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Product Information

Cell-Based ELISA Kit for detecting phospho-EGFR in cultured cell lines
adequate for 96 assays (1 × 96 well plate)

Catalog Number **RAB0151**
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 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 (activated) ELISA kit is a very rapid, convenient, and sensitive assay kit that can monitor the activation or function of important biological pathways in cells. It can be used for measuring the relative amount of EGFR (activated) phosphorylation and screening the effects of various treatments, inhibitors (such as siRNA or chemicals), or activators in cultured human cell lines.

By determining EGFR protein phosphorylation in the experimental model system, pathway activation can be verified in the cell lines without spending excess time and effort in preparing cell lysate and performing a Western Blot analysis. In the Cell-Based EGFR (activated) ELISA kit, cells are seeded into a 96 well tissue culture plate. The cells are fixed after various treatments, inhibitors, or activators. After blocking, Anti-Phospho-EGFR (activated) or Anti-EGFR (primary antibody) is pipetted into the wells and incubated. The wells are washed, and HRP-conjugated anti-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.

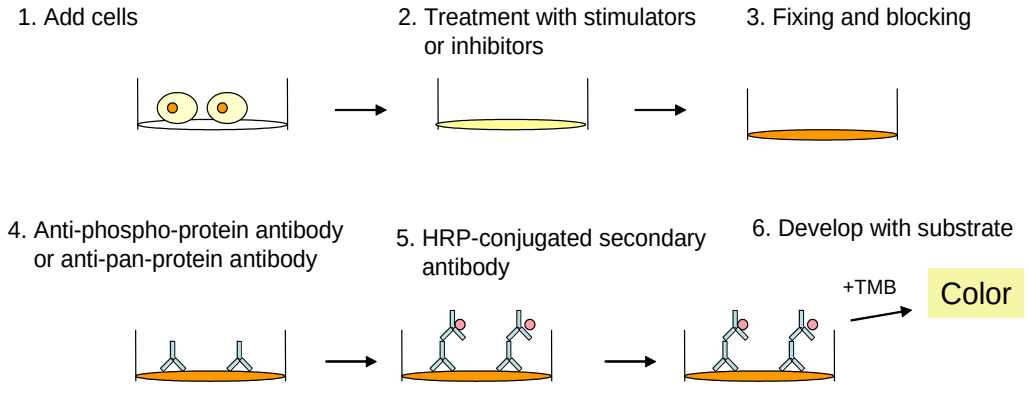


Fig.1. Cell-Based protein phosphorylation procedure

Components

Table 1.

Item	Component	1 Plate Kit	Storage (after initial thaw)*
A	Uncoated Microplate – RABPLATE1	1 plate	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	Phospho-specific Antibody Concentrate – RAB0151	1 vial (10 µL)	–20 °C
H	Pan-EGFR Antibody Concentrate	1 vial (7 µL)	
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	1 vial (12 mL)	2–8 °C
K	Stop Solution - RABSTOP1, contains 0.2 M sulfuric acid	1 vial (14 mL)	

*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 P483.

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.

Item I store 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.

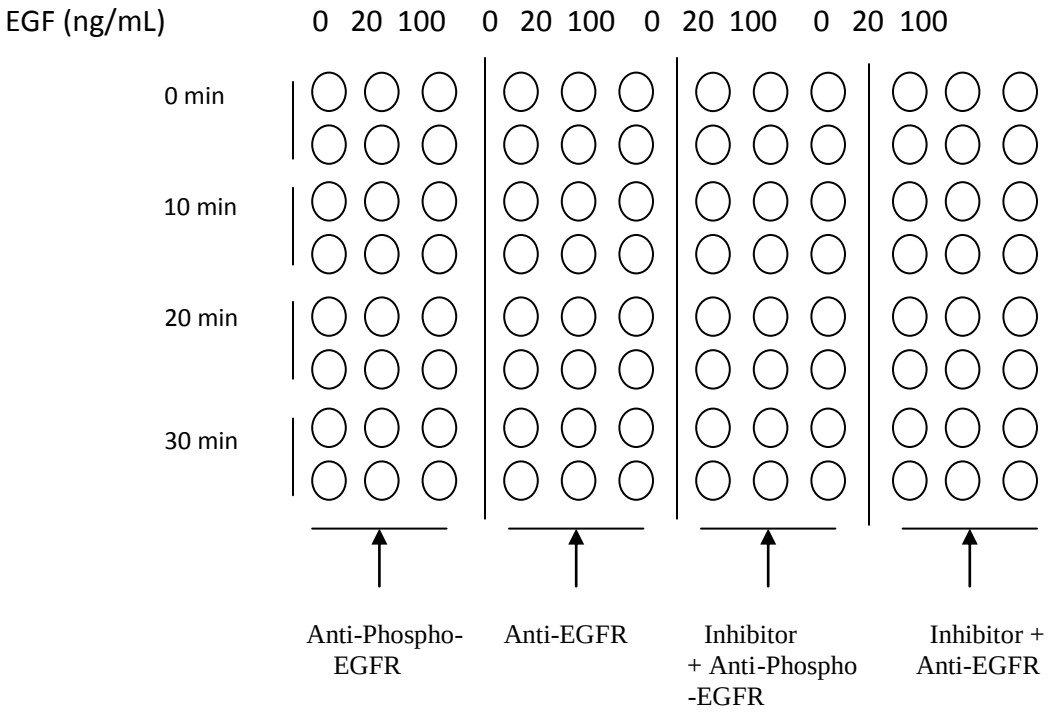
Table 2.

	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	G	Phospho-specific Antibody Concentrate	Dilute 500-fold with 1x Blocking Solution	100 μL of reconstituted stock plus 1,400 μL of 1x Blocking Buffer yields 1.5 mL of 1x working solution
PRIMARY	H	Pan- EGFR Antibody Concentrate	Dilute 1,000-fold with 1x Blocking Solution	10 μL of concentrate plus 9,990 μL of 1x Blocking Solution yields 10 mL of 1x working solution
SECONDARY ANTIBODY	I1	HRP-conjugated Anti-Rabbit IgG Concentrate		
SECONDARY ANTIBODY	I2	HRP-conjugated Anti-Mouse IgG Concentrate		
	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.

Figure 2.
Example of Seeding Cells for Cell-Based Assay



2. Seed 100 µL of 10,000–30,000 cells into each well of a 96 well plate and incubate overnight at 37 °C and 5% CO_2.

   Note: The optimal cell number used is dependent on the cell line and the relative amount of protein phosphorylation. More or less cells may be used.

   Pre-coat the 96 well plate (Item A) by adding 100 µL of poly-L-lysine solution (Catalog Number P4832) into each well and then follow manufacturer's instructions. If seeding HUVECs, HMEC-1, or other loosely attached cells, CellBIND® or poly-L-lysine treated tissue culture plates may be used.

   The cells can be starved 4–24 hours dependent on the cell line prior to treatment with inhibitor or activator.

3. Apply various treatments, inhibitors (such as siRNA or chemicals), or activators according to manufacturer's instructions and incubate. Discard the cell culture medium and wash 3 times with prepared 1x Wash Buffer A (200 µL each). Discard Wash Buffer and then tap the plate upside down to remove all of excess wash buffer.

   Note: Dissolve the inhibitors or activators into serum free cell culture medium and then treat the cells according to manufacturer's instructions.

   To avoid cell loss, do not dispense liquid directly onto the cell surface. Instead, gently touch the pipette tip to the side of the well and gently dispense the liquid down the wall of cell culture wells.

   Flip the plate over a proper receptacle to remove Wash Buffer A and then tap the plate gently onto a paper towel to remove any remaining liquid. Avoid

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 (Item G1 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 Item G (primary antibody). Item I2 is the secondary antibody for Item H (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 are shown:

Note:

1. In Procedure, step 2, A431 cells were seeded into appropriate wells of the microplate. Cells were incubated at 37 °C in 5% CO_2 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, 20, or 30 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.

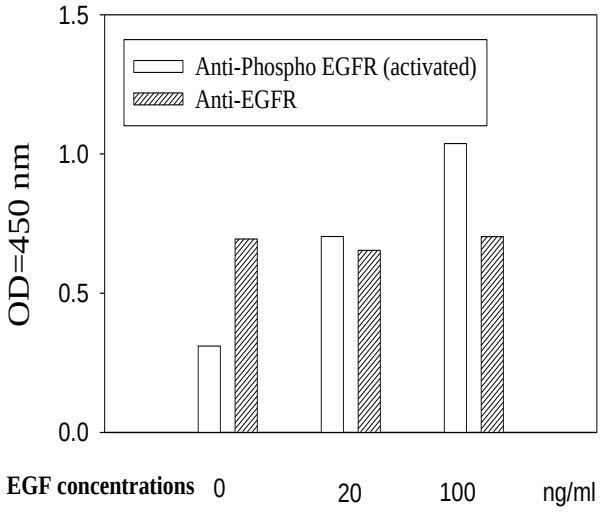


Fig. 3. A431 cells were stimulated by different concentrations of EGF for 20 min at 37^{o}C

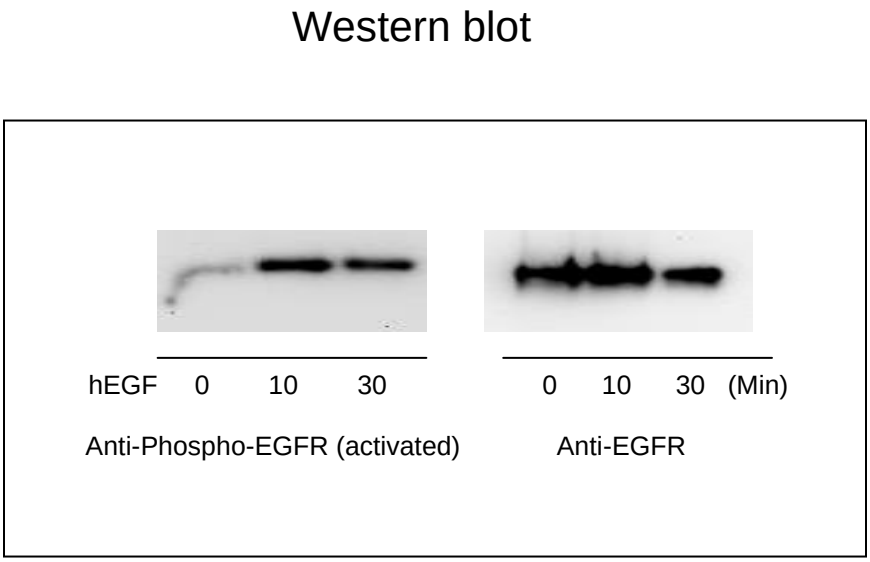


Figure 4.
Western blot analysis of extracts from 100 ng/mL hEGF treated A431 cells. Phospho-EGFR (activated) and EGFR antibodies were used in both detection assays.

References

1. Carpenter, G., Ann. Rev. Biochem., **56**, 881-914 (1987).
2. Hirota, K. et al., J. Biol. Chem., **276**, 25953-25958 (2001).
3. Wang, D. et al., Oncogene, **21**, 2785-2796 (2002).

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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