Product Datasheet

Biological Description

Specificity	Phospho-EGF Receptor (Thr669) Antibody [H18D23] detects endogenous levels of EGFR protein only when phosphorylated at Thr669.
Background	The epidermal growth factor receptor (EGFR) is a 170-kDa transmembrane glycoprotein and a member of the ErbB family of receptor tyrosine kinases (RTKs), which includes EGFR, ErbB2, ErbB3, and ErbB4. These receptors trigger intracellular signaling pathways such as the mitogen-activated protein kinase (MAPK) and phosphatidylinositol 3-kinase (PI3K)/Akt pathways, which regulate cell proliferation, differentiation, and apoptosis. Phosphorylation of EGFR at Thr669 occurs in response to epidermal growth factor (EGF) binding to its extracellular domain. Thr669 is a key regulatory site within the receptor's cytoplasmic domain, located near two proline residues (-Pro-Leu-Thr669-Pro-). Phosphorylation at Thr669 is mediated by extracellular signal-regulated kinase (ERK) and serves as a negative feedback mechanism, reducing EGFR's tyrosine kinase activity. This regulation prevents excessive signaling by suppressing constitutive tyrosine phosphorylation, helping to control receptor activation and ensuring balanced cellular responses. Additionally, phosphorylation at Thr669 and other sites (e.g., Ser-1046/7) contributes to stress signaling via the ERK and p38 pathways.

Usage Information

Application	WB, IP	Dilution	WB: 1:1000; IP: 1:200
Reactivity	Human		
Source	Rabbit Monoclonal Antibody	MW	175 kDa
Storage Buffer	PBS, pH 7.2+50% Glycerol+0.05% BSA+0.01% NaN_3		
Storage (from the date of receipt)	–20°C (avoid freeze-thaw cycles), 2 years		

Experimental Methods

WB

Experimental Protocol:

Sample preparation
1. Tissue: Lyse the tissue sample by adding an appropriate volume of ice-cold **RIPA/NP-40 Lysis Buffer (containing Protease Inhibitor Cocktail, Phosphatase Inhibitor Cocktail)**，and homogenize the tissue at a low temperature or lyse it by sonication on ice, then incubate on ice for 30 minutes.
2. Adherent cell: Aspirate the culture medium and transfer the cells into an EP tube. Wash the cells with ice-cold PBS twice. Add an appropriate volume of **RIPA/NP-40 Lysis Buffer (containing Protease Inhibitor Cocktail, Phosphatase Inhibitor Cocktail)**, sonicate to lyse the cells, and incubate on ice for 30 minutes.
3. Suspension cell: Transfer the culture medium to a pre-cooled centrifuge tube. Centrifuge and aspirate the supernatant. Wash the cells with ice-cold PBS twice.Add an appropriate volume of **RIPA/NP-40 Lysis Buffer (containing Protease Inhibitor Cocktail, Phosphatase Inhibitor Cocktail)**, sonicate to lyse the cells, and incubate on ice for 30 minutes.
4. Place the lysate into a pre-cooled microcentrifuge tube. Centrifuge at 4°C for 15 min. Collect the supernatant;
5. Remove a small volume of lysate to determine the protein concentration;
6. Combine the lysate with protein loading buffer. Boil 20 μL sample under 95-100°C for 5 min. Centrifuge for 5 min after cool down on ice.

Electrophoretic separation
1. According to the concentration of extracted protein, load appropriate amount of protein sample and marker onto SDS-PAGE gels for electrophoresis. **Recommended separating gel (lower gel) concentration: 5%.** Reference Table for Selecting SDS-PAGE Separation Gel Concentrations
2. Power up 80V for 30 minutes. Then the power supply is adjusted (110 V~150 V), the Marker is observed, and the electrophoresis can be stopped when the indicator band of the predyed protein Marker where the protein is located is properly separated. (Note that the current should not be too large when electrophoresis, too large current (more than 150 mA) will cause the temperature to rise, affecting the result of running glue. If high currents cannot be avoided, an ice bath can be used to cool the bath.)

Transfer membrane
1. Take out the converter, soak the clip and consumables in the pre-cooled converter;
2. Activate PVDF membrane with methanol for 1 min and rinse with transfer buffer;
3. Install it in the order of "black edge of clip - sponge - filter paper - filter paper - glue -PVDF membrane - filter paper - filter paper - sponge - white edge of clip";
4. The protein was electrotransferred to PVDF membrane. (0.45 μm PVDF membrane is recommended) Reference Table for Selecting PVDF Membrane Pore Size Specifications
Recommended conditions for wet transfer: **200 mA, 120 min**.
(Note that the transfer conditions can be adjusted according to the protein size. For high-molecular-weight proteins, a higher current and longer transfer time are recommended. However, ensure that the transfer tank remains at a low temperature to prevent gel melting.)

Block
1. After electrotransfer, wash the film with TBST at room temperature for 5 minutes;
2. Incubate the film in the blocking solution **(recommending 5% BSA solution)** for 1 hour at room temperature;
3. Wash the film with TBST for 3 times, 5 minutes each time.

Antibody incubation
1. Use 5% skim milk powder to prepare the primary antibody working liquid (recommended dilution ratio for primary antibody 1:1000), gently shake and incubate with the film at 4°C overnight;
2. Wash the film with TBST 3 times, 5 minutes each time;
3. Add the secondary antibody to the blocking solution and incubate with the film gently at room temperature for 1 hour;
4. After incubation, wash the film with TBST 3 times for 5 minutes each time.

Antibody staining
946. Add the prepared ECL luminescent substrate (or select other color developing substrate according to the second antibody) and mix evenly;
2. Incubate with the film for 1 minute, remove excess substrate (keep the film moist), wrap with plastic film, and expose in the imaging system.

References

https://pubmed.ncbi.nlm.nih.gov/2543683/ https://pubmed.ncbi.nlm.nih.gov/27531070/

Application Data

WB

Validated by Capsid Bio



Lane 1: A-431 (serum starved overnight),
Lane 2: A-431 (serum starved overnight; EGF, 100 ng/ml, 15 min)