Product Information

Phospho-EGFR (pTyr1068) and pan-EGFR ELISA Kit

For detection of human phospho-EGFR (pTyr1068) and pan-EGFR in cell and tissue lysates

RAB0166

Storage Temperature -20 °C

Product Description

The Phospho-EGFR (pTyr1068) and pan-EGFR ELISA kit is an in vitro enzyme-linked immunosorbent assay for the measurement of human phospho-EGFR (pTyr1068) and pan-EGFR, which helps normalize the results of phospho-EGFR from different cell lysates being compared. An anti-EGFR antibody has been coated onto a 96-well plate. Samples are pipetted into the wells and phosphorylated and pan-EGFR present in a sample are bound to the wells by the immobilized antibody. The wells are washed and either anti-phosphorylated EGFR (pTyr1068) or anti-pan-EGFR antibody is used to detect phosphorylated or non-phosphorylated EGFR. After washing away unbound antibody, HRP-conjugated anti-Rabbit IgG or HRP-Streptavidin is pipetted to 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 EGFR (pTyr1068) or pan-EGFR bound. The Stop Solution changes the color from blue to yellow, and the intensity of the color is measured at 450 nm.

Components

- Capture Antibody-Coated Microplate (Item A) - RABEY1068A: 96-wells (12 strips × 8 wells) coated with monoclonal anti-EGFR
- 20x Wash Buffer Concentrate (Item B) - RABWASH5: 25 mL of 20x concentrated solution
- Anti-Phospho-EGFR (pTyr1068) - specific Antibody Concentrate (Item C) - RABE1068C1: 2 vials rabbit anti-phospho-EGFR (pTyr1068) (1 vial is enough to assay half of the microplate)
- HRP-conjugated Anti-Rabbit IgG Concentrate (Item D1) - RABHRP4: 25 µL of 500x concentrated HRP-conjugated Anti-rabbit IgG
- HRP-Streptavidin (Item G) - RABHRP6: 200 µL of 600-fold concentrated HRP-Streptavidin concentrate
- 5x Assay Diluent (Item E) - RABDIL11: 15 mL of 5x concentrated buffer. For diluting cell lysate, antibody (Item F), and HRP-Streptavidin (Item G) diluent
- TMB One-Step Substrate Reagent (Item H) - RABTMB4: 12 mL of 3, 3′, 5, 5′-tetra-methylbenzidine (TMB) in buffered solution
- Phosphorylation ELISA Stop Solution (Item I) - RABSTOP3: 8 mL of 0.2 M sulfuric acid
- 2x Cell Lysate Buffer (Item J) - RABCLB1: 5 mL of 2x Cell Lysate Buffer (not including protease and phosphatase inhibitors)
- Phospho ELISA Lyophilized Positive Control Sample for Phospho-EFGR (pTyr1068) (Item K) - RABSY1068K

Reagents and Equipment Required

(Not Provided)

- Microplate reader capable of measuring absorbance at 450 nm
- Protease and Phosphatase inhibitors
- Shaker
- Precision pipettes to deliver 2 µL to 1 mL volumes
- Adjustable 1-25 mL pipettes for reagent preparation
- 100 mL and 1 L graduated cylinders
- Distilled or deionized water
- Tubes to prepare sample dilutions

Precautions and Disclaimer

For R&D use only. Not for drug, household, or other uses. Please consult the Safety Data Sheet for information regarding hazards and safe handling practices.

Preparation Instructions

Sample Preparation

2x Cell Lysate Buffer should be diluted 2-fold with deionized or distilled water to yield 1x Cell Lysate Buffer (addition of protease and phosphatase inhibitors to 1x 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 Cell Lysate Buffer. Solubilize cells at 4 107 cells/mL in 1x Cell Lysate Buffer. 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, it is recommended to perform serial dilution testing such as 5-fold and 50-fold dilution for the cell lysates with 1x Assay Diluent (Item E) before use.

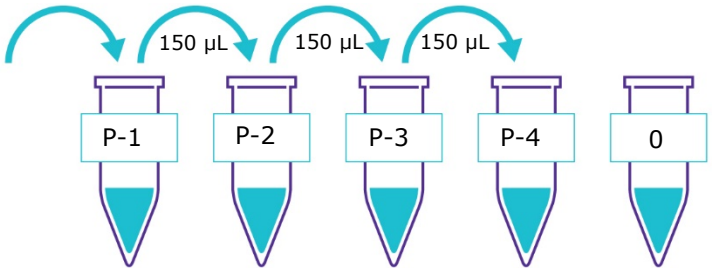
Note: The fold dilution of sample used depends on the abundance of phosphorylated proteins and should be determined empirically. More of the sample can be used if signals are too weak. If signals are too strong, the sample can be diluted further.

Reagent Preparation

1. Bring all reagents and samples to room temperature (18–25 °C) before use.
2. Item E, Assay Diluent should be diluted 5-fold with deionized or distilled water before use.
3. **Preparation of Positive Control**: Briefly spin the Positive Control vial of Item K. Add 400 µL of 1x Assay Diluent (Item E, Assay Diluent should be diluted 5-fold with deionized or distilled water before use) into Item K vial to prepare a Positive Control (P-1) Solution. Dissolve the powder thoroughly by a gentle mix. Pipette 300 µL of 1x Assay Diluent into each tube. Add 100 µL of prepared Positive Control (P-1) into a tube with 300 µL of 1x Assay Diluent to produce a dilution series (see image below). Mix each tube thoroughly before the next transfer. 1x Assay Diluent serves as the background.

Dilution Series for Positive Control

Positive Control Powder + 500 µL of 1x Assay Diluent



4. If the Wash Concentrate (20x) (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 1x Wash Buffer.
5. Briefly spin the anti-phospho-EGFR (pTyr1068) (Item C) before use. Add 100 µL of 1x Assay Diluent into the vial to prepare a detection antibody concentrate. Pipette up and down to mix gently (the concentrate can be stored at 4 °C for 5 days. It can be used for one month if stored at –80 °C. Avoid repeated freeze-thaw cycles). The detection antibody concentrate should further be diluted 60-fold with 1x Assay Diluent and used in Procedure, step 4.
6. Briefly spin the HRP-conjugated anti-rabbit IgG (Item D-1), before use. Pipette up and down to mix gently. HRP-conjugated anti-rabbit IgG concentrate should be diluted 500-folds with 1x Assay Diluent.
7. Briefly spin the vial of rabbit anti-phospho-EGFR (Tyr1068) (Item C) before use. Add 100 µL of 1x Assay Diluent into the vial to prepare a detection antibody concentrate. Pipette up and down to mix gently (the concentrate can be stored at 4 °C for 5 days. It can be used for one month if store at –80 °C. Avoid repeated freeze-thaw cycles). The detection antibody concentrate should be diluted 200-fold with 1x Assay Diluent and used in Procedure, step 4.
8. Briefly spin the HRP-Streptavidin concentrate vial (Item G) and pipette up and down to mix gently before use since precipitation may form during storage. HRP-Streptavidin concentrate should be diluted 600-fold with 1x Assay Diluent.

   For example: Briefly spin the vial (Item G) and pipette up and down to mix gently. Add 20 mL of HRP-Streptavidin concentrate into a tube with 12 mL of 1x Assay Diluent to prepare a 600-fold diluted HRP-Streptavidin solution (don't store the diluted solution for next day use). Mix well.

Storage/Stability

Store the kit at –20 °C Please use within 1 year from the date of shipment. Avoid repeated freeze-thaw cycles.

After initial use, Wash Buffer Concentrate (Item B), HRP-conjugated Anti-rabbit IgG Concentrate (Item D-1), Assay Diluent (Item E), TMB One-Step Substrate Reagent (Item H), HRP-Streptavidin (Item G), Stop Solution (Item I), Cell Lysate Buffer (Item J) and Anti-Phospho-EGFR (pTyr1068) (Item C) should be stored at 2–8 °C to avoid repeated freeze-thaw cycles.

Return unused wells to the pouch containing desiccant pack, reseal along entire edge, and store at –20 °C.

Reconstituted Positive Control (Item K) should be stored at –70 °C.

Procedure

1. Bring all reagents to room temperature (18–25 °C) before use. It is recommended that all samples or Positive Control should be run at least in duplicate.
2. Add 100 µL of each sample or positive control into appropriate wells. Cover well with plate holder and incubate for 2.5 hours at room temperature or overnight at 4 °C with shaking.
3. Discard the solution and wash 4 times with 1x 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 to 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.
4. Add 100 µL of 1x anti-phospho-EGFR (pTyr^{1068}) (see Preparation, step 5) to corresponding well for detecting phospho-EGFR or 100 µL 1x anti-EGFR (see Preparation, step 7) to corresponding well (this helps normalize the results of phospho-EGFR from different cell lysates being compared) for detecting pan-EGFR. Incubate for 1.5 hour at room temperature with shaking.
5. Discard the solution. Repeat the wash as in step 3.
6. Add 100 µL of 1x HRP-conjugated anti-rabbit IgG (see Preparation, step 6) to detect rabbit anti-phospho-EGFR (pTyr^{1068}) (wells to which anti-phospho-EGFR was added). Add 100 µL of 1x HRP-Streptavidin (see Preparation, step 8) to detect pan-EGFR antibody (corresponding well of adding pan-EGFR antibody). Incubate for 1 hour at room temperature with shaking.
7. Discard the solution. Repeat the wash as in step 3.
8. 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 shaking.
9. Add 50 µL of Stop Solution (Item I) to each well. Read at 450 nm immediately.

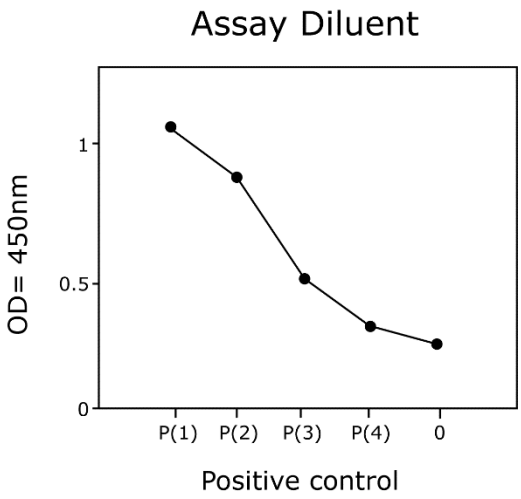
Results

Typical Data

ELISA data analysis: Average the duplicate readings for each sample or positive.

Positive Control

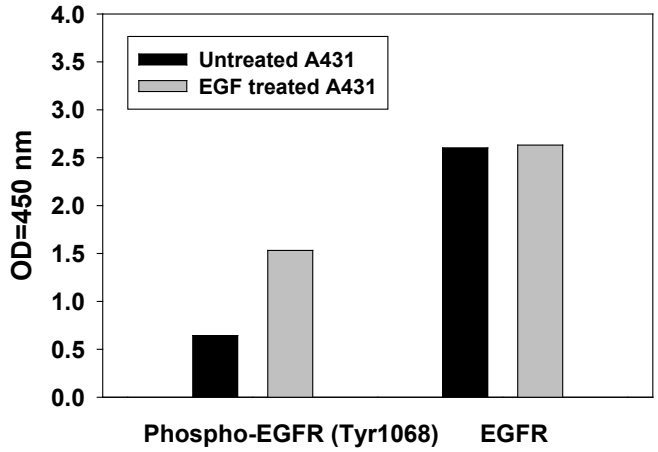
A431 cells were treated with recombinant human EGF at 37 °C for 20 minutes. Solubilize cells at 4 × 10^7 cells/mL in Cell Lysate Buffer. Serial dilutions of cell lysates were analyzed in this ELISA. Please see Preparation, step 3 for detail.



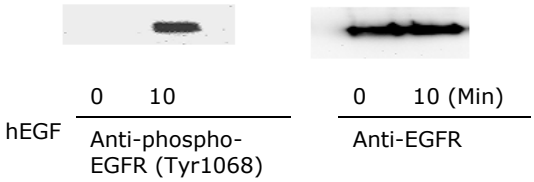
EGF Stimulation of A431 Cell Lines

A431 cells were treated or untreated with 100 ng/mL of recombinant human EGF for 10 minutes. Cell lysates were analyzed using this phospho ELISA and Western blot.

ELISA



Western Blot



References

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2. Zwick, E. et al., Trends Pharmacol. Sci., **20**, 408-412 (1999).
3. Cooper, J.A., and Howell, B., Cell, **73**, 1051-1054 (1993).
4. Riedemann, J. et al., Biochem. Biophys. Res. Commun., **355**, 707 (2007).

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	Too brief incubation times	Ensure sufficient incubation time; Procedure, step 2 may change to over night
	Inadequate reagent volumes or improper dilution	Check pipettes and ensure correct preparation
Large CV	Inaccurate pipetting	Check pipettes
High background	Plate is insufficiently washed	Review the manual for proper wash. If using a plate washer, check 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.
	Stop solution	Stop solution should be added to each well before measurement.

Notice

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