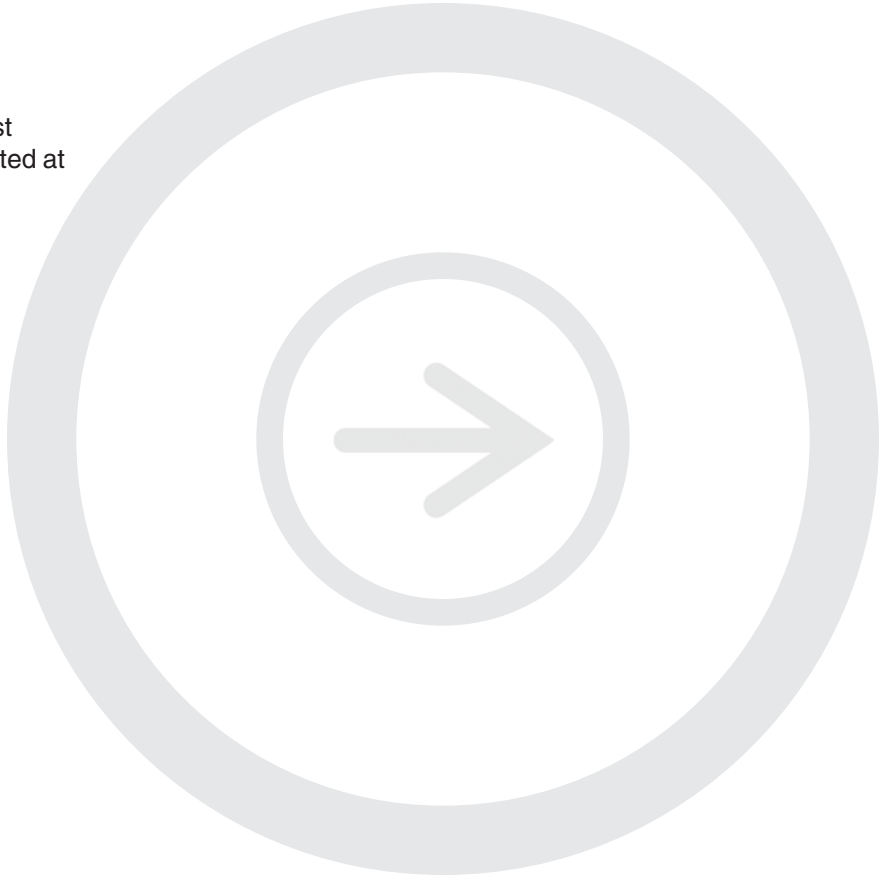
Odyssey®

Infrared Imaging System

In-Cell Western Assay

Complete Sample Protocol for Measuring IC_{50} of Inhibitor PD168393 in A431 Cells Responding to Epidermal Growth Factor

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



LI-COR® Biosciences

Doc# 988-08599

Contents

I. Required Reagents

Odyssey® Reagents

- IRDye™ 800CW- and IRDye™ 680-labeled secondary antibodies (LI-COR)*
- Odyssey® Blocking Buffer (LI-COR, Cat.# 927-40000)

Additional Reagents

- 1X PBS wash buffer
- Tissue culture reagents (serum, D-MEM, trypsin, 1X PBS)
- 20% Tween®-20
- Epidermal Growth Factor (Upstate Group Inc., Cat.# 01-107)
- Protein Tyrosine Kinase Inhibitor PD168393 (CALBIOCHEM®, Cat.# 513033)
- 37% formaldehyde
- 10% Triton® X-100
- Falcon 384-well microplate (Cat.# 353961)
- Primary antibodies

Special Note: Anti-phosphorylated-EGFR and anti-phosphorylated-ERK antibodies are purchased from Cell Signaling Technology and Santa Cruz Biotechnology, respectively. Cell starvation is needed to obtain maximal response when these two phospho-antibodies are used. This is in contrast to use of anti-phospho-ERK from BD Pharmingen and from Cell Signaling Technology.

* 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 A431cell growth in a T75 flask using standard tissue culture procedures until cells reach near confluency (~1.5x10^7 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.
4.	Remove supernatant and disrupt the cell pellet manually by hand tapping the collection tube. Avoid use 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.	Dilute cells with complete media such that 200,000 cells/ml is achieved.
7.	Manually mix the cell suspension thoroughly.
8.	Under sterile conditions dispense 50 µl of the cell suspension per well in Falcon 384-well microplate (10,000 cells plated per well).
9.	Incubate cells and monitor cell density until confluency is achieved with well-to-well consistency; approximately three days.
10.	Warm serum-free media (D-MEM; Gibco) to 37 °C.
11.	Remove complete media from plate wells by aspiration or manual displacement.
12.	Replace media with 50 µl of pre-warmed serum free media per well and incubate 4 to 16 hours.
13.	Warm serum free media (D-MEM; Gibco) to 37 °C.
14.	Dissolve PD168393 in D-MEM to make 3 µM stock. Make two fold serial dilutions of inhibitor using D-MEM so that the final concentration of inhibitor range from 3 µM to 90 pM, as shown in section *IV. Experimental Results*.
15.	Remove media in A431 cell plate.
16.	Add 50 µl of serial diluted inhibitor into cells and incubate 1 to 2 hours.
17.	Remove inhibitor from plate wells by aspiration or manual displacement.
18.	Add either serum free media for resting cells (mock) or serum free media with 100 ng/ml EGF. Use 50 µl of resting/activation media per well.
19.	Allow incubation at 37 °C for 7.5 minutes.

20. Remove activation or stimulation media manually or by aspiration. Immediately fix cells with 4% formaldehyde in 1X PBS for 20 minutes at room temperature.

a. Prepare fresh ***Fixing Solution*** as follows:

1X PBS	45 ml
37% Formaldehyde	5 ml
3.7% Formaldehyde	50 ml

b. Using a multi-channel pipettor, add 150 µl of fresh *Fixing Solution* (room temperature solution, RT). **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 bench top for 20 minutes at RT with no shaking.

21. Wash five times with 1X PBS containing 0.1% Triton X-100 (cell permeabilization) for 5 minutes per wash.

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

1X PBS	495 ml
10% Triton X-100	5 ml
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 *Triton Washing Solution* (RT). **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 at RT.

e. Repeat washing steps 4 more times after removing wash manually.

⚠ **Do not allow cells/wells to become dry during washing. Immediately add the next wash after manual disposal.**

22.	Using a multi-channel pipettor, block cells/wells by adding 50 µl of LI-COR 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 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 Aerius detection. • 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). • 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. • 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.
23.	Allow blocking for 90 minutes at RT with moderate shaking on a rotator.
24.	Add the two primary antibodies into a tube containing Odyssey Blocking Buffer. Choose one of the following primary antibody pairs: • Phospho-ERK (mouse; 1:100 dilution; Santa Cruz Biotechnology, SC-7383) Total ERK1 (Rabbit; 1:200 dilution; Santa Cruz Biotechnology, SC-94) • Phospho-EGFR Tyr1045 (Rabbit; 1:100 dilution; Cell Signaling Technology, 2237) Total ERK2 (Mouse; 1:75 dilution; Santa Cruz Biotechnology, SC-1647) • Phospho-EGFR Tyr1045 (Rabbit; 1:100 dilution; Cell Signaling Technology, 2237) Phospho-ERK (Mouse; 1:100 dilution; Santa Cruz Biotechnology, SC-7383) • Phospho-EGFR Tyr1045 (Rabbit; 1:100 dilution; Cell Signaling Technology, 2237) Total EGFR (Mouse; 1:500 dilution; Biosource International, AHR5062) a. Mix the primary antibody solution well before addition to wells. b. Remove blocking buffer from the blocking step and add 20 µl of the desired primary antibody or antibodies in Odyssey Blocking Buffer to cover the bottom of each well. c. **Make sure to include control wells without primary antibody to serve as a source for background well intensity**. Add 50 µl of Odyssey Blocking Buffer only to control wells.
25.	Incubate with primary antibody overnight with gentle shaking at RT.

26.	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 1X PBS with 0.1% Tween-20 1000 ml b. Using a multi-channel pipettor add 200 µl of *Tween Washing Solution* (RT). **Make sure to carefully add 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 at RT. d. Repeat washing steps 4 more times.
27.	Dilute the fluorescently labeled secondary antibody in Odyssey Blocking Buffer as specified below. To lower background, add Tween-20 to the diluted antibody for a final concentration of 0.2%. Goat anti-rabbit IRDye™ 680 (1:200 dilution; LI-COR Cat.# 926-32221) Goat anti-mouse IRDye™ 800CW (1:800 dilution; LI-COR Cat.# 926-32210) Or Goat anti-mouse IRDye™ 680 (1:200 dilution; LI-COR Cat.# 926-32220) Goat anti-rabbit IRDye™ 800CW (1:800 dilution; LI-COR Cat.# 926-32211) Recommended dilution range is 1:200 to 1:1,200. ⚠ **Avoid prolonged exposure of the antibody vials to light.** **Notes:** • Use IRDye™ 800CW-labeled secondary antibody to detect phosphorylation and IRDye™ 680-labeled secondary antibody to detect total protein.
28.	Mix the antibody solutions well and add 20 µl of the secondary antibody solution to each well. Incubate for 60 minutes with gentle shaking at RT. Protect plate from light during incubation.
29.	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. Using a multi-channel pipettor, add 200 µl of *Tween Washing Solution* at RT (see step 26). **Make sure to carefully add 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 at RT. c. Repeat washing steps 4 more times after removing wash manually. ⚠ Protect plate from light during washing.
30.	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).
31.	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.

32.	Scan the plate with detection in both the 700 and 800 channels using the Odyssey Infrared Imaging System (700 nm detection for IRDye™ 680 antibody and 800 nm detection for IRDye™ 800CW antibody). Use medium 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

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.

- 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 96- and 382-well plates are recommended by LI-COR Biosciences:

96 well format	Nunc™ (Part Number 161093, 165305)
96 well format	Falcon™ (Part Number 353075, 353948)
384 well format	Nunc™ (Part Number 164688, 164730)
384 well format	Falcon™ (Part Number 353961, 353962)

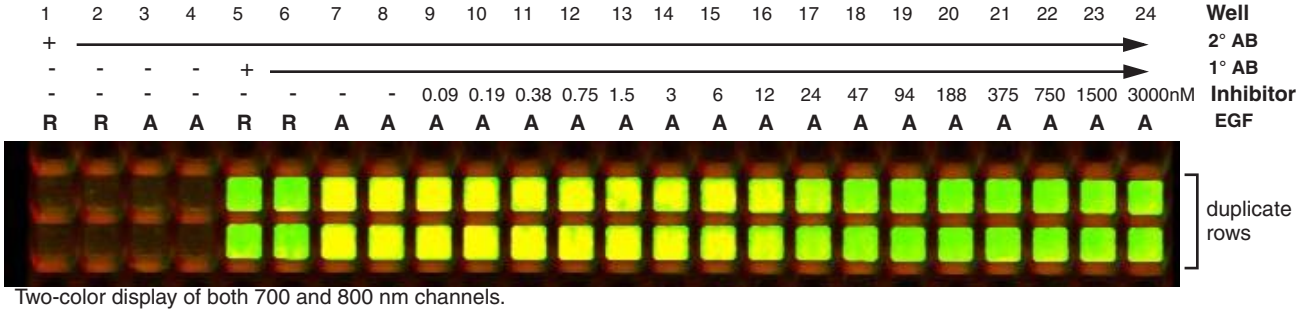
- The Odyssey Imager requires that microplates have a maximum 4.0 mm distance from the Odyssey scanning surface to the target detection area of the plate. When using the plates specified above for In-Cell Western assays, the recommended focus offset is 3.0 mm.
- If you use plates other than the plates recommended above, 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 results.

IV. Experimental Results

Simultaneous measurement of the effect of PD168393 on the phosphorylation of EGFR and downstream ERK.

Four different experiments were conducted on the same microplate. (Color images can be seen at http://biosupport.licor.com/support.)

Experiment 1. Effect on the phosphorylation of EGFR (normalized against total EGFR).

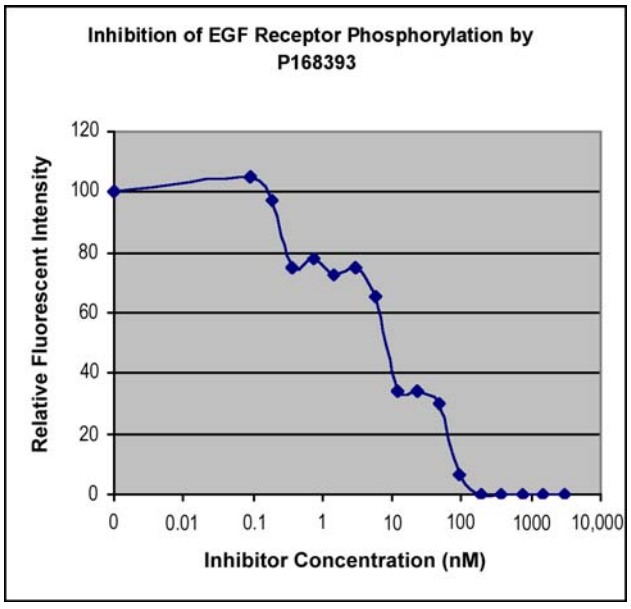


Two-color display of both 700 and 800 nm channels.

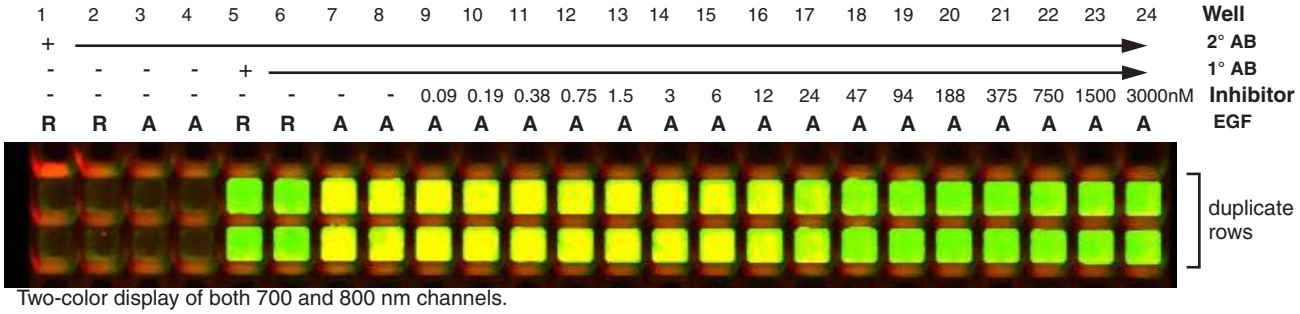
700 nm image (phosphorylated EGFR).

800 nm image (total EGFR).

The level of EGFR phosphorylation was assessed in the 700 nm channel. A dramatic increase of EGFR phosphorylation in response to EGF stimulation in the 7th and 8th wells was seen, compared to the basal level of phosphorylation in the 5th and 6th wells (without EGF). Dose-dependent inhibition of EGFR phosphorylation by PD168393 was observed in the 9th to 24th wells. Similar amounts of EGFR are present in all wells, as indicated by the 800 channel. The 1st to 4th wells, reacting only with the secondary antibodies, serve as a negative control and background subtraction. R = resting cells; A = activated cells.



Experiment 2. Effect on the phosphorylation of EGFR (normalized against total ERK).

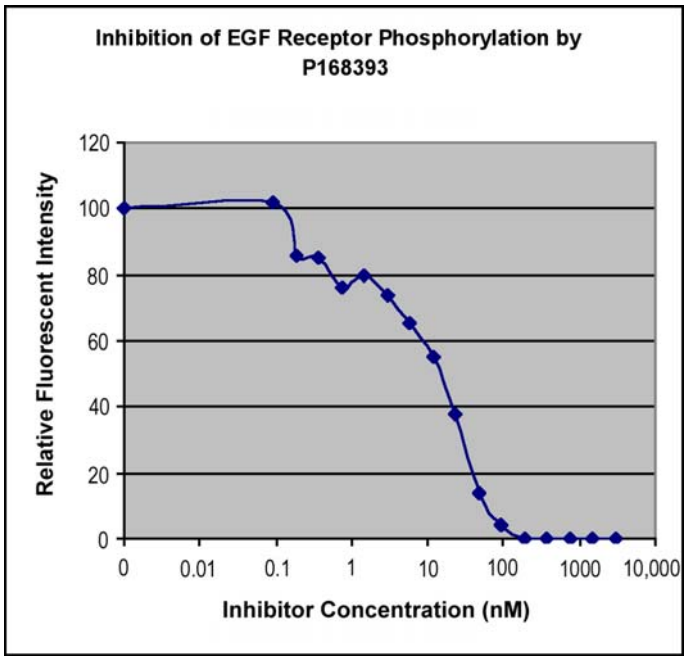


Two-color display of both 700 and 800 nm channels.

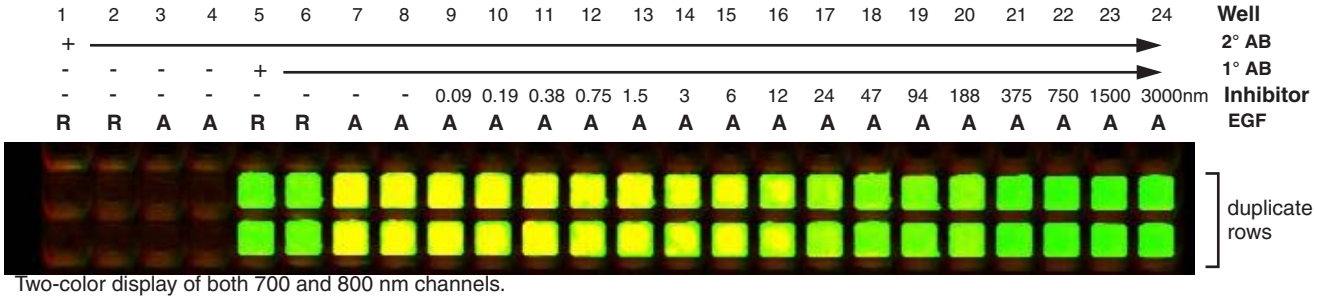
700 nm image (phosphorylated EGFR).

800 nm image (total ERK).

The level of EGFR phosphorylation was assessed in the 700 nm channel. A dramatic increase of EGFR phosphorylation in response to EGF stimulation in the 7th and 8th wells was seen, compared to the basal level of phosphorylation in the 5th and 6th wells (without EGF). Dose-dependent inhibition of EGFR phosphorylation by PD168393 was observed in the 9th to 24th wells. Similar amounts of ERK are present in all wells, as indicated by the 800 channel. The 1st to 4th wells reacting only with the secondary antibodies, serve as a negative control and background subtraction. R = resting cells; A = activated cells.



Experiment 3. Effect on the phosphorylation of ERK (normalized against total ERK).

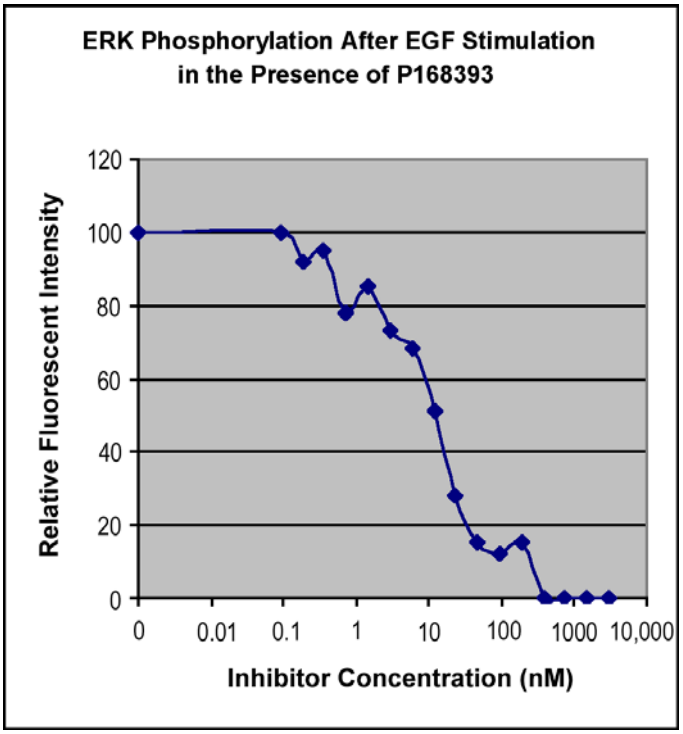


Two-color display of both 700 and 800 nm channels.

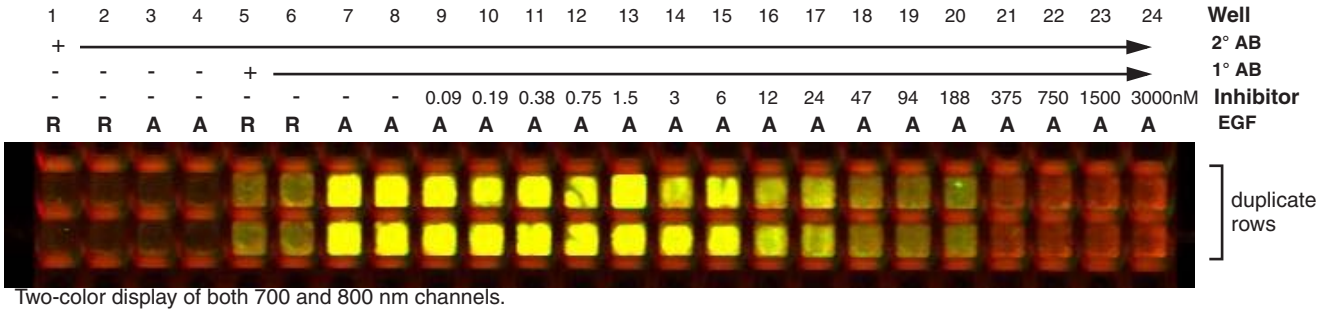
700 nm image (phosphorylated ERK).

800 nm image (total ERK).

The level of ERK phosphorylation was assessed in the 700 nm channel. A dramatic increase of ERK phosphorylation in response to EGF stimulation in the 7th and 8th wells was seen, compared to the basal level of phosphorylation in the 5th and 6th wells (without EGF). Dose-dependent inhibition of ERK phosphorylation by PD168393 was observed in the 9th to 24th wells. Similar amounts of ERK are present in all wells, as indicated by the 800 channel. The 1st to 4th wells reacting only with the secondary antibodies, serve as a negative control and background subtraction. R = resting cells; A = activated cells.



Experiment 4. Effect on the phosphorylation of EGFR and ERK.

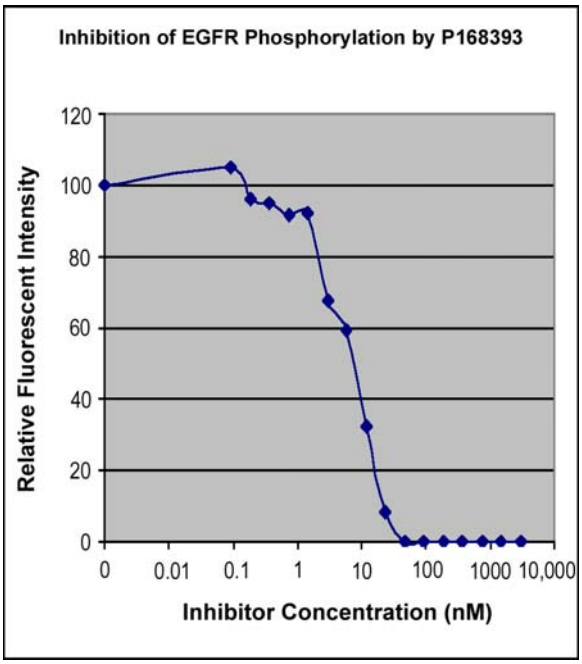


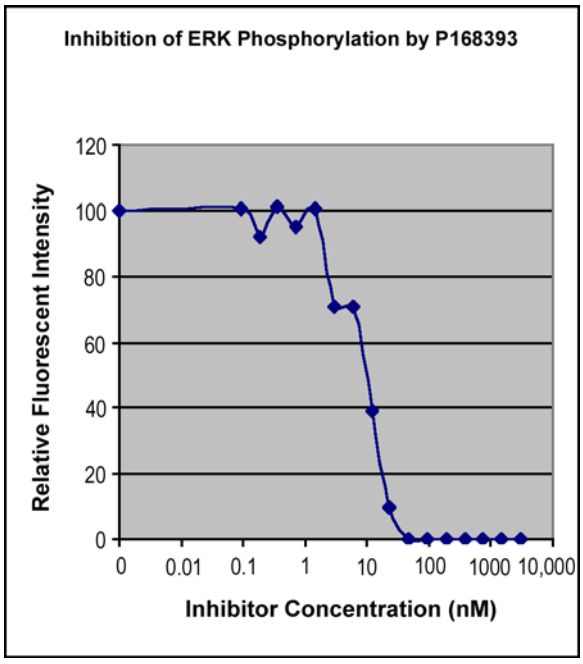
Two-color display of both 700 and 800 nm channels.

700 nm image (phosphorylated EGFR).

800 nm image (phosphorylated ERK).

The degree of phosphorylation of EGFR and ERK was assessed in the 700 nm (red) and 800 nm (green) channels, respectively. A dramatic increase of phosphorylation of EGFR and ERK in response to EGF stimulation in the 7th and 8th wells was seen, compared to the basal level phosphorylation in the 5th and 6th wells (without EGF). Dose-dependent inhibition of phosphorylation of EGFR and ERK by PD168393 was observed in 9th to 24th wells. The 1st to 4th wells reacting only with the secondary antibodies, serve as a negative control and background subtraction. R = resting cells; A = activated cells.





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