

Product Information

Cell-Based ELISA Kit for detecting phospho-EGFR(pTyr⁸⁴⁵), phospho-EGFR(pTyr⁹⁹²), phospho-EGFR(pTyr^{1,068}), and phospho-EGFR in cultured cell lines

adequate for 192 assays (2 × 96 well plate)

Catalog Number **RAB0159**

Storage Temperature -20 °C

TECHNICAL BULLETIN

Product Description

Protein phosphorylation is instrumental in the regulation of protein activity within a cell. It plays important roles in the living cells including proliferation, differentiation, and metabolism. A large number of protein kinases and phosphatases have been extensively investigated, and have been shown to be involved in signal transduction pathways.

The Cell-Based EGFR ELISA Sampler Kit is a very rapid, convenient, and sensitive assay kit which can monitor the activation or function of important biological pathways in cells. It can be used for measuring the relative amount of EGFR (Tyr⁸⁴⁵), EGFR (Tyr⁹⁹²), EGFR (Tyr^{1,068}) and EGFR (activated) phosphorylation and screening the effect of various inhibitors (such as siRNA or chemicals) or activators **in cultured human, mouse, and rat cell lines. EGFR (Tyr^{1,068}) and EGFR (activated) antibodies only can detect human cell lines.**

By determining specific protein phosphorylation in the experimental model system, pathway activation can be verified in the cell lines without spending excess time and effort in preparing a cell lysate and performing Western Blot analysis. In the Cell-Based EGFR ELISA Sampler Kit, cells are seeded into a 96 well tissue culture plate. The cells are fixed after various treatments, such as inhibitors or activators. After blocking, anti-phosphoprotein specific antibody or anti-pan-protein specific antibody (primary antibody) is pipetted into the wells and incubated. The wells are washed, HRP-conjugated anti-rabbit or mouse IgG (secondary antibody) is added to the wells. The wells are washed again, a TMB substrate solution is added to the wells and color develops in proportion to the amount of protein. The Stop Solution changes the color from blue to yellow, and the intensity of the color is measured at 450 nm.

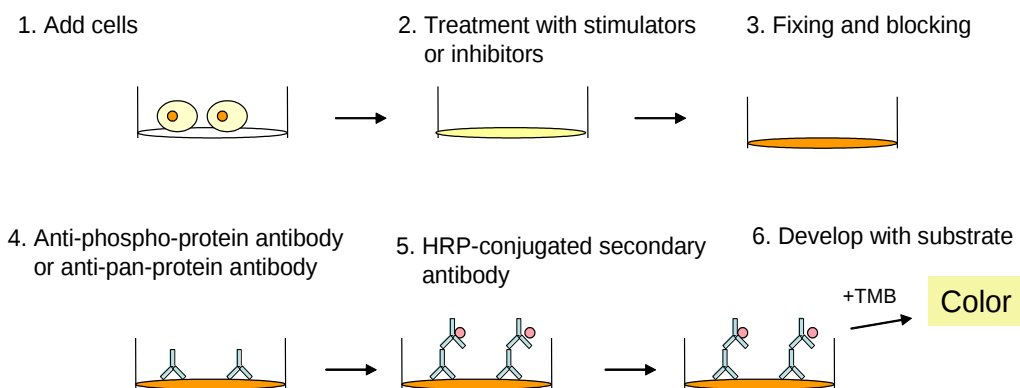


Fig.1. Cell-Based protein phosphorylation procedure

Components

Table 1.

Item	Component	2 Plate Kit	Storage (after initial thaw)*
A	Uncoated Microplate – RABPLATE1	2 plates	Room Temperature
B	20x Wash Buffer Concentrate A – RABWASH1	1 vial (30 mL)	2–8 °C
C	20x Wash Buffer Concentrate B – RABWASH2	1 vial (30 mL)	
D	Fixing Solution - RABFIX1	1 vial (30 mL)	
E	Quenching Solution for Cell-based ELISA Assay - RABQUENCH	1 vial (2 mL)	
F	5x Blocking Solution - RABBLOCK	1 vial (20 mL)	2–8 °C (1 month)
G-1	Phospho-specific Antibody Concentrate - RABE845G (Mouse Anti-phospho (Tyr ⁸⁴⁵) EGFR Concentrate)	1 vial	–20 °C
G-2	Phospho-specific Antibody Concentrate - RABE992G (Rabbit Anti-phospho (Tyr ⁹⁹²) EGFR Concentrate)	1 vial	
G-3	Phospho-specific Antibody Concentrate - RABE1068G (Rabbit Anti-phospho (Tyr ^{1,068}) EGFR Concentrate)	1 vial	
G-4	Phospho-specific Antibody Concentrate - RABEACTG (Mouse Anti-phospho (activated) EGFR Concentrate)	1 vial	
H	Pan EGFR Antibody Concentrate - RABEGFRH	1 vial	
I1	HRP Conjugated Anti-Rabbit IgG Concentrate - RABHRP2	1 vial (10 µL)	
I2	HRP-conjugated Anti-Mouse IgG Concentrate - RABHRP1	1 vial (10 µL)	
J	TMB Substrate Reagent - RABTMB1	2 vials (12 mL/ea)	2–8 °C
K	Stop Solution - RABSTOP1, contains 0.2 M sulfuric acid	1 vial (14 mL/ea)	

*For up to 3 months (unless otherwise stated) or until expiration date.

Reagents and Equipment Required but Not Provided.

1. A model cell line, protein tyrosine kinase inhibitors, growth factors, or cytokines.
2. Microplate reader capable of measuring absorbance at 450 nm.
3. 37 °C incubator.
4. Precision pipettes to deliver 2 µL to 1 mL volumes.
5. Adjustable 1-25 mL pipettes for reagent preparation.
6. 100 mL and 1 liter graduated cylinders.
7. Absorbent paper.
8. Distilled or deionized water.
9. Orbital shaker or oscillating rocker.
10. Poly-L-lysine solution, Catalog Number P4832.

Precautions and Disclaimer

This product is for Research Use Only. Not for Use in Diagnostic Procedures. Please consult the Safety Data Sheet for information regarding hazards and safe handling practices.

Storage/Stability

Upon receipt, the kit should be stored at –20 °C. Please use within 6 months from the date of shipment.

Store Items I1 and I2 at 2–8 °C for up to one month (store at –20 °C for up to 6 months, avoid repeated freeze-thaw cycles).

For storage of components after initial thaw, see Table 1.

Preparation Instructions

Notes: Thaw all reagents to room temperature immediately before use. If wash buffers contain visible crystals, warm to room temperature and mix gently until dissolved.

Briefly centrifuge (~1,000 x g) Items G, H, and I before opening to ensure maximum recovery.

Table 2.

Item	Component	Preparation	Example
A	Uncoated Microplate	No Preparation	N/A
B	20x Wash Buffer Concentrate A	Dilute 20-fold with distilled or deionized water	25 mL of concentrate plus 475 mL of water yields 500 mL of 1x working solution
C	20x Wash Buffer Concentrate B		
D	Fixing Solution	No Preparation	N/A
E	Quenching Solution for Cell-based ELISA Assay	Dilute 30-fold with 1x Wash Buffer A	1 mL of concentrate plus 29 mL of wash buffer yields 30 mL of 1x working solution
F	5x Blocking Solution	Dilute 5-fold with distilled or deionized water	20 mL of concentrate plus 80 mL of water yields 100 mL of 1x working solution
PRIMARY ANTIBODY	G-1 Phospho-specific Antibody Concentrate	1. Reconstitute by adding 100 μL of 1x Blocking Buffer to each vial. 2. Pipette up and down to mix. 3. Dilute reconstituted stock 20-fold with 1x Blocking Buffer	10 μ L of reconstituted stock plus 190 μ L of 1x Blocking Buffer = 200 μ L of 1x working solution
	G-2 Phospho-specific Antibody Concentrate		
	G-3 Phospho-specific Antibody Concentrate		
	G-4 Phospho-specific Antibody Concentrate		
H	Pan EGFR Antibody Concentrate	1. Reconstitute by adding 100 μL of 1x Blocking Buffer to each vial. 2. Pipette up and down to mix. 3. Dilute reconstituted stock 80-fold with 1x Blocking Buffer	10 μ L of reconstituted stock plus 790 μ L of 1x Blocking Buffer = 800 μ L of 1x working solution
SECONDARY ANTIBODY	I1 HRP-conjugated Anti-Rabbit IgG Concentrate	Dilute 2000-fold with 1x Blocking Buffer for Item G3 and Item H	2 μ L of concentrate plus 3,998 μ L of 1x Blocking Buffer = 4 mL of 1x working solution
		Dilute 5000-fold with 1x Blocking Buffer for Item G2	2 μ L of concentrate plus 9,998 μ L of 1x Blocking Buffer = 10 mL of 1x working solution
	I2 HRP-conjugated Anti-Mouse IgG Concentrate	Dilute 2000-fold with 1x Blocking Buffer for Item G1	2 μ L of concentrate plus 3,998 μ L of 1x Blocking Buffer = 4 mL of 1x working solution
		Dilute 1000-fold with 1x Blocking Buffer for Item G4	2 μ L of concentrate plus 1,998 μ L of 1x Blocking Buffer = 2 mL of 1x working solution
J	TMB Substrate Reagent	No preparation	N/A
K	Stop Solution , contains 0.2 M sulfuric acid		

Procedure

Note: All incubations and wash steps must be performed under gentle rocking or rotation (1–2 cycles/sec).

1. Design the experiment, see Figure 2.

the use of vacuum suction to remove solutions from the plate.

4. Add 100 μL of Fixing Solution (Item D) into each well and incubate for 20 minutes at room temperature with shaking. The fixing solution is used to permeabilize the cells.
5. Wash the plate 3 times with 1x Wash Buffer A (200 μL each), then tap the plate upside down to remove all of wash buffer.
6. Add 200 μL of prepared 1x Quenching Buffer (Item E) and incubate 20 minutes at room temperature. The quenching buffer is used to minimize the background response.
7. Wash the plate 4 times with 1x Wash Buffer A (200 μL each), then tap the plate upside down to remove all of wash buffer.
8. Add 200 μL of prepared 1x Blocking Solution (Item F) and incubate for 1 hour at 37 °C.
9. Wash 3 times with prepared 1x Wash Buffer B (200 μL each), then tap the plate upside down to remove all of excess wash buffer.
Note: The plate may be stored at –70 °C for several days.
10. Add 50 μL of 1x primary antibody (Items G1, G2, G3, G4, or H) to the corresponding well and incubate for 2 hours at room temperature with shaking.
11. Wash 4 times with 1x Wash Buffer B (200 μL each), then tap the plate upside down to remove all of excess wash buffer.
12. Add 50 μL of 1x HRP-conjugated secondary antibody (Item I1 or I2) and incubate for 1 hour at room temperature.
Note: Item I1 is the secondary antibody for Items G2, G3, and H (primary antibody). Item I2 is the secondary antibody for Items G1 and G4 (primary antibody).
13. Wash 4 times with 1x Wash Buffer B (200 μL each), then tap the plate upside down to remove all of excess wash buffer.

14. Add 100 μL of TMB Substrate Reagent (Item J) to each well and incubate for 30 minutes with shaking at room temperature in the dark.

15. Add 50 μL of Stop Solution (Item K) to each well and read at 450 nm, measure OD immediately.

Results

Representative results Cell-Based EGFR (Tyr992) are shown:

Notes:

1. In Procedure, step 2 – 20,000 A431 cells were seeded into appropriate wells of the microplate. Cells were incubated at 37 °C in 5% CO₂ overnight.
2. Added 50 μL of different concentrations of stimulators (rhEGF concentration for A431 cells: 0, 20 or 100 ng/mL in serum free DMEM) to appropriate wells (see Figure 3). Then incubated for 10 minutes at 37 °C.
3. Discarded the solution and washed 3 times with 1x Wash Buffer A (200 μL each) immediately. Then tapped the plate upside down to remove all of excess wash buffer and followed with Procedure, steps 4–15.

Figures 3-1 to 3-4.

A431 cells were stimulated by different concentrations of EGF.

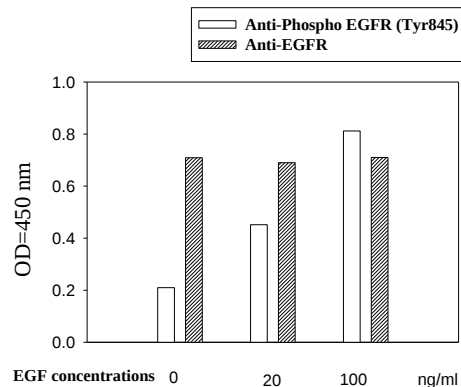


Fig. 3-1. A431 cells were stimulated by different concentrations of EGF for 10 min at 37°C

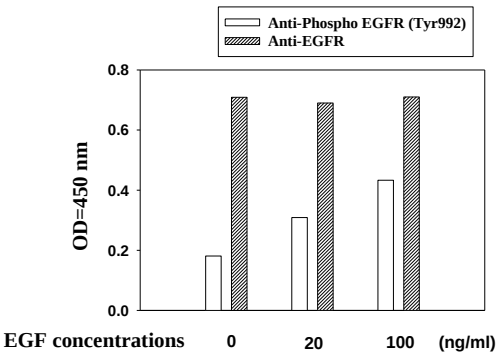


Fig. 3-2. A431 cells were stimulated by different concentrations of EGF for 10 min at 37°C

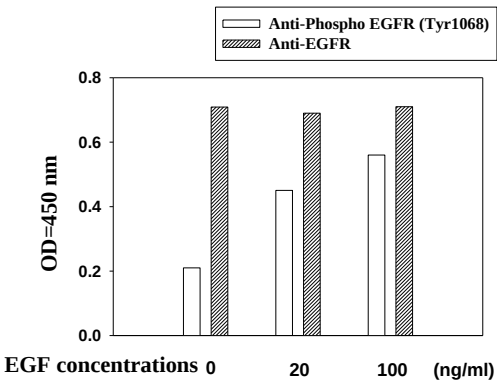


Fig. 3-3. A431 cells were stimulated by different concentrations of EGF for 10 min at 37°C

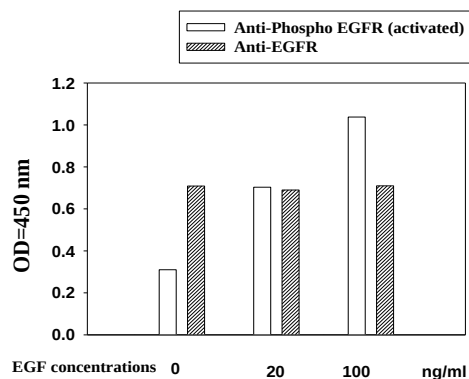


Fig. 3-4. A431 cells were stimulated by different concentrations of EGF for 20 min at 37°C

Western blots

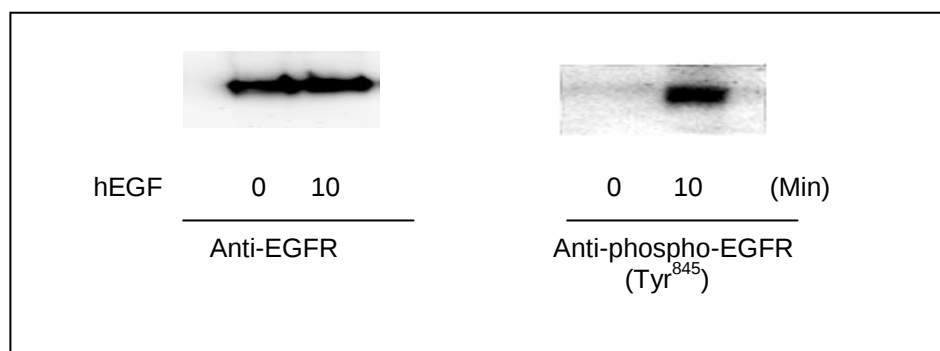


Figure 4-1.

Western blot analysis of extracts from 100 ng/mL hEGF treated A431 cells. Phospho-EGFR (Tyr⁸⁴⁵) and EGFR antibodies were used in both detection assays.

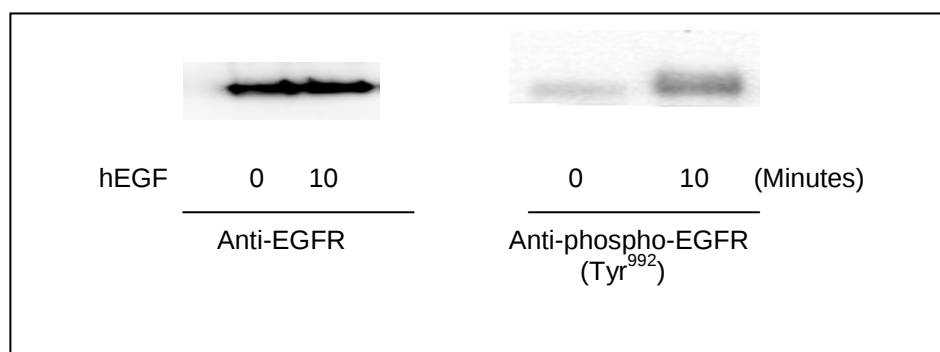


Figure 4-2.

Western blot analysis of extracts from 100 ng/mL hEGF treated A431 cells. Phospho-EGFR (Tyr⁹⁹²) and EGFR antibodies were used in both detection assays

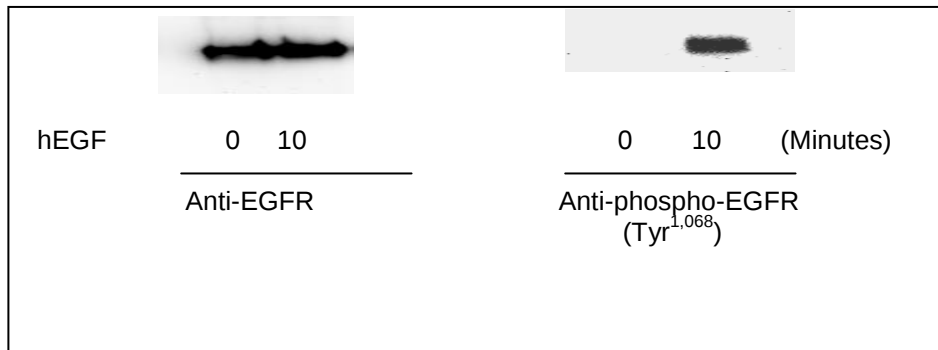


Figure 4-3.

Western blot analysis of extracts from 100 ng/mL hEGF treated A431 cells. Phospho-EGFR (Tyr^{1,068}) and EGFR antibodies were used in both detection assays.

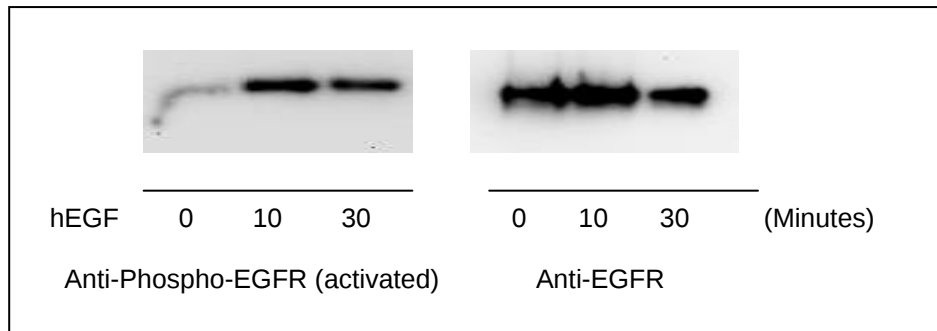


Figure 4-4.

Western blot analysis of extracts from 100 ng/mL hEGF treated A431 cells. Phospho-EGFR (activated) and EGFR antibodies were used in both detection assays.

References

1. Michael J. Clemens and Michael C. in Protein Phosphorylation in Cell Growth Regulation, 1st edition, 1997.
2. Fu, X.Y. et al. Cell, 74, 1135 (1993).
3. Darnell, J.E., Science, 277, 1630 (1997).
4. Kanai, M. et al., Oncogene, 22, 548-554 (2003).
5. Smith, P.D., and Crompton, M.R., Biochem. J., 331, 381 (1998).

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Appendix
Troubleshooting Guide

Problem	Cause	Solution
Low signal	Improper storage of the ELISA kit	Store all of components according to manual instructions. Keep TMB substrate solution in dark
	Improper dilution	Ensure correct preparation of antibody and reagents
	Cells drop off from the wells	Some of treatments may make cells drop off from the wells. Reduce inhibitor or activator concentration.
High background	Inadequate washing	Be sure to remove all of washing solution and follow the recommendation for washing
	Too many cells	Reduce the cell number
Large CV	Inaccurate pipetting	Check pipette
	Remaining wash buffer in the well	Remove all of wash buffer
	Cells drop off from the wells	Please don't directly contact the cells with tips when adding reagents or wash buffer.

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