BioLegend®

Enabling Legendary Discovery™

Human Phospho-EGFR (Tyr845)

ELISA MAX™ *Deluxe Set*

Cat. No. 449804

BioLegend's ELISA MAX™ Deluxe Set contains the components necessary for the accurate quantification of natural and recombinant human phospho-EGFR (Tyr845). The set is designed for cost-effective and accurate quantification of human phospho-EGFR (Tyr845) in cell lysate samples. BioLegend's ELISA MAX™ Deluxe Sets are sensitive, accurate, and robust.

It is highly recommended that this instruction sheet be read in its entirety before using this product. Do not use this set beyond the expiration date.

Materials Provided

1. Hu Phospho-EGFR (Tyr845) ELISA MAX™ Capture Ab (200X)
2. Hu Phospho-EGFR (Tyr845) ELISA MAX™ Detection Ab (200X)
3. Hu Phospho-EGFR (Tyr845) Lyophilized Standard
4. Avidin-HRP (1000X)
5. Substrate Solution F
6. Coating Buffer A (5X)
7. Assay Diluent B (5X)

Introduction

EGFR (Epidermal Growth Factor Receptor) is a member of the ErbB family of cell surface receptors. It is an oncoprotein which has been found to have increased expression and activity in multiple cancer types. However, low activity of EGFR can result in degenerative diseases such as Alzheimer's disease. EGFR signaling pathway starts when it binds to the ligand EGF, resulting in receptor dimerization, transphosphorylation of the C-terminal tails, and propagation of the signal downstream. Each phosphorylation sites can serve as a docking site for different proteins of different signaling pathways. Phosphorylation of tyrosine 845 is associated with regulation of receptor function and tumor progression via c-Src in a Zn^{2+}-dependent manner.

Principle of the Test

BioLegend ELISA MAX™ Deluxe Set contains pre-optimized essential reagents and additional buffers and solutions for Sandwich ELISA assay. A human EGFR specific capture antibody is first coated on a 96-well plate. Standards and samples are added to the wells, and human phospho-EGFR (Tyr845) binds to the immobilized capture antibody. Next, a biotinylated anti-human phospho-EGFR (Tyr845) detection antibody is added, producing an antibody-antigen-antibody "sandwich". The Avidin-HRP reagent is subsequently added, followed by TMB Substrate, producing a blue color in proportion to the concentration of phospho-EGFR (Tyr845) present in wells. Then Stop Solution should be added to wells to terminate the reaction. This step changes the reaction color from blue to yellow. The absorbance in wells should be read at 450nm and 570nm using a microplate reader.

For research purposes only. Not for use in diagnostic or therapeutic procedures.

Materials to be Provided by the End-User

- Microwell plates: BioLegend Cat. No. 423501 is recommended
- Wash Buffer: BioLegend Cat. No. 421601 is recommended, or PBS + 0.05% Tween-20
- Stop Solution: BioLegend Cat. No. 423001 is recommended, or acid solution, e.g. 2N H_2SO_4
- Plate Sealers: BioLegend Cat. No. 423601 is recommended
- PBS (Phosphate-Buffered Saline): 8.0 g NaCl, 1.16 g Na_2HPO_4, 0.2 g KH_2PO_4, 0.2 g KCl, add deionized water to 1 L; pH to 7.4, 0.2 µm filtered
- Deionized (DI) water
- A microplate reader capable of measuring absorbance at 450 nm
- Adjustable pipettes to measure volumes ranging from 2 µL to 1 mL
- Wash bottle or automated microplate washer
- Log-Log graph paper or software for data analysis
- Tubes to prepare standard dilutions
- Timer

Storage Information

- Store kit components between 2°C and 8°C.
- After reconstitution of the lyophilized standard with 1X Assay Diluent B, aliquot into polypropylene vials and store at -70°C for up to one month. Avoid repeated freeze/thaw cycles.
- Prior to use, bring all components to room temperature (18°C-25°C). Upon assay completion return all components to appropriate storage conditions.

Health Hazard Warnings

1. Reagents that contain preservatives may be harmful if ingested, inhaled or absorbed through the skin. Refer to the MSDS online for details (www.biolegend.com/support/#msds).
2. Substrate Solution F is harmful if ingested. Additionally, avoid skin, eye or clothing contact.
3. To reduce the likelihood of blood-borne transmission of infectious agents, handle all serum and/or plasma in accordance with NCCLS regulations.

Specimen Collection and Handling

Cell Lysates: Rinse cells 2 times with PBS, and remove liquid entirely after the second rinse. To ensure high detectability, solubilize at least $6x10^6$ cells / mL in cold lysis buffer (e.g. 1% NP-40 Alternative, 1X Halt Protease/ Phosphotase Inhibitor*, 137 mM NaCl, 20 mM TRIS (pH 8.0), 10% Glycerol, 2 mM EDTA). Vortex then allow to sit on ice for 30 minutes. Centrifuge at 14000 x g for 5 minutes at 4°C to remove insoluble material. Collect the supernatant and then quantify total protein concentration via a protein assay (e.g. Lowry protein assay). Assay immediately or store at -20°C. Avoid repeated freeze/thaw cycles.

*Halt™ Protease and Phosphatase Inhibitor Cocktail (100X): ThermoFisher Cat. No. 78440 is recommended

Reagent and Sample Preparation

Do not mix reagents from different sets or lots. Reagents and/or antibodies from different manufacturers should not be used with this set. All reagents should be diluted immediately prior to use.

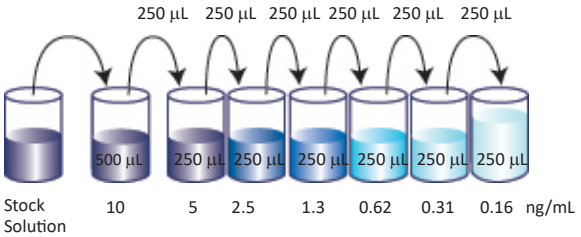
NOTE: Precipitation of 5X Assay Diluent B may be observed when stored long term between 2°C and 8°C. The precipitation does not alter the performance of the Buffer. If heavy precipitation is observed after the dilution to 1X Assay Diluent B, it can be filtered to clarify the solution.

Preparation of 1X Reagent for 1 Plate

Material	Dilute with
2.4 mL of Coating Buffer A (5X)	9.6 mL of Deionized Water
60 µL of Capture Antibody (200X)	12 mL of 1X Coating Buffer A
12 mL of Assay Diluent B (5X)	48 mL of PBS
60 µL of Detection Antibody (200X)	12 mL of 1X Assay Diluent B
12 µL of Avidin-HRP (1,000X)	12 mL of 1X Assay Diluent B

Lyophilized vials are under vacuum pressure. Reconstitute the Human Phospho-EGFR (Tyr845) Lyophilized Standard by adding the volume of 1X Assay Diluent B to make the 50 ng/mL standard stock solution (Refer to ELISA MAX Kit Lot-Specific Certificate of Analysis/ELISA MAX Kit Protocol). Allow the reconstituted standard to sit for 15 minutes at room temperature, then mix gently prior to making dilutions.

Prior to use, prepare 500 µL of the top standard at a concentration of 10 ng/mL from the stock solution in 1X Assay Diluent B. Perform six two-fold serial dilutions of the 10 ng/mL top standard with 1X Assay Diluent B in separate tubes. After diluting, the Human Phospho-EGFR (Tyr845) standard concentrations are 10 ng/mL, 5 ng/mL, 2.5 ng/mL, 1.3 ng/mL, 0.62 ng/mL, 0.31 ng/mL, and 0.16 ng/mL, respectively. 1X Assay Diluent B serves as the zero standard (0 pg/mL).



Samples: To ensure high detectability in cell lysate samples, it is recommended to lyse at least 6 x 10^6 cells/ 1 mL lysis buffer. Perform an assay to measure total protein concentration (e.g. Lowry protein assay). Then, dilute cell lysate samples down to 50 µg/mL total protein concentration before adding to the wells. If more dilution is required, samples should be diluted in 1X Assay Diluent B.

Assay Procedure

Do not use sodium azide in any solutions as it inhibits the activity of the horseradish-peroxidase enzyme.

1. One day prior to running the ELISA, dilute Capture Antibody in 1X Coating Buffer A as described in Reagent Preparation. Add 100 µL of this Capture Antibody solution to all wells of a 96-well plate. Seal plate and incubate overnight (16-18 hrs) between 2°C and 8°C.
2. Bring all reagents to room temperature (RT) prior to use. It is strongly recommended that all standards and samples be run in duplicate or triplicate. A standard curve is required for each assay
3. Wash plate 4 times with at least 300 µL Wash Buffer per well and blot residual buffer by firmly tapping plate upside down on absorbent paper. **All subsequent washes should be performed similarly.**
4. To block non-specific binding and reduce background, add 200 µL 1X Assay Diluent B per well.
5. Seal plate and incubate at RT for 1 hour with shaking on a plate shaker

(e.g. 500 rpm with a 0.3 cm circular orbit). All subsequent incubation with shaking should be performed similarly.

6. While plate is being blocked, prepare the appropriate sample dilutions (if necessary) and standards.
7. Wash plate 4 times with Wash Buffer.
8. Add 100 µL/well of standards or samples to the appropriate wells. If dilution is required, samples should be diluted in 1X Assay Diluent B before adding to the wells.
9. Seal plate and incubate at RT for 2 hours with shaking.
10. Wash plate 4 times with Wash Buffer.
11. Add 100 µL of diluted Detection Antibody solution to each well, seal plate and incubate at RT for 1 hour with shaking.
12. Wash plate 4 times with Wash Buffer.
13. Add 100 µL of diluted Avidin-HRP solution to each well.
14. Seal plate and incubate at RT for 30 minutes with shaking.
15. Wash plate 5 times with Wash Buffer. For this final wash, soak wells in Wash Buffer for 30 seconds to 1 minute for each wash. This will help minimize background.
16. Add 100 µL of TMB Substrate Solution and incubate **in the dark** for 15 minutes*. Positive wells should turn blue in color. It is not necessary to seal the plate during this step.
17. Stop reaction by adding 100 µL of Stop Solution to each well. Positive wells should turn from blue to yellow.
18. Read absorbance at 450 nm within 30 minutes. If the reader can read at 570 nm, the absorbance at 570 nm can be subtracted from the absorbance at 450 nm.

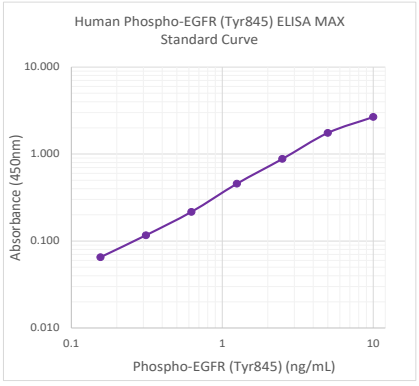
***Optimal substrate incubation time depends on laboratory conditions**

Calculation of Results

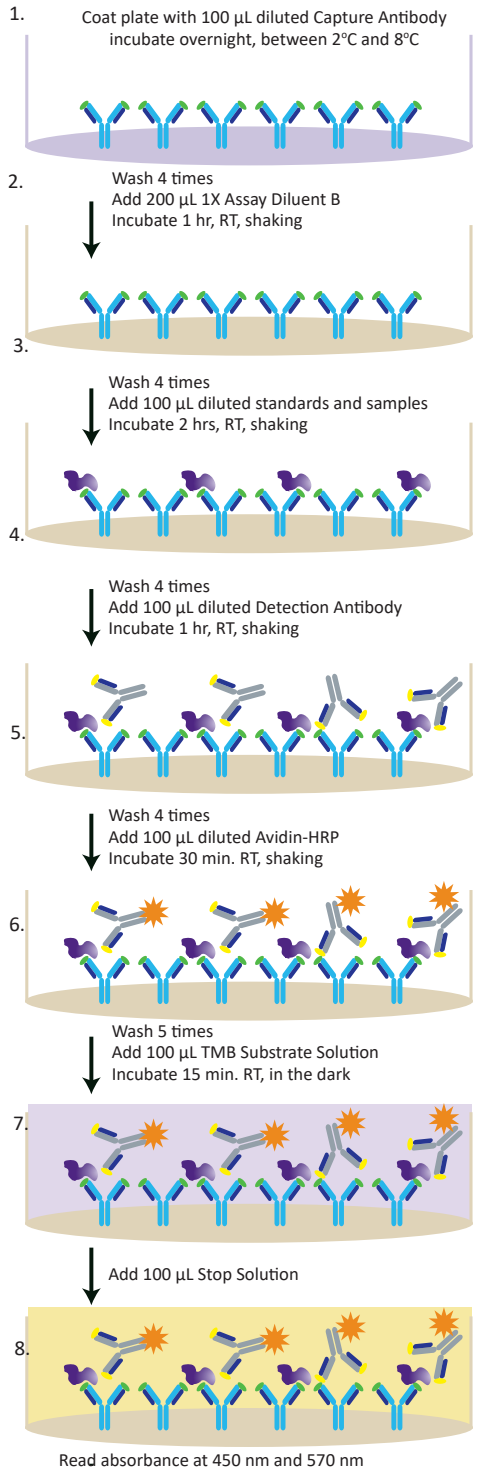
Plot the standard curve on log-log axis graph paper with analyte concentration on the x-axis and absorbance on the y-axis. Draw a best fit line through the standard points. To determine the unknown analyte concentrations in the samples, find the absorbance value of the unknown on the y-axis and draw a horizontal line to the standard curve. At the point of intersection, draw a vertical line to the x-axis and read the corresponding analyte concentration. If the samples were diluted, multiply by the appropriate dilution factor. The data is best calculated with computer-based curve-fitting software using a 5- or 4-parameter logistics curve-fitting algorithm. If a test sample's absorbance value falls outside the standard curve ranges, that test sample needs to be reanalyzed at a higher or lower dilution as appropriate.

Typical Data

Standard Curve: This standard curve was generated at BioLegend for demonstration purposes only. A standard curve must be run with each assay.



Assay Procedure Summary



Performance Characteristics

Sensitivity: The expected minimum detectable concentration of human Phospho-EGFR (Tyr845) for this set is 0.064 ± 0.036 ng/mL.

Specificity: No cross reactivity was observed when this kit was used to analyze unphosphorylated EGFR, as well as less than 1% cross reactivity with phospho-EGFR (Tyr1173).

Troubleshooting

High Background:

- Background wells were contaminated.
- Matrix used had endogenous analyte.
- Plate was insufficiently washed.
- TMB Substrate Solution was contaminated.

No signal:

- Incorrect or no antibodies were added.
- Avidin-HRP was not added.
- Substrate solution was not added.
- Wash buffer contains sodium azide.

Low or poor signal for the standard curve:

- Standard was incompletely reconstituted or was stored improperly.
- Reagents were added to wells with incorrect concentrations.
- Plate was incubated with improper temperature, timing or agitation.

Signal too high, standard curves saturated:

- Standard was reconstituted with less volume than required.
- One or more reagent incubation steps were too long.
- Plate was incubated with inappropriate temperature, timing, or agitation.

Sample readings out of range:

- Samples contain no or below detectable levels of analyte.
- Samples contain analyte concentrations greater than highest standard point.

High variations in samples and/or standards:

- Pipetting errors may have occurred.
- Plate washing was inadequate or nonuniform.
- Samples were not homogenous.
- Samples or standard wells were contaminated.

BioLegend, Inc.

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