3050 Spruce Street, St. Louis, MO 63103 USA
Tel: (800) 521-8956 (314) 771-5765 Fax: (800) 325-5052 (314) 771-5757
email: techservice@sial.com sigma-aldrich.com

Product Information

Phospho-EGFR (pTyr^{992}) ELISA Kit
for detection of human phospho-EGFR (pTyr^{992}) in cell and tissue lysates

Catalog Number **RAB0169**
Storage Temperature –20 °C

TECHNICAL BULLETIN

Product Description

The Phospho-EGFR (pTyr^{992}) ELISA kit is a very rapid, convenient, and sensitive assay kit that can monitor the activation or function of important biological pathways in human and mouse cell lysates. By determining phosphorylated EGFR protein in an experimental model system, one can verify pathway activation in cell lysates. One can simultaneously measure numerous different cell lysates without spending excess time and effort in performing a Western blot analysis.

This Sandwich ELISA kit is an *in vitro* enzyme-linked immunosorbent assay for the measurement of human and mouse phospho-EGFR. An anti-pan EGFR antibody has been coated onto a 96 well plate. Samples are pipetted into the wells and EGFR present in a sample is bound to the wells by the immobilized antibody. The wells are washed and rabbit anti-phospho-EGFR (pTyr^{992}) antibody is used to detect phosphorylated EGFR. After washing away unbound antibody, HRP-conjugated anti-rabbit IgG is pipetted into the wells. The wells are again washed, a TMB substrate solution is added to the wells and color develops in proportion to the amount of phospho-EGFR (pTyr^{992}) bound. The Stop Solution changes the color from blue to yellow, and the intensity of the color is measured at 450 nm.

Components

1. Pan EGFR Microplate (Item A): 96 wells (12 strips × 8 wells) coated with anti-pan-EGFR.
2. Wash Buffer Concentrate (20x) (Item B): 25 mL of 20× concentrated solution.
3. Positive Control-A431S003-1 (Item K): 1 vial of lyophilized powder from 100 µL of cell lysate.
4. Phospho Detection Antibody EGFR (pTyr^{992}) (Item C-1): 2 vials of rabbit anti-phospho-EGFR (pTyr^{992}) (1 vial is enough to assay half of the microplate).
5. HRP-conjugated anti-rabbit IgG concentrate (Item D-1): 1 vial (25 µL) of 500× concentrated HRP-conjugated anti-rabbit IgG.
6. TMB One-Step Substrate Reagent (Item H): 12 mL of 3,3′,5,5′-tetramethylbenzidine (TMB) in buffer solution.
7. Stop Solution (Item I): 8 mL of 0.2 M sulfuric acid.
8. Assay Diluent (Item E): 15 mL of 5× concentrated buffer. For diluting cell lysate samples, detection antibody (Item C-1), and HRP-conjugated anti-rabbit IgG concentrate.
9. Cell Lysate Buffer (Item J): 10 mL of 2× cell lysis buffer (does not include protease and phosphatase inhibitors).

Reagents and Equipment Required but Not Provided.

1. Microplate reader capable of measuring absorbance at 450 nm
2. Protease and Phosphatase inhibitors
3. Shaker
4. Precision pipettes to deliver 2 µL to 1 mL volumes
5. Adjustable 1-25 mL pipettes for reagent preparation
6. Absorbent paper
7. 100 mL and 1 liter graduated cylinders
8. Distilled or deionized wate.
9. Log-log graph paper or computer and software for ELISA data analysis
10. Tubes to prepare the positive control or sample dilutions

Precautions and Disclaimer

This product is for Research Use Only. Not for Use in Diagnostic Procedures. Please consult the Safety Data Sheet for information regarding hazards and safe handling practices.

Preparation Instructions

Sample Preparation

2× Cell Lysate Buffer should be diluted 2-fold with deionized or distilled water to yield 1× Cell Lysate Buffer (addition of protease and phosphatase inhibitors to 1× Cell Lysate Buffer is recommended prior to sample preparation).

Cell lysates – Rinse cells with PBS, making sure to remove any remaining PBS before adding the lysis buffer. Solubilize cells at 4×10^7 cells/mL in prepared Cell Lysate Buffer (Item J) (see Preparation, step 3). Pipette up and down to resuspend and incubate the lysates with shaking at 2–8 °C for 30 minutes. Microcentrifuge at 13,000 rpm for 10 minutes at 2–8 °C, and transfer the supernatants into a clean test tube. Lysates should be used immediately, or aliquoted and stored at –70 °C. Avoid repeated freeze-thaw cycles. Thawed lysates should be kept on ice prior to use.

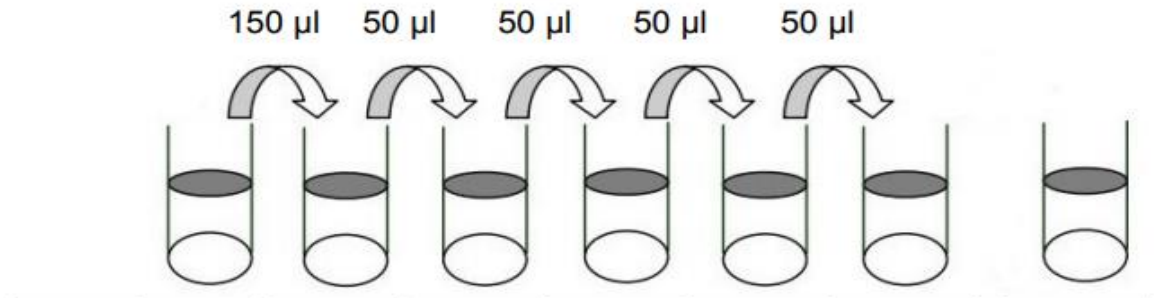
For the initial experiment, a serial dilution, such as a 5-fold to 100-fold dilution, of cell lysates with prepared Assay Diluent (Item E) (see Preparation, step 2) is recommended before use.

Note: The fold dilution of sample used depends on the abundance of phosphorylated proteins and should be determined empirically. More of the sample can be used if signals are too weak. If signals are too strong, the sample can be diluted further.

Reagent Preparation

1. Bring all reagents and samples to room temperature (18–25 °C) before use.
2. 5× Assay Diluent (Item E) should be diluted 5-fold with deionized or distilled water before use.
3. Cell lysate buffer (Item J) should be diluted 2-fold with deionized or distilled water (for cell lysate and tissue lysate). Addition of protease and phosphatase inhibitors (not included) to the lysis buffer prior to use is recommended.
4. Preparation of Positive Control: Briefly spin the Positive Control Vial (Item K). Add 500 µL of prepared 1× Assay Diluent (Item E) into Item K to prepare a Positive Control Stock solution. Gently mix the powder to allow it to dissolve thoroughly. If a precipitate is seen in the solution after mixing, this can be removed by a quick centrifuge of the positive control vial, and then pipetting the supernatant only for the assay. Add 150 µL of the prepared Positive Control Stock Solution from the vial of Item K into a tube with 300 µL of 1× Assay Diluent to prepare Positive Control (P-1). Pipette 450 µL of 1× Assay Diluent into each tube. Use the Positive Control (P-1) solution to produce a dilution series (see Figure 1). Mix each tube thoroughly before the next transfer. 1× Assay Diluent serves as the blank (P-0).

Figure 1.
Dilution Series for Positive Control



	Stock	P-1	P-2	P-3	P-4	P-5	Blank (P-0)
Diluent volume	Item K + 500 µl	300 µl	450 µl	450 µl	450 µl	450 µl	450 µl

5. If the Wash Concentrate (20×) (Item B) contains visible crystals, warm to room temperature and mix gently until dissolved. Dilute 20 mL of Wash Buffer Concentrate into deionized or distilled water to yield 400 mL of 1× Wash Buffer.
6. Preparation of rabbit anti-phospho-EGFR ($pTyr^{992}$) antibody: Briefly spin the vial of rabbit anti-phospho-EGFR ($pTyr^{992}$) (Item C-1). Add 100 µL of 1× Assay Diluent into the vial to prepare a phospho detection antibody concentrate. Pipette up and down to mix gently (the concentrate can be stored at 4 °C for 5 days or at –80 °C for one month). The concentrate should then be diluted 55-fold with 1× Assay Diluent and used in the Procedure, step 4.
7. Preparation of HRP-conjugated anti-rabbit IgG: Briefly spin the vial of HRP-conjugated anti-rabbit IgG concentrate (Item D-1) before use. HRP-conjugated anti-rabbit IgG should be diluted 500-fold with 1× Assay Diluent and used in the Procedure, step 7.

   For example: Briefly spin the vial (Item D-1) and pipette up and down to mix gently. Add 10 µL of HRP-conjugated anti-rabbit IgG concentrate into a tube with 5.0 mL of 1× Assay Diluent to prepare a 500-fold diluted HRP-conjugated anti-rabbit IgG solution.

Storage/Stability

Store the kit at –20 °C. It remains active for up to 1 year. Avoid repeated freeze-thaw cycles.

The reconstituted standard should be stored at –20 °C or –70 °C (–70 °C is recommended). Opened microplate strips or reagents may be store for up to 1 month at 2–8 °C. Return unused wells to the pouch containing desiccant pack and reseal along entire edge.

Procedure

1. Bring all reagents to room temperature (18–25 °C) before use. It is strongly recommended to run all positive controls and samples in at least duplicate.
2. Label removable 8 well strips as appropriate for the experiment.
3. Add 100 µL of positive control (see Preparation, step 4) or sample into appropriate wells. Cover the wells and incubate for 2.5 hours at room temperature or overnight at 4 °C with gentle shaking.
4. Discard the solution and wash 4 times with 1 Wash Solution. Wash by filling each well with Wash Buffer (300 µL) using a multichannel pipette or autowasher. Complete removal of liquid at each step is essential for good performance. After the last wash, remove any remaining Wash Buffer by aspirating or decanting. Invert the plate and blot it against clean paper towels.
5. Add 100 µL of prepared 1× rabbit anti-phospho-EGFR ($pTyr^{992}$) (see Preparation, step 6) to each well. Incubate for 1 hour at room temperature with gentle shaking.
6. Discard the solution. Repeat the wash as in step 4.
7. Add 100 µL of prepared HRP-conjugated anti-rabbit IgG solution (see Preparation, step 7) to each well. Incubate for overnight at 4 °C with gentle shaking.
8. Discard the solution. Repeat the wash as in step 4.
9. Add 100 µL of TMB One-Step Substrate Reagent (Item H) to each well. Incubate for 30 minutes at room temperature in the dark with gentle shaking.
10. Add 50 µL of Stop Solution (Item I) to each well. Read at 450 nm immediately.

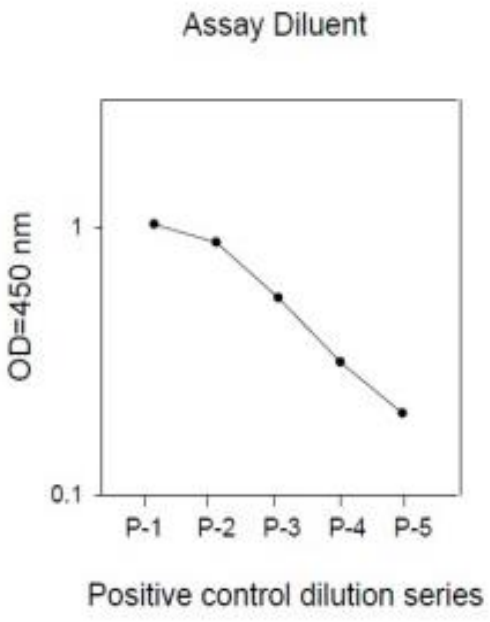
Results

Typical Data

Calculate the mean absorbance for each set of duplicate positive controls and samples, and then subtract the average zero (blank) optical density.

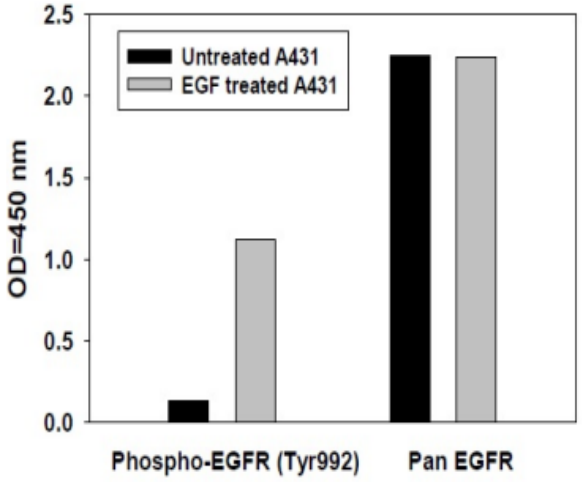
Positive Control:

A431 cells were treated with recombinant human EGF at 37 °C for 20 minutes. Cells were solubilzed at 4×10^7 cells/mL in Cell Lysate Buffer. Serial dilutions of lysates were analyzed using this phosphoELISA kit (see Preparation, step 3).

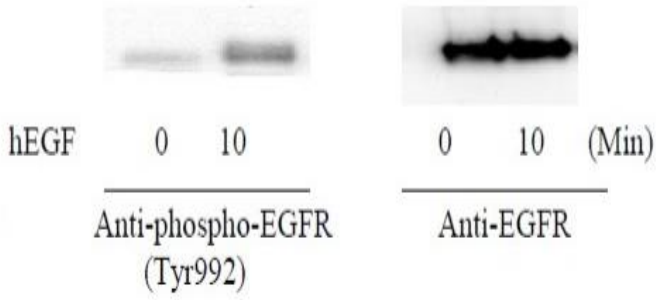


Recombinant Human EGF Stimulation of A431 Cell Lines:
A431 cells were untreated or treated with 100 ng/mL of recombinant human EGF for 10 minutes. Cell lysates were analyzed using this phosphoELISA kit and Western blot.

ELISA

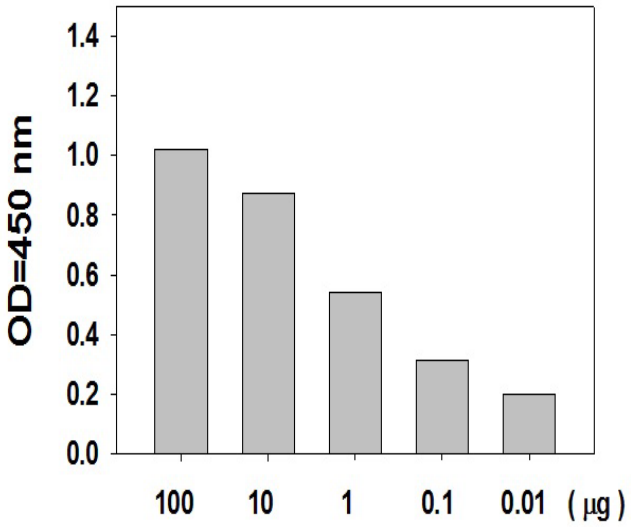


Western blot



Sensitivity
The A431 cells were treated with 100 ng/mL of recombinant human EGF for 20 minutes to induce phosphorylation of EGFR. Serial dilutions of lysates were analyzed using this phosphoELISA kit.

ELISA



Appendix

Troubleshooting Guide

Problem	Cause	Solution
Poor standard curve	Inaccurate pipetting	Check pipettes
	Improper standard dilution	Ensure a brief spin of Item C and dissolve the powder thoroughly with gentle mixing.
Low signal in samples	Sample concentration is too low	Increase sample concentration
	Improper preparation of detection antibody	Briefly spin down vials before opening. Dissolve the powder thoroughly.
	Too brief incubation times	Ensure sufficient incubation time; Procedure, step 3 may change to over night
	Inadequate reagent volumes or improper dilution	Check pipettes and ensure correct preparation
Large CV	Inaccurate pipetting	Check pipettes
	Air bubbles in wells	Remove bubbles in wells
High background	Plate is insufficiently washed	Review the manual for proper wash. If using a plate washer, ensure that all ports are unobstructed.
	Contaminated wash buffer	Make fresh wash buffer
Low sensitivity	Improper storage of the ELISA kit	Store the standard at <–20 °C after reconstitution, others at 4 °C. Keep substrate solution protected from light
	Improper primary or secondary antibody dilution	Ensure correct dilution
	Stop solution	Add stop solution to each well before reading plate.

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