Version 1 Last updated 24 October 2019

ab126438
Human EGFR (pY1068) ELISA Kit

For the quantitative measurement of Human EGFR (pY1068) in human cell lysates.

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

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1. Overview

Human EGFR ELISA Kit (pY1068) (ab126438) 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 (pY1068). An anti-EGFR antibody has been coated onto a 96-well plate. Samples are pipetted into the wells and phosphorylated and unphosphorylated EGFR present in a sample is bound to the wells by the immobilized antibody. The wells are washed and anti-phosphorylated EGFR (pY1068) antibodies are used to detect phosphorylated EGFR. After washing away unbound antibody, HRP-conjugated anti-Rabbit IgG (secondary antibody) 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 (pY1068) bound. The Stop Solution changes the color from blue to yellow, and the intensity of the color is measured at 450 nm.

2. Protocol Summary

Prepare all reagents, samples, and positive control as instructed.

Add 100 µL positive control or sample to each well. Incubate 2.5 hours at room temperature or overnight at 4°C with gentle shaking. Discard the solution and wash 4 times with 1X Wash Solution.

Add 100 µL prepared detection antibody to each well. Incubate 1.5 hours at room temperature. Discard the solution and wash 4 times with 1X Wash Solution.

Add 100 µL prepared HRP-conjugated solution. Incubate overnight at 4°C with gentle shaking. Discard the solution and wash 4 times with 1X Wash Solution.

Add 100 µL TMB Substrate Reagent to each well. Incubate 30 minutes at room temperature. Discard the solution and wash 4 times with 1X Wash Solution.

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

3. Precautions

Please read these instructions carefully prior to beginning the assay.

- All ELISA 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 handle 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 pipette 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.

4. Storage and Stability

Entire ELISA kit at may be stored at -20°C for up to 6 months from the date of shipment.

Avoid repeated freeze-thaw cycles. For extended storage, it is recommended to store at -80°C.

Observe the storage conditions for individual prepared components in the Reagent Preparation section 9.

5. Limitations

- ELISA 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	Quantity	Storage Condition
500X HRP-conjugated anti-rabbit IgG	25 µl	-20°C
20X Wash Buffer Concentrate	25 ml	-20°C
2X Cell Lysis Buffer	5 ml	-20°C
5X Assay Diluent	15 ml	-20°C
Anti-phospho-EGFR (Y1068)	2 x 6 µl	-20°C
EGFR Microplate (12 strips x 8 wells) coated with monoclonal anti-EGFR	1 unit	-20°C
Positive Control: lyophilized powder from A431 cell lysate	1 vial	-20°C
Stop Solution	8 ml	-20°C
TMB One-Step Substrate Reagent	12 ml	-20°C

7. Materials Required, Not Supplied

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

- Microplate reader capable of measuring absorbance at 450 or 600 nm.
- Deionized water.
- Multi- and single-channel pipettes.
- Tubes for standard dilution or sample dilutions.
- 100 mL and 1 L graduated cylinders.
- Protease and phosphatase inhibitors.

8. Technical Hints

- 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.
- Complete removal of all solutions and buffers during wash steps is necessary to minimize background.
- All samples should be mixed thoroughly and gently.
- Avoid multiple freeze/thaw of samples.
- When generating positive control samples, it is advisable to change pipette tips after each step.
- 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.

9. Reagent Preparation

- Equilibrate all reagents to room temperature (18-25°C) prior to use. The kit contains enough reagents for 96 wells.
- Prepare only as much reagent as is needed on the day of the experiment.

9.1 5X Assay Diluent:

Dilute 5X Assay Diluent 5-fold with deionized or distilled water before use.

9.2 Cell lysis buffer:

Dilute 2-fold with deionized or distilled water (for cell lysate and tissue lysate). We also recommend the addition of protease and phosphatase inhibitors (not included) to the lysis buffer prior to use.

9.3 Anti-phospho-EGFR (Y1068):

Briefly spin the vial of rabbit anti-phospho-human EGFR (Y1068). Add 100 µL of 1X 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 1X Assay Diluent for use in the assay.

9.4 20X Wash Buffer:

If the Wash Concentrate (20X) 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.

9.5 500X HRP-conjugated anti-rabbit IgG:

Briefly spin the vial of HRP-conjugated anti-rabbit IgG concentrate before use. HRP conjugated anti-rabbit IgG should be diluted 500-fold with 1X Assay Diluent.

10. Positive Control Preparation

- Always prepare a fresh set of standards for every use.
- Discard working standard dilutions after use as they do not store well.
- The following section describes the preparation of a standard curve for duplicate measurements (recommended).

10.1 Briefly spin the Positive Control Vial.

10.2 Add 400 µL of prepared 1X Assay Diluent into Positive Control Vial to prepare a Positive Control 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.

10.3 Pipette 300 µL 1X Assay Diluent into each tube.

10.4 Use the Positive Control solution to produce a dilution series. Adding100 µL from #1 to #2, then from #2 to #3, etc.

10.5 Mix each tube thoroughly before the next transfer.

10.6 Tube #6 contains no protein and is the Blank control.

Tube #	Volume to dilute	Volume of Assay Diluent
1	Positive Control Stock Solution	-
2	100 µL of tube #1	300 µL
3	100 µL of tube #2	300 µL
4	100 µL of tube #3	300 µL
5	100 µL of tube #4	300 µL
6 (Control)	-	300 µL

11. Sample Preparation

Cell Lysate Preparation:

- Rinse the cells with PBS, making sure to remove any remaining PBS before adding the lysis buffer.
- Solubilize cells at 4 x 10^7 cells/ml in prepared Cell Lysate Buffer (see Reagent Preparation section). Pipette up and down to resuspend the pellet.
- 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, we recommend a serial dilution, such as a 5-fold to 100-fold dilution, for your cell lysates with prepared Assay Diluent (see Reagent Preparation) before use.

Δ 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.

12. 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.

12.1 Label removable 8-well strips as appropriate for your experiment.

12.2 Add 100 µL of positive control 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.

12.3 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 auto-washer. 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.

12.4 Add 100 µL of prepared 1X rabbit anti-phospho-human EGFR (Y1068) antibody (see Reagent Preparation) into the wells designated to detect phosphorylated protein. Incubate for 1.5 hours at room temperature with gentle shaking.

12.5 Discard the solution. Repeat the wash as in step 12.3.

12.6 Add 100 µL of prepared HRP-conjugated anti-rabbit IgG solution (see Reagent Preparation) to each well. Incubate overnight at 4 °C with gentle shaking.

12.7 Discard the solution. Repeat the wash as in step 12.3.

12.8 Add 100 µL of TMB One-Step Substrate Reagent to each well. Incubate for 30 minutes at room temperature in the dark with gentle shaking.

12.9 Add 50 µL of Stop Solution (Item I) to each well. Read at 450 nm immediately.

13. Calculations

13.1 Calculate the mean absorbance for each set of duplicate standards, controls and samples.
13.2 Subtract the average zero standard optical density.
13.3 Plot the standard curve on log-log, with standard concentration on the x-axis and absorbance on the y-axis.
13.4 Draw the best-fit straight line through the standard points.

14. Typical Data

Typical standard curve – data provided **for demonstration purposes only.** A new standard curve must be generated for each assay performed.

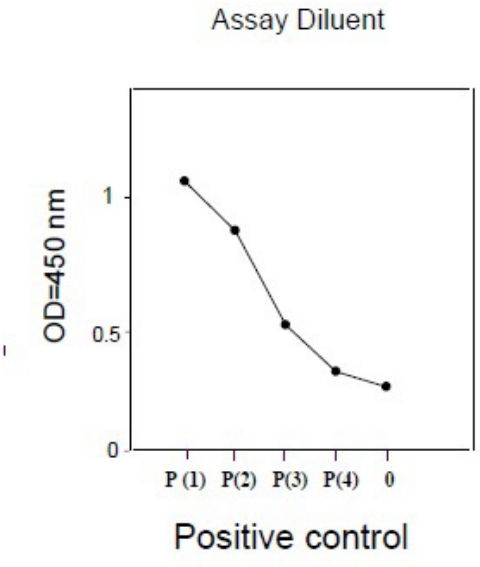

Figure 1. Human EGFR (pY1068) ELISA kit (ab126438) Standard curve.

15. Troubleshooting

Problem	Reason	Solution
Poor standard curve	Inaccurate Pipetting	Check pipettes
	Improper standard dilution	Prior to opening, briefly spin the stock standard tube and dissolve the powder thoroughly by gentle mixing
Low Signal	Improper preparation of standard antibody	Briefly spin down vials before opening. Dissolve the powder thoroughly.
	Too brief incubation times	Ensure sufficient incubation time. Sample and standard addition may be done overnight at 4°C with gentle shaking (note: may increase overall signals including background).
	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 your standard at <-70°C after reconstitution, others at 4°C. Keep substrate solution protected from light.
	Stop solution	Add stop solution to each well before reading plate

16. Notes

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