Complete Sample Protocol for Measuring IC50 of Inhibitor PD168393 in A431 Cells Responding to Epidermal Growth Factor

LI-COR Biosciences

Abstract

Developed for:

Aerius,
Odyssey® Classic,
Odyssey CLx, and
Odyssey Sa
Infrared Imaging Systems

Citation: LI-COR Biosciences Complete Sample Protocol for Measuring IC50 of Inhibitor PD168393 in A431 Cells Responding to Epidermal Growth Factor. **protocols.io**
dx.doi.org/10.17504/protocols.io.grxbv7n
Published: 26 Jun 2018

Guidelines

Required Reagents

LI-COR Reagents

- IRDye® 800CW Goat anti-Mouse Secondary Antibody (LI-COR P/N 925-32210 or 926-32210)
- IRDye 680RD Goat anti-Rabbit Secondary Antibody (LI-COR P/N 925-68071 or 926-68071)
- Odyssey® Blocking Buffer (LI-COR P/N 927-40000 or 927-50000)

Additional Reagents

- 1X PBS wash buffer
- Tissue culture reagents (serum, DMEM, trypsin, 1X PBS)
- Epidermal Growth Factor (Millipore, P/N 01-107)
- Protein Tyrosine Kinase Inhibitor PD168393 (CALBIOCHEM®, P/N 513033)
- Primary antibodies
- 20% Tween® 20
- 37% formaldehyde
- 10% Triton® X-100
- Clear or black 96-well or 384-well microplates (See IX. Experimental Considerations)

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.

Experimental Considerations

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

- In-Cell Western analyses use detection at the well surface with minimal 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 walls, since autofluorescence from the white surface will create significant noise.
- In-Cell Western assays require sterile plates for tissue culture growth. The following plates are recommended by LI-COR Biosciences:

  96-well format, Eppendorf (P/Ns 0030730119 or 0030741030)
  96-well, clear, Nunc® (P/Ns 167008, 161093)
  96-well, clear, FalconTM (P/Ns 353075, 353948)
  96-well, black with clear bottom, Nunc (P/N 165305)
  96-well format, Corning (P/N 3603)

  384-well, clear, Nunc (P/Ns 164688, 164730)
  384-well, clear, Falcon (P/Ns 353229, 353289)
  384-well, black with clear bottom, Nunc (P/N 142761)
  384-well format, Corning (P/N 3712)

- **Focus Offset Optimization** – If plates other than those recommended are used, the focus offset can be determined empirically by scanning a plate containing experimental and control samples using the following focus offset settings.

Instrument	Focus Offset Determination (mm)
Odyssey Classic & Odyssey CLx	1.0, 1.5, 2.0, 2.5, 3.0, 3.5, & 4.0
Odyssey Sa & Aerius	1.5, 2.0, 2.5, 3.0 & 3.95

Use the same intensity settings for each scan. After reviewing the scans, use the focus offset with the highest signal-to-noise ratio for experiments. The actual minimum and maximum focus offset will vary with each instrument. Alternatively, the plate manufacturer can be consulted to obtain the measured distance from the skirt bottom to the bottom of the plate.

- All Aerius and Odyssey® Imaging systems (excluding Odyssey Fc) require microplates that have a maximum 4.0 mm distance from the base of the microplate to the target detection area of the plate (actual maximum focus offset varies with each Aerius and Odyssey Sa instrument and is found by choosing Settings > System Administration, then clicking Scanner Information). When using plates specified for In-Cell Western assays, the recommended focus offset is 3.5 mm or higher.
- **Intensity Setting Optimization -**

Instrument	Initial Intensity Setting (700/800 nm)	Intensity Settings: Weak Signal (700/800 nm)	Intensity Settings: Saturated Signal (700/800 nm)
Odyssey® Classic	5 / 5	7.5 / 7.5	2.5 / 2.5
Odyssey CLx	5 / 5	7.5 / 7.5	2.5 / 2.5
	AutoScan*	-	-
Odyssey Sa	7 / 7	8 / 8	4 / 4
Aerius	7 / 7	8 / 8	4 / 4

*The Odyssey CLx AutoScan function alleviates the need to scan the plate at multiple intensity settings.

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

Experimental Results

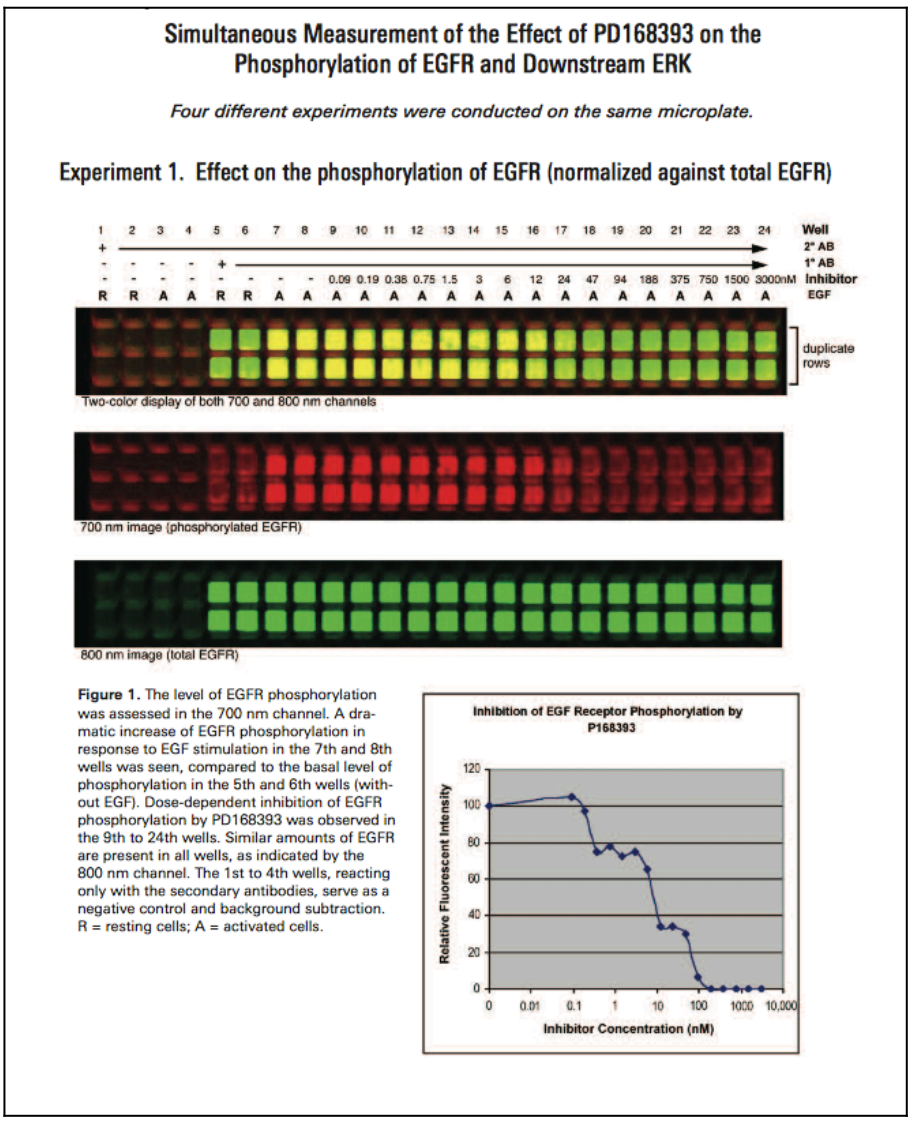


Figure 1. The level of EGFR phosphorylation was assessed in the 700 nm channel. A dramatic increase of EGFR phosphorylation in response to EGF stimulation in the 7th and 8th wells was seen, compared to the basal level of phosphorylation in the 5th and 6th wells (without EGF). Dose-dependent inhibition 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 nm 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.

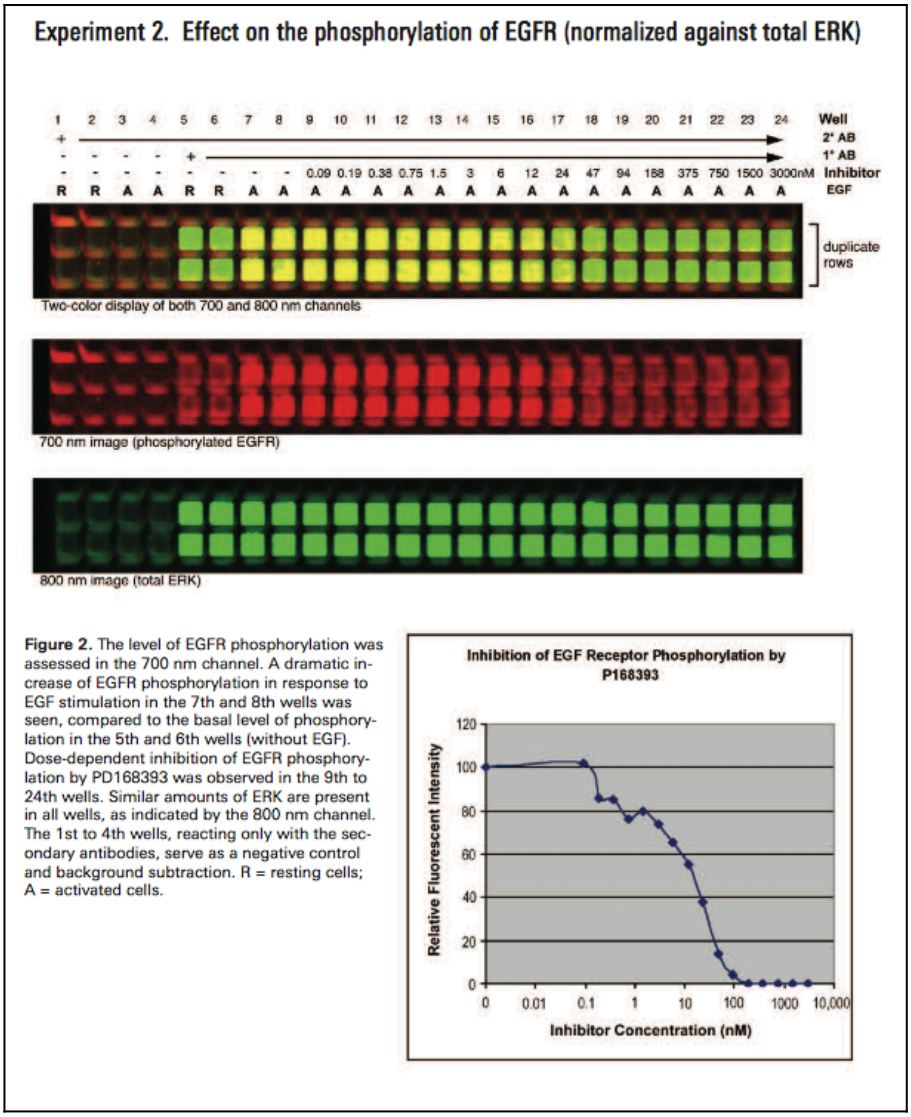


Figure 2. The level of EGFR phosphorylation was assessed in the 700 nm channel. A dramatic increase of EGFR phosphorylation in response to EGF stimulation in the 7th and 8th wells was seen, compared to the basal level of phosphorylation in the 5th and 6th wells (without EGF). Dose-dependent inhibition 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 nm 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)

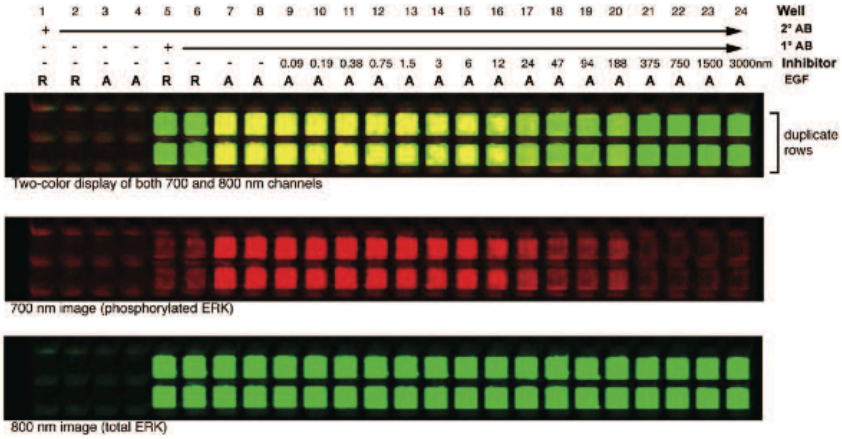
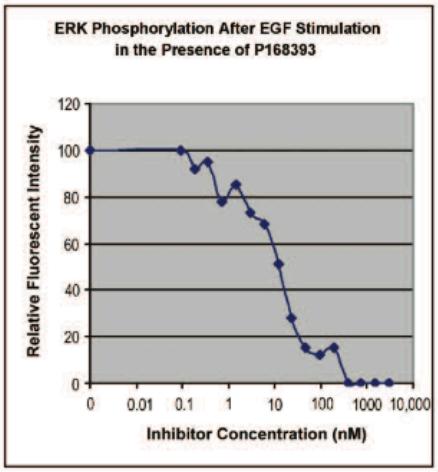


Figure 3. The level of ERK phosphorylation was assessed in the 700 nm channel. A dramatic increase of ERK phosphorylation in response to EGF stimulation in the 7th and 8th wells was seen, compared to the basal level of phosphorylation in the 5th and 6th wells (without EGF). Dose-dependent inhibition 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 nm 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.



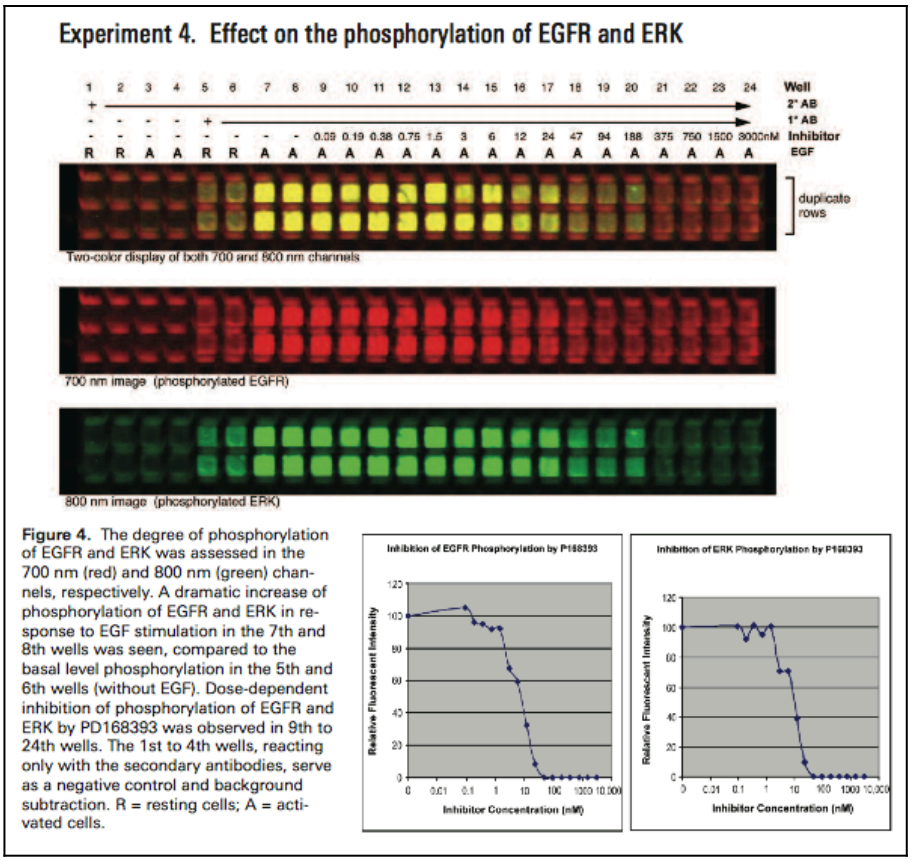


Figure 4. The degree of phosphorylation of EGFR and ERK was assessed in the 700 nm (red) and 800 nm (green) channels, respectively. A dramatic increase of phosphorylation of EGFR and ERK in response to EGF stimulation in the 7th and 8th wells was seen, compared to the basal level phosphorylation in the 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.

Materials

Odyssey® Blocking Buffer (PBS) 927-40000 927-40100 by LI-COR

Odyssey Blocking Buffer (TBS) 927-50000 927-50100 by LI-COR

IRDye® 800CW Goat anti-Mouse Secondary Antibody 925-32210 926-32210 by LI-COR

IRDye 680RD Goat anti-Rabbit Secondary Antibody 925-668071 926-68071 by LI-COR

Epidermal Growth Factor 01-107 by Emd Millipore

Protein Tyrosine Kinase Inhibitor PD168393 513033 by Emd Millipore

Protocol

Cell Preparation

Step 1.

Allow A431 cell growth in a T75 flask using standard tissue culture procedures until cells reach near confluency (1.5 x 10^7 cells; DMEM, 10% FBS; Gibco®).

Cell Preparation

Step 2.

Remove growth media, wash cells with sterile 1X PBS, and trypsinize cells for displacement.

Cell Preparation

Step 3.

Neutralize displaced cells with culture media and clarify by centrifugation.

Cell Preparation

Step 4.

Remove supernatant and disrupt the cell pellet manually by hand-tapping the collection tube.

To maintain cell integrity, do not pipet or vortex during pellet disruption.

Cell Preparation

Step 5.

Resuspend cells in 20 mL of complete media and count cells using a hemocytometer.

AMOUNT

20 ml Additional info:
complete media

Cell Preparation

Step 6.

Dilute cells with complete media to achieve concentration of 200,000 cells/mL.

Cell Preparation

Step 7.

Manually mix the cell suspension thoroughly.

Cell Preparation

Step 8.

Under sterile conditions, dispense 50 µL of the cell suspension per well in Falcon 384-well microplate. 10,000 cells plated per well.

AMOUNT

50 µl Additional info: cell suspension per well

NOTES

Ashley Humphrey 04 Jun 2018

see 'Experimental Considerations' in Guidelines for product specifications.

Cell Preparation

Step 9.

Incubate cells and monitor cell density until confluency is achieved with well-to-well consistency (approximately three days).

DURATION

12:00:00

Cell Treatment

Step 10.

Warm serum-free media (DMEM, Gibco®) to 37 °C.

TEMPERATURE

37 °C Additional info: warming

Cell Treatment

Step 11.

Remove complete media from the microwell plate by aspiration or manual displacement.

Cell Treatment

Step 12.

Carefully replace media with 50 µL of pre-warmed, serum-free media per well, and incubate 4 to 16 hours.

AMOUNT

50 µl Additional info: serum free media per well

DURATION

16:00:00

Cell Treatment

Step 13.

Warm serum-free media (DMEM; Gibco) to 37 °C.

TEMPERATURE

37 °C Additional info: serum-free media

Cell Treatment

Step 14.

- Dissolve PD168393 in DMEM to make 3 µM stock.
- Make two-fold serial dilutions of inhibitor using DMEM so that the final concentration of inhibitor range from 3 µM to 90 pM, as shown in 'Experimental Results' (see guidelines).

Cell Treatment

Step 15.

Remove media in A431 cell plate.

Cell Treatment

Step 16.

Add 50 µL of serial diluted inhibitor into cells and incubate 1 to 2 hours.

AMOUNT

50 µl Additional info:
inhibitor

DURATION

02:00:00

Cell Treatment

Step 17.

Remove inhibitor from plate wells by aspiration or manual displacement.

Cell Treatment

Step 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.

AMOUNT

50 µl Additional info:
resting/activation media
per well

Cell Treatment

Step 19.

Allow incubation at 37 °C for 7.5 minutes.

TEMPERATURE

37 °C Additional info:
incubation temperature

DURATION

00:07:30

Fixing/Permeabilizing Cells

Step 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. Detailed in steps 21-23

Fixing/Permeabilizing Cells

Step 21.

Prepare fresh Fixing Solution as follows:

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

Fixing/Permeabilizing Cells

Step 22.

Using a multi-channel pipettor, add 150µL of fresh *Fixing Solution* (room temperature solution, RT). **Add the solution by pipetting down the sides of the wells carefully to avoid detaching the cells from the well bottom.**

AMOUNT

150 µl Additional info:
Fixing Solution

Fixing/Permeabilizing Cells

Step 23.

Allow incubation on bench top for 20 minutes at RT with no shaking.

DURATION

00:20:00

Fixing/Permeabilizing Cells

Step 24.

Wash 5 times with 1X PBS containing 0.1% Triton® X-100 (cell permeabilization) for 5 minutes per wash. Detailed in steps 25-41.

NOTES

James Thornton Jr 11 Jan 2017

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

Fixing/Permeabilizing Cells

Step 25.

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

Fixing/Permeabilizing Cells

Step 26.

Remove Fixing Solution to an appropriate waste container (contains formaldehyde).

Fixing/Permeabilizing Cells

Step 27.

Using a multi-channel pipettor, add 200 µL of *Triton Washing Solution* (RT). **Make sure to add the solution down the sides of the wells carefully to avoid detaching the cells.** (wash 1/5)

AMOUNT

200 µl Additional info:
Triton Washing Solution

Fixing/Permeabilizing Cells

Step 28.

Allow wash to shake on a plate shaker for 5 minutes at RT. (wash 1/5)

DURATION

00:05:00

Fixing/Permeabilizing Cells

Step 29.

Remove wash manually. (wash 1/5)

Fixing/Permeabilizing Cells

Step 30.

Using a multi-channel pipettor, add 200 µL of *Triton Washing Solution* (RT). **Make sure to add the solution down the sides of the wells carefully to avoid detaching the cells.** (wash 2/5)

AMOUNT

200 µl Additional info:
Triton Washing Solution

Fixing/Permeabilizing Cells

Step 31.

Allow wash to shake on a plate shaker for 5 minutes at RT. (wash 2/5)

DURATION

00:05:00

Fixing/Permeabilizing Cells

Step 32.

Remove wash manually. (wash 2/5)

Fixing/Permeabilizing Cells

Step 33.

Using a multi-channel pipettor, add 200 µL of *Triton Washing Solution* (RT). **Make sure to add the solution down the sides of the wells carefully to avoid detaching the cells.** (wash 3/5)

AMOUNT

200 µl Additional info:
Triton Washing Solution

Fixing/Permeabilizing Cells

Step 34.

Allow wash to shake on a plate shaker for 5 minutes at RT. (wash 3/5)

DURATION

00:05:00

Fixing/Permeabilizing Cells

Step 35.

Remove wash manually. (wash 3/5)

Fixing/Permeabilizing Cells

Step 36.

Using a multi-channel pipettor, add 200 µL of *Triton Washing Solution* (RT). **Make sure to add the solution down the sides of the wells carefully to avoid detaching the cells.** (wash 4/5)

AMOUNT

200 µl Additional info:
Triton Washing Solution

Fixing/Permeabilizing Cells

Step 37.

Allow wash to shake on a plate shaker for 5 minutes at RT. (wash 4/5)

DURATION

00:05:00

Fixing/Permeabilizing Cells

Step 38.
Remove wash manually. (wash 4/5)

Fixing/Permeabilizing Cells

Step 39.
Using a multi-channel pipettor, add 200 µL of *Triton Washing Solution* (RT). **Make sure to add the solution down the sides of the wells carefully to avoid detaching the cells.** (wash 5/5)

AMOUNT

200 µl Additional info:
Triton Washing Solution

Fixing/Permeabilizing Cells

Step 40.
Allow wash to shake on a plate shaker for 5 minutes at RT. (wash 5/5)

DURATION

00:05:00

Fixing/Permeabilizing Cells

Step 41.
Remove wash manually. (wash 5/5)

Blocking Cells

Step 42.
Using a multi-channel pipettor, block cells/wells by adding 50 µL of Odyssey® Blocking Buffer to each well. **Add the solution by pipetting down the sides of the wells carefully to avoid detaching the cells.**

AMOUNT

50 µl Additional info:
Odyssey® Blocking Buffer
per well

NOTES

James Thornton Jr 11 Jan 2017

No single blocking reagent will be optimal for every antigen-antibody pair. Some primary antibodies may exhibit greatly reduced signal or different non-specific binding 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 infrared fluorescent detection.

James Thornton Jr 11 Jan 2017

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 reused for more than a few days. If using casein, a 0.1% solution in 0.2X PBS buffer is recommended (Hammarstengrade casein is not required).

James Thornton Jr 11 Jan 2017

Blocking solutions containing BSA can be used, but in some cases 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.

Blocking Cells

Step 43.

Allow blocking for 90 minutes at RT with moderate shaking on a plate shaker.

DURATION

01:30:00

Primary Antibodies

Step 44.

Add the two primary antibodies into a tube containing Odyssey® Blocking Buffer. Choose one of the following primary antibody pairs:

- Phospho-EGFR Tyr1045 (Rabbit; 1:100 dilution; Cell Signaling Technology, P/N 2237) | Total EGFR (Mouse; 1:500 dilution; Biosource International, P/N AHR5062)
- Phospho-EGFR Tyr1045 (Rabbit; 1:100 dilution; Cell Signaling Technology, P/N 2237) | Total ERK2 (Mouse; 1:75 dilution; Santa Cruz Biotechnology, P/N SC-1647)
- Phospho-ERK (Mouse; 1:100 dilution; Santa Cruz Biotechnology, P/N SC-7383) | Total ERK1 (Rabbit; 1:200 dilution; Santa Cruz Biotechnology, P/N SC-94)
- Phospho-EGFR Tyr1045 (Rabbit; 1:100 dilution; Cell Signaling Technology, P/N 2237) | Phospho-ERK (Mouse; 1:100 dilution; Santa Cruz Biotechnology, P/N SC-7383)

Primary Antibodies

Step 45.

Mix the primary antibody solution thoroughly before adding to wells.

Primary Antibodies

Step 46.

Remove the 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.

AMOUNT

20 µl Additional info:
primary antibody or
antibodies

Primary Antibodies

Step 47.

- 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.

AMOUNT

50 µl Additional info:
Odyssey Blocking Buffer

Primary Antibodies

Step 48.

Incubate with primary antibody overnight with gentle shaking at RT.

NOTES

James Thornton Jr 11 Jan 2017

For greatest sensitivity, continue incubation overnight at 4 °C with no shaking.

James Thornton Jr 11 Jan 2017

If left overnight, cover the plates to prevent cells from drying out.

Primary Antibodies

Step 49.

Wash the plate 5 times with 1X PBS + 0.1% Tween® 20 for 5 minutes at RT with gentle shaking, using a generous amount of buffer.

Primary Antibodies

Step 50.

Prepare Tween Washing Solution as follows:

1X PBS	995 mL
20% Tween 20	5 mL
1X PBS with 0.1% Tween 20	1,000 mL

Primary Antibodies

Step 51.

Using a multi-channel pipettor, add 200 µL of *Tween Washing Solution* (RT). **Make sure to add solution down the sides of the wells carefully to avoid detaching the cells from the well bottom**. (wash 1/5)

AMOUNT

200 µl Additional info:
Tween Washing Solution

Primary Antibodies

Step 52.

Allow wash to shake on a plate shaker for 5 minutes at RT. (wash 1/5)

DURATION

00:05:00

Primary Antibodies

Step 53.

Remove wash manually.(wash 1/5)

Primary Antibodies

Step 54.

Using a multi-channel pipettor, add 200 µL of *Tween Washing Solution* (RT). **Make sure to add solution down the sides of the wells carefully to avoid detaching the cells from the well bottom**. (wash 2/5)

AMOUNT

200 µl Additional info:
Tween Washing Solution

Primary Antibodies

Step 55.

Allow wash to shake on a plate shaker for 5 minutes at RT. (wash 2/5)

Primary Antibodies

Step 56.

Remove wash manually. (wash 2/5)

Primary Antibodies

Step 57.

Using a multi-channel pipettor, add 200 µL of *Tween Washing Solution* (RT). **Make sure to add solution down the sides of the wells carefully to avoid detaching the cells from the well bottom**. (wash 3/5)

AMOUNT

200 µl Additional info:
Tween Washing Solution

Primary Antibodies

Step 58.

Allow wash to shake on a plate shaker for 5 minutes at RT. (wash 3/5)

DURATION

00:05:00

Primary Antibodies

Step 59.

Remove wash manually. (wash 3/5)

Primary Antibodies

Step 60.
Using a multi-channel pipettor, add 200 µL of *Tween Washing Solution* (RT). **Make sure to add solution down the sides of the wells carefully to avoid detaching the cells from the well bottom**. (wash 4/5)

AMOUNT

200 µl Additional info:
Tween Washing Solution

Primary Antibodies

Step 61.
Allow wash to shake on a plate shaker for 5 minutes at RT. (wash 4/5)

DURATION

00:05:00

Primary Antibodies

Step 62.
Remove wash manually.(wash 4/5)

Primary Antibodies

Step 63.
Using a multi-channel pipettor, add 200 µL of *Tween Washing Solution* (RT). **Make sure to add solution down the sides of the wells carefully to avoid detaching the cells from the well bottom**. (wash 5/5)

AMOUNT

200 µl Additional info:
Tween Washing Solution

Primary Antibodies

Step 64.
Allow wash to shake on a plate shaker for 5 minutes at RT. (wash 5/5)

DURATION

00:05:00

Primary Antibodies

Step 65.
Remove wash manually. (wash 5/5)

Secondary Antibodies

Step 66.
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%.

- IRDye® 680RD Goat anti-Rabbit (1:800 dilution)
- IRDye 800CW Goat anti-Mouse (1:800 dilution)

Or:

- IRDye 680RD Goat anti-Mouse (1:800 dilution)
- IRDye 800CW Goat anti-Rabbit (1:800 dilution)

NOTES

James Thornton Jr 11 Jan 2017

Recommended dilution range is 1:200 to 1:1,200.

James Thornton Jr 11 Jan 2017

Avoid prolonged exposure of the dye-labeled secondary antibody vials to light.

James Thornton Jr 11 Jan 2017

NOTE: Use IRDye 800CW secondary antibody to detect phosphorylation and IRDye 680RD secondary antibody to detect total protein.

Secondary Antibodies

Step 67.

Mix the antibody solutions thoroughly and add 20 µL of the secondary antibody solution to each well.

AMOUNT

20 µl Additional info:
secondary antibody
solution

Secondary Antibodies

Step 68.

Incubate for 60 minutes with gentle shaking at RT. Protect plate from light during incubation.

DURATION

01:00:00

Secondary Antibodies

Step 69.

Wash the plate 5 times with 1X PBS + 0.1% Tween 20 for 5 minutes at RT with gentle shaking, using a generous amount of buffer.

NOTES

James Thornton Jr 11 Jan 2017

Protect plate from light during washing.

James Thornton Jr 11 Jan 2017

NOTE: If using an Eppendorf glass-bottom plate (P/N 0030741030), rinse briefly with PBS, then wash once with PBS on a rotator for 5 minutes at RT.

Secondary Antibodies

Step 70.

Using a multi-channel pipettor, add 200 µL of *Tween Washing Solution* at RT (Primary Antibodies, step 50). **Make sure to add solution down the sides of the wells carefully to avoid detaching the cells from the well bottom**. (wash 1/5)

AMOUNT

200 µl Additional info:
Tween Washing Solution

Secondary Antibodies

Step 71.

Allow wash to shake on a plate shaker for 5 minutes at RT. (wash 1/5)

DURATION

00:05:00

Secondary Antibodies

Step 72.

Remove wash manually.(wash 1/5)

Secondary Antibodies

Step 73.

Using a multi-channel pipettor, add 200 µL of *Tween Washing Solution* at RT (VI. Primary Antibodies, step 50). **Make sure to add solution down the sides of the wells carefully to avoid detaching the cells from the well bottom**. (wash 2/5)

AMOUNT

200 µl Additional info:
Tween Washing Solution

Secondary Antibodies

Step 74.

Allow wash to shake on a plate shaker for 5 minutes at RT. (wash 2/5)

DURATION

00:05:00

Secondary Antibodies

Step 75.

Remove wash manually.(wash 2/5)

Secondary Antibodies

Step 76.

Using a multi-channel pipettor, add 200 µL of *Tween Washing Solution* at RT (Primary Antibodies, step 50).

Make sure to add solution down the sides of the wells carefully to avoid detaching the cells from the well bottom. (wash 3/5)

AMOUNT

200 µl Additional info:
Tween Washing Solution

Secondary Antibodies

Step 77.
Allow wash to shake on a plate shaker for 5 minutes at RT. (wash 3/5)

DURATION

00:05:00

Secondary Antibodies

Step 78.
Remove wash manually.(wash 3/5)

Secondary Antibodies

Step 79.
Using a multi-channel pipettor, add 200 µL of *Tween Washing Solution* at RT (Primary Antibodies, step 50). **Make sure to add solution down the sides of the wells carefully to avoid detaching the cells from the well bottom**. (wash 4/5)

AMOUNT

200 µl Additional info:
Tween Washing Solution

Secondary Antibodies

Step 80.
Allow wash to shake on a plate shaker for 5 minutes at RT. (wash 4/5)

DURATION

00:05:00

Secondary Antibodies

Step 81.
Using a multi-channel pipettor, add 200 µL of *Tween Washing Solution* at RT (Primary Antibodies, step 50). **Make sure to add solution down the sides of the wells carefully to avoid detaching the cells from the well bottom**. (wash 5/5)

AMOUNT

200 µl Additional info:
Tween Washing Solution

Secondary Antibodies

Step 82.
Allow wash to shake on a plate shaker for 5 minutes at RT. (wash 5/5)

DURATION

00:05:00

Secondary Antibodies

Step 83.
Remove wash manually.(wash 5/5)

Imaging

Step 84.
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.

Imaging

Step 85.
For best results, scan plate immediately; plates may also be stored at 4 °C up to several weeks (protected from light).

TEMPERATURE

4 °C Additional info:
Storage temperature

Imaging

Step 86.
Before scanning, clean the bottom plate surface and the Odyssey Infrared Imager scanning bed (if applicable) with moist, lint-free paper to avoid obstructions during scanning.

Imaging

Step 87.
Scan plate with detection in both 700 and 800 nm channels using an Odyssey or Aerius System described following:

Instrument	Resolution*	Focus Offset	Scan Quality*	Intensity Setting (700/800)	Scan Time Medium Quality
Odyssey® Classic	169 µm	3.5	medium-lowest	5 / 5	7 min
Odyssey CLx	169 µm	3.5	medium-lowest	5 / 5	7 min
	169 µm	3.5	medium-lowest	AutoScan	16 min
Odyssey Sa	200 µm	3.5	medium-lowest	7 / 7	3 min
Aerius	200 µm	3.5	medium-lowest	7 / 7	3 min

**Higher resolution or scan quality may be used, but scan time will increase.*

Warnings

See SDS for safety and warnings.