

Version 1 Last updated 23 June 2017

# **ab207463**

## **Phospho-EGFR (Y1173)**

### **+ Total In-Cell ELISA Kit**

For the quantitative measurement of total and Y1173 phosphorylated EGFR in adherent and non-adherent cells.

This product is for research use only and is not intended for diagnostic use.

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# 1. Overview

Phospho-EGFR (Y1173) + Total In-Cell ELISA Kit (ab207463) provides a simple, efficient, cell-based method to monitor proteins activated by phosphorylation. This kit is designed specifically to quantify activated (phosphorylated) EGFR and/or total EGFR. Cells are cultured in 96-well plates and stimulated to induce the pathway of interest. Following stimulation, the cells are rapidly fixed to preserve activation-specific protein modifications. Each well is then incubated with a primary antibody that recognizes either phosphorylated EGFR or total EGFR. Subsequent incubation with secondary HRP-conjugated antibody and developing solution provides an easily quantified colorimetric readout. The relative number of cells in each well is then determined using the provided Crystal Violet solution. The 96-well plate format is suitable for high-throughput screening applications.

The Phospho-EGFR (Y1173) + Total In-Cell ELISA Kit (ab207463) contains two 96-well plates and two primary antibodies. The phospho-EGFR (Tyr1173) antibody was raised in rabbit against a synthetic phospho-peptide corresponding to residues surrounding phosphorylated Tyr1173 of human EGF receptor. The phospho-EGFR (Tyr1173) antibody recognizes EGFR only when phosphorylated at Tyr1173. The total-EGFR antibody recognizes EGFR protein regardless of its phosphorylation site. This kit can be used to study phosphorylated EGFR relative to cell number or be used to determine EGFR phosphorylation relative to the total EGFR protein found in the cells. Once the phospho-EGFR and total-EGFR signals have been normalized for cell number, a comparison of the ratio of phosphorylated EGFR to total EGFR for each of the cell growth conditions can be made. The provided total-EGFR antibody can be used as a positive control to demonstrate that the cells contain EGFR, the kit reagents are functional and that the protocol is performed correctly. Also, because fixed cells are stable for several weeks, you can prepare many plates simultaneously and then perform the assay when desired.

Epidermal Growth Factor (EGF) Receptor (EGFR, HER-1, c-ErbB-1) is a member of the Epidermal Growth Factor Receptor family of Receptor Tyrosine Kinases. These cell surface receptors play an important role in the flow of information from the outside of a cell to the inside. Upon binding of EGF to the extracellular domain, the receptor undergoes dimerization and becomes phosphorylated on several tyrosine residues

within the cytoplasmic domain. This results in EGFR activation and increased tyrosine kinase activity toward a variety of intracellular substrates. Autophosphorylation of tyrosine 845, 992 and 1173 are critical to EGFR signaling. Phosphorylation at tyrosine 992 creates a direct binding site for phospholipase C-g (PLC-g). PLC-g binding to EGFR (Y992) results in the activation of protein kinase C and the subsequent activation of downstream signaling cascades. Phosphorylation at 1173 creates a major binding site for the protein tyrosine phosphatase SHP-1, which can dephosphorylate the EGFR and thereby block EGFR-induced activation of the ERK1/2 signaling pathway. Tyrosine 845 phosphorylation is mediated by integrin engagement and Src, and regulates receptor function and tumor progression. Aberrant regulation of EGFR signaling has been associated with many types of cancer including mammary, ovarian, non-small cell lung, glioblastoma, prostate, pancreas and head and neck. Thus, methods to rapidly quantify receptor activation are in high demand.

The Epidermal Growth Factor Receptor (EGFR, HER-1, c-ErbB-1) is a 170 kDa member of the Epidermal Growth Factor (EGF) Receptor family of Receptor Tyrosine Kinases (RTKs) which includes ErbB-2 (Her2/neu), ErbB-3 (HER3) and ErbB-4 (HER4). These cell-surface receptors are glycoproteins comprised of an extracellular ligand-binding domain, a single transmembrane domain and an intracellular domain with ligand-activated tyrosine kinase activity. These proteins modulate critical cellular processes such as mitogenesis, cell death, angiogenesis and cell differentiation.

Proteins of the EGFR family of RTKs are activated by ligand binding. This results in receptor dimerization, autophosphorylation, activation of downstream signaling molecules and ultimately, lysosomal degradation of the receptor. Natural ligands that activate EGFR are EGF and TGF-alpha.

EGFR activity is modulated to a large extent through phosphorylation of several phosphorylation and autophosphorylation sites.

Autophosphorylation at Tyr992 creates a direct binding site for the SH2 domain of phospholipase C-g (PLC-g). PLC-g binding to EGFR Y992 results in the activation of protein kinase C (PKC) and subsequent activation of the MAP kinase/ERK signaling cascades. These cascades in turn regulate a variety of proteins, including cytosolic proteins involved in protein translation and nuclear proteins such as the transcription factors Elk-1, c-Jun, STAT1 and 3, Ets-1, c-Myc, ER, CREB and PPARg.

Phosphorylation at Tyr1173 permits binding of the N-terminal SH2 domain for the protein tyrosine phosphatase SHP-1. SHP-1 has the capacity to activate the cytoplasmic tyrosine kinase c-Src by dephosphorylation of the inhibitory phosphotyrosine at the C-terminus, an event that may be related to SHP-1-dependent activation of the MAP kinase cascade. Tyrosine 845, a major autophosphorylation site in EGFR, is mediated by integrin engagement and Src, and regulates receptor function and tumor progression. Phosphorylation of Tyr845 in the kinase domain may stabilize the activation loop, maintain the enzyme in an active state and provide a binding surface for substrate proteins. c-Src is involved in phosphorylation of Tyr845.

Aberrant control of EGFR is implicated in development, progression and severity of several human cancers. For example, EGFR overexpression correlates with poor prognosis in breast, ovarian and head/neck cancers. Several mutant forms of the coding gene have also been found. DGEFR, a deletion mutation lacking exons 2-7, is most common and is associated with glioblastoma.

## 2. Protocol Summary

Culture cells and treat as desired



Fix cells by replacing the growth medium with 4% (adherent cells) or 8% (non-adherent cells) formaldehyde in PBS for 20 minutes at RT. Wash cells



Quench endogenous peroxide by incubating in Quenching Buffer for 20 minutes at RT. Wash cells



Incubate with Antibody Blocking Buffer for 1 hour at RT. Wash cells



Incubate with primary antibody overnight at +4°C. Wash cells



Incubate with secondary antibody for 1 hour at RT. Wash cells



Add developing solution, incubate for 2-20 minutes at RT in the dark



Add stop solution and read absorbance at 450 nm within 5 minutes



Crystal Violet cell staining (Optional)

### 3. Precautions

**Please read these instructions carefully prior to beginning the assay.**

- All kit components have been formulated and quality control tested to function successfully as a kit.
- We understand that, occasionally, experimental protocols might need to be modified to meet unique experimental circumstances. However, we cannot guarantee the performance of the product outside the conditions detailed in this protocol booklet.
- Reagents should be treated as possible mutagens and should be handled with care and disposed of properly. Please review the Safety Datasheet (SDS) provided with the product for information on the specific components.
- Observe good laboratory practices. Gloves, lab coat, and protective eyewear should always be worn. Never pipet by mouth. Do not eat, drink or smoke in the laboratory areas.
- All biological materials should be treated as potentially hazardous and handled as such. They should be disposed of in accordance with established safety procedures.
- Sodium Azide and Formaldehyde are highly toxic chemicals. Appropriate safety precautions (gloves and eye protection) should be used. In addition, formaldehyde is highly toxic by inhalation and should be used only in a ventilated hood.

### 4. Storage and Stability

**Store kit at -20°C immediately upon receipt. Kit has a storage time of 1 year from receipt, providing components have not been reconstituted.**

Refer to list of materials supplied for storage conditions of individual components. Observe the storage conditions for individual prepared components in the Materials Supplied section.

## 5. Limitations

- Assay kit intended for research use only. Not for use in diagnostic procedures.
- Do not mix or substitute reagents or materials from other kit lots or vendors. Kits are QC tested as a set of components and performance cannot be guaranteed if utilized separately or substituted.

## 6. Materials Supplied

Item	1 x 96 tests	5 x 96 tests	Storage Condition
Phospho-EGFR (Y1173) antibody	2 x 11 µL	10 x 11 µL	-20°C
Total-EGFR antibody	2 x 11 µL	10 x 11 µL	+4°C
Anti-rabbit HRP-conjugated IgG	2 x 11 µL	10 x 11 µL	+4°C
1X Antibody Blocking Buffer	1 x 22 mL	5 x 22 mL	-20°C
1X Antibody Dilution Buffer	1 x 30 mL	5 x 30 mL	-20°C
10X PBS	1 x 120 mL	5 x 120 mL	RT
10% Triton X-100	1 x 10 mL	5 x 10 mL	RT
Crystal Violet Solution	1 x 22 mL	5 x 22 mL	+4°C
Developing Solution	2 x 11 mL	10 x 11 mL	+4°C
Stop Solution	2 x 11 mL	10 x 11 mL	+4°C
1% SDS Solution	1 x 22 mL	5 x 22 mL	RT
96-well tissue culture plate	2	10	RT
Plate sealing tape	2	10	RT

## 7. Materials Required, Not Supplied

These materials are not included in the kit, but will be required to successfully perform this assay:

- Multi-channel pipettor.
- Multi-channel pipettor reservoirs.
- Rocking platform.
- Parafilm.
- Microplate spectrophotometer capable of reading at 450 nm and at 595 nm for Crystal Violet staining.
- Fresh 10% hydrogen peroxide ( $\text{H}_2\text{O}_2$ ) in  $\text{dH}_2\text{O}$  (3 mL are required).
- 10  $\mu\text{g}/\text{mL}$  poly-L-Lysine (if using non-adherent cells).
- 10% Sodium Azide ( $\text{NaN}_3$ ) in  $\text{dH}_2\text{O}$  (250  $\mu\text{L}$  are required).
- 37% Formaldehyde (2.5 mL are required for adherent cells; 5.0 mL are required for non-adherent cells).

## 8. Technical Hints

- This kit is sold based on number of tests. A 'test' simply refers to a single assay well. The number of wells that contain sample, control or standard will vary by product. Review the protocol completely to confirm this kit meets your requirements. Please contact our Technical Support staff with any questions.
- Selected components in this kit are supplied in surplus amount to account for additional dilutions, evaporation, or instrumentation settings where higher volumes are required. They should be disposed of in accordance with established safety procedures.
- Make sure all buffers and solutions are at room temperature before starting the experiment.
- Samples generating values higher than the highest standard should be further diluted in the appropriate sample dilution buffers.
- Avoid foaming or bubbles when mixing or reconstituting components.
- Avoid cross contamination of samples or reagents by changing tips between sample, standard and reagent additions.
- Ensure plates are properly sealed or covered during incubation steps.
- Make sure you have the right type of plate for your detection method of choice.
- Make sure the heat block/water bath and microplate reader are switched on before starting the experiment.

## 9. Reagent Preparation

- Equilibrate all reagents to room temperature (18-25°C) prior to use.
- Prepare only as much reagent as is needed on the day of the experiment. Capture and Detector Antibodies have only been tested for stability in the provided 10X formulations.
- We provide an excess of buffer components in order to perform one 96-well assay with the phospho-EGFR antibody and one 96-well assay with the total-EGFR antibody.

### 9.1 1X PBS:

Prepare 1X PBS by adding 1 volume of 10X PBS (pH 7.4) to 9 volumes of dH<sub>2</sub>O and mixing thoroughly.

### 9.2 Preparation of Fixing Buffer (4% or 8% Formaldehyde in PBS):

Fixing Buffer is used to fix cells after cell culturing. Prepare by adding formaldehyde to 1X PBS and mixing well. 4% formaldehyde is used with adherent cells; 8% formaldehyde is used with non-adherent cells. The recipe in the Quick Chart for Preparing Buffers is written for use with a stock solution of 37% formaldehyde.

### 9.3 Preparation of Wash Buffer (0.1% Triton X-100 in PBS):

Prepare Wash Buffer by adding the provided 10% Triton X-100 solution to 1X PBS and mixing thoroughly.

### 9.4 Quenching Buffer (Wash Buffer containing 1% H<sub>2</sub>O<sub>2</sub> and 0.1% Sodium Azide):

Quenching Buffer is used to inactivate the cells' endogenous peroxidase activity. Prepare by adding fresh Sodium Azide and fresh hydrogen peroxide to the Wash Buffer.

### 9.5 Blocking Buffer:

Supplied ready-to-use. A small amount of white precipitate may form if thawed in a warm water bath. This does not interfere with buffer function.

### 9.6 Antibody Dilution Buffer:

Supplied ready-to-use. A small amount of white precipitate may form if thawed in a warm water bath. This does not interfere with buffer function.

**9.7 Diluted phospho-EGFR antibody:**

Prepare by diluting the supplied antibody 1/200 in Antibody Dilution Buffer (see the Quick Chart for Preparing Buffers).

**9.8 Diluted total-EGFR antibody:**

Prepare by diluting the supplied antibody 1/200 in Antibody Dilution Buffer (see the Quick Chart for Preparing Buffers).

**9.9 Diluted HRP-conjugated secondary antibody:**

Prepare by diluting the supplied antibody 1/1000 in Antibody Dilution Buffer (see the Quick Chart for Preparing Buffers).

**9.10 Preparation of Developing Solution:**

The Developing Solution must be warmed to room temperature before use. This solution is light sensitive, therefore, we recommend avoiding direct exposure to intense light during storage. The Developing Solution may develop a yellow hue over time. This does not affect product performance. A blue color present in the solution indicates that it has been contaminated and must be discarded. Prior to use, transfer the amount of Developing Solution required for the assay into a secondary container (see the Quick Chart for Preparing Buffers in this section), avoid direct exposure to intense light and leave at room temperature for at least 1 hour. After use, discard any remaining solution that was transferred into the secondary container.

**9.11 Preparation of Stop Solution**

Prior to use, transfer the amount of Stop Solution required for the assay into a secondary container (see the Quick Chart for Preparing Buffers in this section). After use, discard any remaining Stop Solution that was transferred into the secondary container.

**Δ Note:** The Stop Solution is corrosive. Wear personal protective equipment when handling, i.e. lab coat, gloves and eye protection.

**9.12 1% SDS Solution:**

Supplied ready-to-use. 1% SDS Solution is used in the Crystal Violet counting procedure to solubilize cells and release the dye for subsequent quantification at 595 nm.

**9.13 Crystal Violet Solution:**

Supplied ready-to-use. Crystal Violet is used to estimate the relative number of cells in each well. This stain binds to cell nuclei and gives an OD<sub>595</sub> reading that is proportional to cell number.

## Quick Chart for Preparing Buffers

Reagents to prepare	Components	1 well	48 wells	96 wells	192 wells
Fixing Buffer for adherent cells	1X PBS	98 $\mu$ L	4.7 mL	9.41 mL	18.82 mL
	37% Formaldehyde	12 $\mu$ L	576 $\mu$ L	1.15 mL	2.30 mL
Fixing Buffer for non-adherent cells	1X PBS	86 $\mu$ L	4.13 mL	8.26 mL	16.51 mL
	37% Formaldehyde	24 $\mu$ L	1.15 mL	2.30 mL	4.61 mL
Wash Buffer	1X PBS	3.376 mL	162 mL	310 mL	620 mL
	10% Triton X-100	34.1 $\mu$ L	1.64 mL	3.13 mL	6.26 mL
Quenching Buffer	Wash Buffer	97.9 $\mu$ L	4.7mL	9.40 mL	18.8 mL
	10% H <sub>2</sub> O <sub>2</sub>	11 $\mu$ L	528 $\mu$ L	1.06 mL	2.11 mL
	10% Sodium Azide	1.1 $\mu$ L	52.8 $\mu$ L	106 $\mu$ L	211 $\mu$ L
Diluted total-EGFR antibody	Antibody Dilution Buffer	45 $\mu$ L	2160 $\mu$ L	4320 $\mu$ L	-
	Total-EGFR antibody	0.225 $\mu$ L	10.8 $\mu$ L	21.6 $\mu$ L	-
Diluted phospho-EGFR antibody	Antibody Dilution Buffer	45 $\mu$ L	2160 $\mu$ L	4320 $\mu$ L	-
	Phospho-EGFR antibody	0.225 $\mu$ L	10.8 $\mu$ L	21.6 $\mu$ L	-
Diluted HRP-conjugated secondary antibody	Antibody Dilution Buffer	110 $\mu$ L	5280 $\mu$ L	10.56 mL	21.12 mL
	HRP-conjugated secondary antibody	0.11 $\mu$ L	5.28 $\mu$ L	10.56 $\mu$ L	21.12 $\mu$ L
1X PBS (for wash steps)	10X PBS	154 $\mu$ L	7.39 mL	14.11 mL	28.22 mL
	dH <sub>2</sub> O	1.39 mL	66.53 mL	127.01 mL	254.02 mL

## 10. Sample Preparation

### 10.1 Adherent cell protocol:

10.1.1 Seed cells in the 96-well plate so that they will be approximately 80% confluent at the time of fixing, after they have been treated as desired. The growth area in each well of the 96-well plate is 0.32 cm<sup>2</sup>. The provided plates are sterile and treated for tissue culture.

10.1.2 Grow and treat cells as desired.

10.1.3 Fix cells by replacing the growth medium with 100 µL of 4% formaldehyde in PBS. To minimize the escape of formaldehyde vapors, place a 10 cm x 17 cm piece of parafilm over the plate and then cover the plate with the lid. The covered plate can also be placed in a zip-lock bag. Incubate for 20 minutes at room temperature.

**Δ Note:** Formaldehyde is highly toxic. Confine vapors to a chemical hood and wear appropriate gloves and eye protection when using this chemical.

### 10.2 Non-Adherent Cell Protocol:

The protocol is suitable for use with non-adherent cells if the cells are cultured and fixed as follows:

10.2.1 Treat the 96-well culture plate with 10 µg/ml poly-L-Lysine for 30 minutes at 37°C. Wash twice for 5 minutes with PBS.

10.2.2 Seed 17,000 cells/well, or whatever amount is appropriate for your particular cell line.

10.2.3 Grow and treat cells as desired.

10.2.4 Fix cells by replacing the growth medium with 100 µL of 8% formaldehyde in PBS. Incubate for 20 minutes at room temperature.

**Δ Note:** Fixed cells should be stored refrigerated in a zip-lock or heat-sealed bag with the formaldehyde solution in the wells.

## 11. Assay Procedure

- Equilibrate all materials and prepared reagents to room temperature prior to use.
- We recommend that you assay all standards, controls and samples in duplicate.
- Prepare all reagents, working standards, and samples as directed in the previous sections.

### 11.1 Block cells

- 11.1.1 Remove formaldehyde solution and wash cells 3 times with 200  $\mu$ L Wash Buffer. Each wash step should be performed for 5 minutes with gentle shaking.
- 11.1.2 Remove Wash Buffer, add 100  $\mu$ L Quenching Buffer and incubate for 20 minutes at room temperature.
- 11.1.3 Remove Quenching Buffer and wash cells 2 times for 5 minutes each with 200  $\mu$ L Wash Buffer.
- 11.1.4 Remove Wash Buffer, add 100  $\mu$ L Antibody Blocking Buffer and incubate 1 hour at room temperature.

### 11.2 Binding of primary and secondary antibodies

**$\Delta$  Note:** Depending on experiment design, some wells may be incubated with diluted phospho-EGFR antibody, some with total-EGFR antibody and some with secondary antibody alone (negative controls). For negative control wells, incubate with 40  $\mu$ L Antibody Dilution Buffer during primary antibody incubation step.

- 11.2.1 Remove Antibody Blocking Buffer and wash cells 2 times with 200  $\mu$ L Wash Buffer.
- 11.2.2 Remove Wash Buffer, add 40  $\mu$ L of diluted primary antibody (or Antibody Dilution Buffer for negative control wells) and seal plate with sealing tape. Place a 10 cm x 17 cm piece of parafilm over the plate, cover with lid and incubate overnight at 4°C. Be sure that the plate is level and that each well is tightly sealed with the sealing tape to prevent evaporation.  
 **$\Delta$  Note:** In cells known to generate high amounts of phosphorylated EGFR, a 3-hour primary antibody incubation is sufficient. For maximum sensitivity an overnight incubation is recommended.
- 11.2.3 Remove primary antibody, wash cells 3 times for 5 minutes each with 200  $\mu$ L Wash Buffer.

- 11.2.4 Remove Wash Buffer, add 100  $\mu$ L diluted secondary antibody, cover plate with tissue culture plate lid or sealing tape, and incubate 1 hour at room temperature.
- 11.2.5 During this incubation, transfer the amount of Developing Solution required for the assay into a secondary container and leave at room temperature for at least an hour (avoid light).
- 11.3 **Colorimetric detection**
- 11.3.1 Remove secondary antibody, wash cells 3 times for 5 minutes with 200  $\mu$ L Wash Buffer and then 2 times for 5 minutes with 200  $\mu$ L 1X PBS.
- 11.3.2 Remove PBS from plate wells and add 100  $\mu$ L room temperature Developing Solution to each well.
- 11.3.3 Incubate 2-20 minutes at room temperature protected from direct light. Monitor the blue color development until the darkest-staining wells are medium- to dark-blue. Do not overdevelop.
- 11.3.4 Add 100  $\mu$ L Stop Solution. This acidic solution turns the blue color to yellow. Take care with pipetting to ensure that each well is developed for the same amount of time.  
 **$\Delta$  Note:** The Stop Solution is corrosive. Wear personal protective equipment when handling, i.e. lab coat, gloves and eye protection.
- 11.3.5 Read absorbance on a spectrophotometer within 5 minutes at 450 nm with an optional reference wavelength of 655 nm.  
 **$\Delta$  Note:** The phospho-EGFR and total-EGFR antibodies can be used on equivalent cell cultures to determine the effects of various cell treatments on the ratio of phosphorylated EGFR to total EGFR. However, if the signals with the phospho-EGFR antibody and the total-EGFR antibody are identical, one cannot conclude that the treatment resulted in phosphorylation of 100% of the EGFR.
- 11.4 **OPTIONAL - Crystal Violet cell staining**  
Crystal Violet is an intense stain that binds to the cell nuclei and gives an OD<sub>595</sub> reading that is proportional to cell number. If you wish to normalize your readings from above, simply follow the steps below.
- 11.4.1 After reading, wash wells twice with 200  $\mu$ L Wash Buffer and 2 times with 200  $\mu$ L 1X PBS. Tap plates onto paper towels to remove excess liquid from wells and air-dry at room temperature for 5 minutes.

- 11.4.2 Add 100  $\mu$ L Crystal Violet solution to each well and incubate 30 minutes at room temperature.  
**Δ Note:** Crystal Violet is an intense stain. Avoid contact with skin and clothing.
- 11.4.3 Wash wells 3 times with 200  $\mu$ L 1X PBS for 5 minutes each.
- 11.4.4 Add 100  $\mu$ L of 1% SDS Solution to each well and incubate on shaker for 1 hour at room temperature.
- 11.4.5 Read absorbance on a spectrophotometer at 595 nm. If the signals obtained are greater than the range of your spectrophotometer, the signal can be reduced by removing some (*e.g.* 50  $\mu$ L) of the liquid from each well and replacing with an equivalent volume of dH<sub>2</sub>O.
- 11.4.6 The measured OD<sub>450</sub> readings are corrected for cell number by dividing the OD<sub>450</sub> reading for a given well by the OD<sub>595</sub> reading for that well. This relative cell number is then used to normalize each reading from Step 11.3.

## 12. Assay Specificity

The phospho-EGFR (Tyr1173) antibody was raised in rabbit against a synthetic phospho-peptide corresponding to residues surrounding phosphorylated Tyr1173 of human EGF receptor. The phospho-EGFR (Tyr1173) antibody recognizes EGFR only when phosphorylated at Tyr1173. The total-EGFR antibody recognizes EGFR protein regardless of its phosphorylation site.

## 13. Species Reactivity

This kit recognizes phosphorylated and total EGFR from human, mouse and rat origin.

## 14. Troubleshooting

Problem	Reason	Solution
<b>No signal or weak signal in wells incubated with either phospho-EGFR antibody or total-EGFR antibody.</b>	Omission of key reagent	Check that all reagents have been added in the correct order
	Substrate or conjugate is no longer active	Test conjugate and substrate for activity
	Enzyme inhibitor present	Sodium azide will inhibit the peroxidase reaction, follow our recommendations to prepare buffers
	Plate reader or CCD camera settings not optimal	Verify the wavelength (measurement mode) and filter settings in the plate reader
	Developing Solution was cold	Bring Developing Solution to room temperature
	Inadequate volume of Developing Solution	Check to make sure that correct volume is delivered by pipette
	Cells do not contain detectable levels of phospho EGFR and/or total EGFR	Use Western blotting to confirm that cells contain detectable levels of protein(s) of interest. If you do not require all of the included antibodies, they can be used in Colorimetric Western blotting at a 1/400 dilution for the total and a 1/1000 for the phospho antibody
	Insufficient number of cells were plated	Plate cells so that they are 80% confluent at time of fixing
	Cells did not adhere correctly to plate	Follow protocol for use of non-adherent cells
	Cells are not from correct origin	Refer to species reactivity information
	Excessive washing	Wash steps should be 5 minutes each
	Incubation of secondary antibody was too long	Incubate secondary antibody for 1 hour

<b>High background in all wells</b>	Developing time too long	Stop enzymatic reaction as soon as the positive wells turn medium-dark blue
	Concentration of antibodies too high	Perform antibody titration to determine optimal working concentration. Start using 1/200 for the phospho- and the total-antibody and 1/1000 for the secondary antibody. The sensitivity of the assay will be decreased
	Inadequate washing	Ensure all wells are filled with Wash Buffer and follow washing recommendations
	Inadequate quenching or blocking	Ensure that quenching and blocking steps were performed according to the protocol
<b>Uneven signal development</b>	Incomplete washing of wells	Ensure all wells are filled with Wash Buffer and follow washing recommendations
	Well cross-contamination	Follow washing recommendations
<b>No signal or weak signal in wells incubated with phospho-EGFR antibody</b>	Cell culture conditions did not induce phosphorylation of EGFR	Perform Western blot with phospho-EGFR antibody to confirm that cells contain detectable levels of phosphorylated EGFR
<b>Antibody solution evaporates from well during overnight incubation with primary antibody</b>	Sealing tape was incorrectly applied	Ensure that each well is sealed when sealing tape is applied and ensure that the parafilm sheet covers the plate completely before the lid is placed on the plate. The plate can also be placed in a zip-lock or heat-sealed bag
<b>Insufficient sensitivity</b>	Antibody concentration incorrect	If the cells studied have very low levels of the protein of interest, the sensitivity of detection may be improved by increasing the concentration of primary antibody used and by minimizing the incubation volume. It is possible to

		perform the overnight incubation in as little as 25 $\mu$ L, however, this will make multichannel pipetting difficult and requires the plate be carefully sealed and incubated on a level surface. Alternatively, if the cells have easily detectable levels of the phosphorylated protein and the detection of small changes in phosphorylation is desired, sensitivity of the assay may be improved by decreasing the concentration of the phospho antibody used
<b>Poor precision</b>	Cross-well read through	The 96-well plates provided are designed to minimize signal cross-well contamination. If possible, do not use the phospho and total antibodies in adjoining wells. If this is not possible, use the total antibody at a higher dilution

# 15. Notes

# Technical Support

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