Version 2a Last updated 07 Dec 2023

ab126439 - EGFR (pY1068) + total EGFR Human ELISA

Instructions for Use

For the quantitative measurement of human phosphorylated EGFR (Tyr1068) + total EGFR Human concentrations in cell lysates.

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

Table of Contents

1. Introduction

ab126439 is a very rapid, convenient and sensitive assay kit that can monitor the activation or function of important biological pathways in cell lysates. By determining phosphorylated EGFR protein in your experimental model system, you can verify pathway activation in your cell lysates. You 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 phospho-EGFR (Tyr 1068) and total EGFR (help normalize the results of phospho-EGFR from different cell lysate being compared). An anti-EGFR antibody has been coated onto a 96-well plate. Samples are pipetted into the wells and phosphorylated and total EGFR present in a sample is bound to the wells by the immobilized antibody. The wells are washed and anti-phosphorylated EGFR (Tyr 1068) or anti-total-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 (Tyr 1068) or total EGFR bound. The Stop Solution changes the color from blue to yellow, and the intensity of the color is measured at 450 nm.

2. Assay Summary

Prepare all reagents, samples and standards as instructed.

Add 100 µl sample or positive control to each well. Incubate 2.5 hours at room temperature or overnight at 4°C.

Add 100 µl prepared primary antibody to each well. Incubate 1.5 hours at room temperature.

Add 100 µl prepared secondary antibody solution. Incubate for overnight (corresponding well of adding Rabbit phospho-EGFR) or incubate for 1 hour at room temperature (corresponding well of adding HRP Strepavidin).

Add 100 µl TMB One-Step Substrate Reagent to each well. Incubate for 30 minutes at room temperature.

Add 50 µl Stop Solution to each well. Read at 450 nm immediately.

3. Kit Contents

- Pan EGFR Microplate (Item A): 96 wells (12 strips x 8 wells) coated with monoclonal anti-EGFR antibody.
- Wash Buffer Concentrate (20x) (Item B): 25 ml of 20x concentrated solution.
- Anti-phospho EGFR (Tyr 1068) (Item C): 1 vial of rabbit anti phospho human EGFR (Tyr 1068).
- HRP-conjugated Anti-rabbit IgG (Item D-1): 25 µl of HRP-conjugated anti-rabbit IgG.
- Pan Detection Antibody EGFR: (Item L): 1 vial of Goat anti-pan EGFR
- HRP-Streptavidin concentrate (Item G): 200 µl of 600 fold concentrated HRP-Streptavidin concentrate.
- Assay Diluent (Item E): 15 ml of 5x concentrated buffer. For diluting cell lysate sample, Pan Detection Antibody EGFR (Item L) and HRP-Strepavidin (Item G).
- TMB One-Step Substrate Reagent (Item H): 12 ml of 3,3',5,5'-tetramethylbenzidine (TMB) in buffered solution.
- Stop Solution (Item I): 8 ml of 0.2 M sulfuric acid.

- Cell Lysis Buffer (Item J): 5 ml 2x cell lysis buffer (not including protease and phosphatase inhibitors).

- Positive Control (Item K): 1 vial of lyophilized powder from A431 cell lysate.

4. Storage and Handling

Upon receipt, the kit should be stored at –20°C. After initial use, Wash Buffer Concentrate (Item B), HRP-conjugated Anti-rabbit IgG (Item D-1), Assay Diluent (Item E), TMB One-Step Substrate Reagent (Item H), Stop Solution (Item I), Cell Lysis Buffer (Item J) and Pan Detection Antibody EGFR (Item L) should be stored at 4°C to avoid repeated freeze-thaw cycles. Anti-phospho EGFR (Tyr 1068) (Item

C) and HRP-Strepavidin concentrate (Item G) should be stored at –20°C. 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 -80 °C.

5. Additional Materials Required

- 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.

6. Preparation of Samples

Cell lysates:

Cell Lysis Buffer should be diluted 2-fold with deionized or distilled water before use (recommend to add protease and phosphatase inhibitors).

Rinse cells with PBS, making sure to remove any remaining PBS before adding the Cell Lysis Buffer. Solubilize cells at 4 x 107 cells/ml in 1x Cell Lysis Buffer (we recommend adding protease and phosphatase inhibitors to Cell Lysis Buffer prior to sample preparation). 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 supernates 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, we recommend to do a serial dilution testing such as 5-fold and 50-fold dilution for your cell lysates with 1x Assay Diluent (Item E) before use.

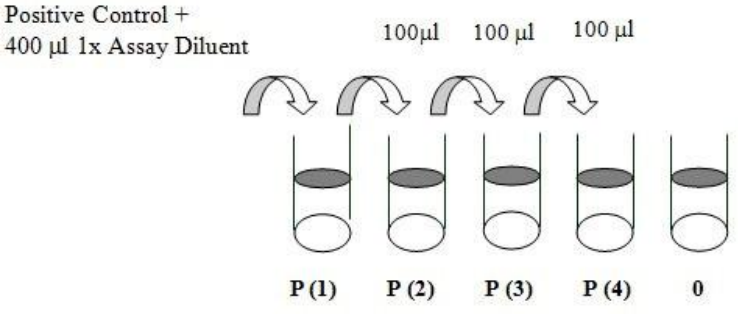
Note: The fold dilution of sample used depends on the abundance of phosphorylated proteins and should be determined empirically. More of the sample can be used if signals are too weak. If signals are too strong, the sample can be diluted further.

7. Preparation of Reagents

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 (Item K). Add 400 µl 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 Positive Control P-1 (See i. Positive Control of Data Analysis section for a typical result). Dissolve the powder thoroughly by a gentle mix. Label 4 additional tubes P-2, P-3, P-4 and 0. Pipette 300 µl 1x Assay Diluent into each tube. Add 100 µl prepared Positive Control P-1 into tube P-2 with 300 µl 1x Assay Diluent to produce a dilution series (shown below). Mix each tube thoroughly before the next transfer. Standard 0 (1x Assay Diluent) serves as the background.



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 (Tyr 1068) (Item C) before use. Add 100ul of 1x Assay Diluent into the vial to prepare a stock solution. 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 stock solution should then be diluted 55-fold with 1x Assay Diluent for use in step 4 of the Assay Procedure.

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-fold with 1x Assay Diluent.

7. Briefly spin the Pan Detection Antibody EGFR vial (Item L) before use. Add 100 µl of 1x Assay Diluent to the vial to prepare the Pan Detection Antibody EGFR stock solution. Pipette up and down to mix gently (the stock solution can be stored at 4°C for 5 days. It can be used within one month if stored at -80°C. Avoid repeated freeze-thaw cycles). The Pan Detection Antibody EGFR concentrate should be diluted 55-fold with 1x Assay Diluent and used as described in Step 4 of the Assay Procedure.

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. A*dd 20µl of HRP-Streptavidin concentrate into a tube with 12 ml 1x Assay Diluent to prepare a 600-fold diluted HRP-Streptavidin solution (don't store the diluted solution for next day use). Mix well.*

9. Cell Lysis Buffer should be diluted 2-fold with deionized or distilled water before use (recommend to add protease and phosphatase inhibitors).

8. Assay Method

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. It is also recommended to run the positive controls in singlet for each of the pan and phospho-specific antibodies.

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 multi-channel 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 prepared 1x rabbit anti-phospho-human EGFR (Tyr 1068) (Preparation of Reagents step 5) to corresponding wells designated to detect phosphorylated protein. Add 100 µl of prepared Pan Detection Antibody EGFR (Preparation of Reagents step 7) to the remaining wells to detect pan protein (total EGFR). Incubate for 1.5 hour at room temperature with gentle shaking.

5. Discard the solution. Repeat the wash as in step 3.

6. Add 100 µl of the prepared 500-fold diluted HRP-conjugated anti-rabbit IgG (see Preparation of Reagents step 6) to the wells corresponding with rabbit anti-phospho-human EGFR (Tyr1068) in order to detect phosphorylated protein. To the remaining wells (corresponding with goat anti-pan-EGFR), add 100 µl of prepared 600-fold diluted HRP-Streptavidin solution (see Reagent Preparation step 8) in order to detect pan protein. Incubate for 1 hour at room temperature with gentle 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.

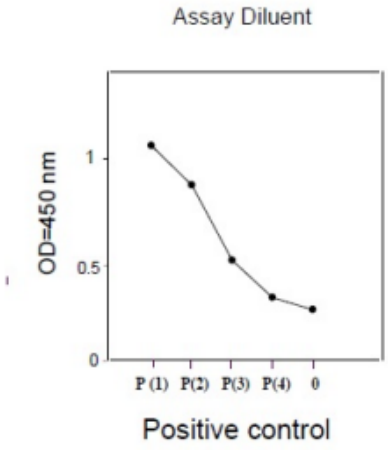
9. Add 50 µl of Stop Solution (Item I) to each well. Read at 450 nm immediately. ELISA data analysis: Average the duplicate readings for each sample or positive control then subtract the average blank optical density.

10. Data Analysis

ELISA data analysis: Average the duplicate readings for each sample or positive control then subtract the average blank optical density.

i. Positive Control

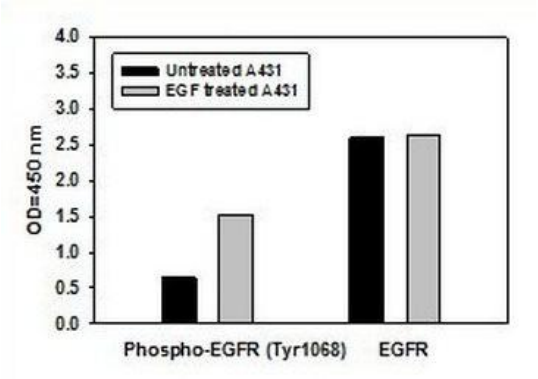
A431 cells were treated with recombinant human EGF at 37°C for 10 min. Solubilize cells at 4 x 107 cells/ml in Cell Lysis Buffer. Serial dilutions of lysates were analyzed in this ELISA. Please see step 3 of Preparation of Reagents for detail.



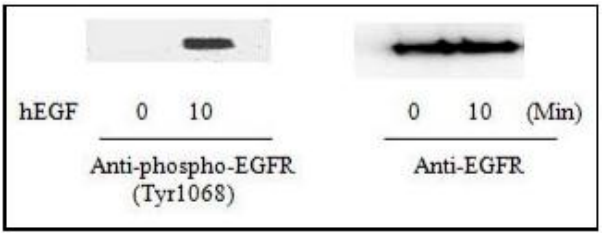
ii. Recombinant Human EGF Stimulation of A431 Cell Lines

A431 cells were treated or untreated with 100 ng/ml recombinant human EGF for 10 min. Cell lysates were analyzed using this phosphoELISA and Western Blot.

a) ELISA



b) Western Blot Analysis



11. Troubleshooting

Problem	Cause	Solution
Low signal in samples	- Sample concentration is too low - Improper preparation of detection antibody - Too brief incubation times - Inadequate reagent volumes or improper dilution	- Increase sample concentration - Briefly spin down vials before opening. Dissolve the powder thoroughly. - Ensure sufficient incubation time; assay procedure step 3 may be done overnight - Check pipettes and ensure correct preparation
High signal in samples	- Sample concentration is too high	- Reduce sample concentration
Large CV	- Inaccurate pipetting - Air bubbles in wells	- Check pipettes - Remove bubbles in wells
High background	- Plate is insufficiently washed - Contaminated wash buffer	- Review the manual for proper wash. If using a plate washer, ensure that all ports are unobstructed. - Make fresh wash buffer
Low sensitivity	- Improper storage of the ELISA kit - Stop solution - Improper primary or secondary antibody dilution	- Store your positive control at <-70°C after reconstitution, others at 4°C. Keep substrate solution protected from light. - Add stop solution to each well before reading plate - Ensure correct dilution

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