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# Product Information

**Cell-Based ELISA for detecting phospho-ERK1/2 ($pThr^{202}/pTyr^{204}$) in cultured cell lines**

Catalog Number **RAB0350**
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 Human/Mouse/Rat ERK1/2 ($Thr^{202}/Tyr^{204}$) 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 ERK1/2 ($Thr^{202}/Tyr^{204}$) phosphorylation and screening the effects of various treatments, inhibitors (such as siRNA or chemicals), or activators in cultured human, mouse, and rat cell lines.

By determining ERK1/2 protein phosphorylation in the experimental model system, one can verify pathway activation in the cell lines without spending excess time and effort in preparing cell lysate and performing an analysis of Western blot. In the Cell-Based ERK1/2 ($Thr^{202}/Tyr^{204}$) 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-ERK1/2 ($Thr^{202}/Tyr^{204}$) or Anti-ERK1/2 is pipetted into the wells and incubated. The wells are washed, and HRP conjugated anti-mouse IgG 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.

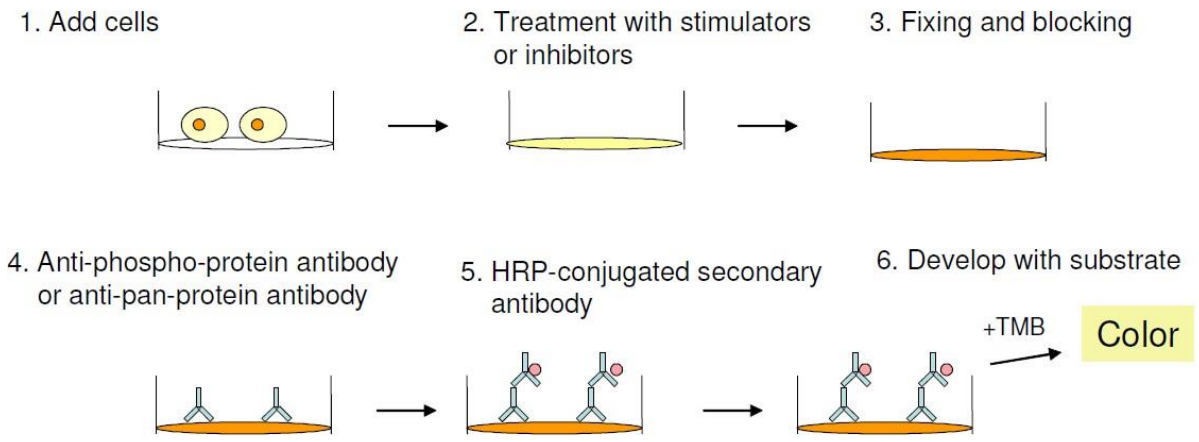



**Fig.1. Cell-Based protein phosphorylation procedure**

**Components**
Adequate components are supplied for 96 assays (96 well plate)

**Table 1.**

| Item | Component | 1 Plate Kit | Storage (after initial thaw)* |
|---|---|---|---|
| A | Uncoated 96 Well Microplate – RABPLATE1 | 1 plate | Room Temperature |
| B | 20× Wash Buffer Concentrate A – RABWASH1 | 1 vial (30 mL) | 2–8 °C |
| C | 20× 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 | 5× Blocking Solution - RABBLOCK | 1 vial (20 mL) | 2–8 °C (1 month) |
| G | Phospho-specific Antibody Concentrate – RABE204G | 1 vial (7 µL) | –20 °C |
| H | Pan ERK Antibody Concentrate - RABERKH | 1 vial (7 µL) | |
| I2 | HRP-conjugated Anti-Mouse IgG Concentrate – RABHRP2 | 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

**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**

Note: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 × *g*) Items G, H, and I before opening to ensure maximum recovery.

**Table 2.**

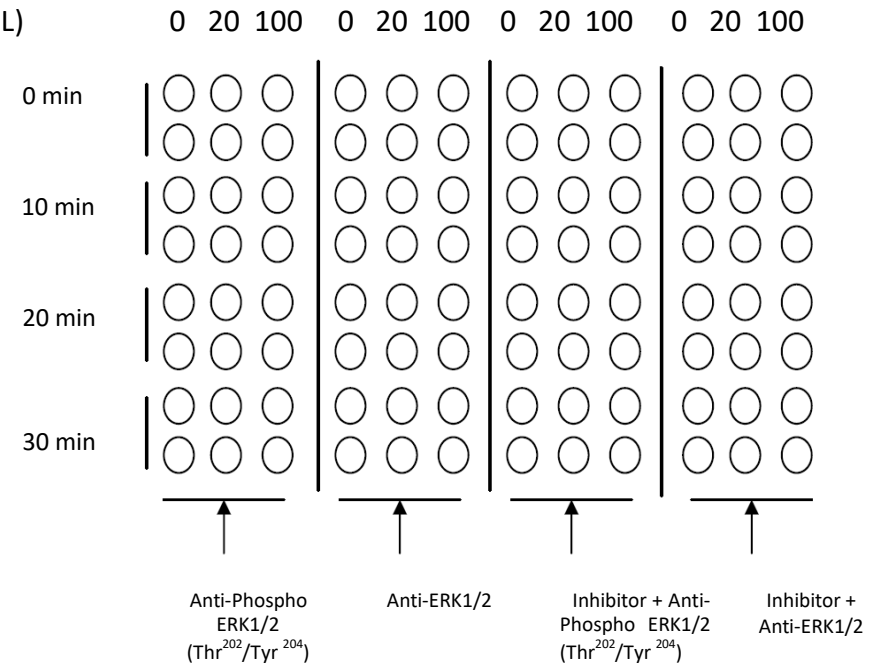
| | Item | Component | Preparation | Example |
|---|---|---|---|---|
| | A | Uncoated 96 Well Microplate | No Preparation | N/A |
| | B | 20× Wash Buffer Concentrate A | Dilute 20-fold with distilled or deionized water | 25 mL of concentrate plus 475 mL of water yields 500 mL of 1× working solution |
| | C | 20× Wash Buffer Concentrate B | | |
| | D | Fixing Solution | No Preparation | N/A |
| | E | Quenching Solution for Cell-based ELISA Assay | Dilute 30-fold with 1× Wash Buffer A | 1 mL of concentrate plus 29 mL of wash buffer yields 30 mL of 1× working solution |
| | F | 5× Blocking Solution | Dilute 5-fold with distilled or deionized water | 20 mL of concentrate plus 80 mL of water yields 100 mL of 1× working solution |
| PRIMARY ANTIBODY | G | Phospho-specific Antibody Concentrate | Dilute 1000-fold with 1× Blocking Solution | 7 µL of concentrate plus 6993 µL of 1× Blocking Buffer yields 7 mL of 1x working solution |
| PRIMARY ANTIBODY | H | Pan ERK Antibody Concentrate | | |
| SECONDARY | I2 | HRP-conjugated Anti-Mouse IgG Concentrate | | 10 µL of concentrate plus 9,990 µL of 1× Blocking Solution yields 10 mL of 1× working solution |
| | J | TMB Substrate Reagent | No Preparation | N/A |
| | K | Stop Solution | | |

**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 below.

**Figure 2.**
Example of plate layout for cell-based assay




2. Seed 100 µL of 30,000 cells into each well of the Uncoated 96 well Microplate (Item A) provided and incubate overnight at 37 °C and 5% $CO_2$.

   Notes: The optimal cell number used will vary depending on the cell line and the relative amount of protein phosphorylation. More or less cells may be used but this must be determined empirically.

   The cells can be starved 4-24 hours (depending on cell line) prior to treatment with inhibitors or activators.

3. Apply various treatments, inhibitors (such as siRNA or chemicals), or activators according to manufacturer's instructions and incubate for the desired time points.

   Note: It is recommended to dissolve inhibitors or activators in serum-free cell culture medium before treating the cells (unless otherwise stated in the manufacturer's instructions.)

4. Discard the cell culture medium by flipping the microplate upside down and gently tapping the bottom of the microplate over a sink.

5. Wash by pipetting 200 µL of the prepared 1× Wash Buffer A (Item B) into each well. Discard the wash buffer (same as step 4) and wash 2 more times for a total of 3 washes using fresh wash buffer each time. After the final wash, gently blot the microplate onto a paper towel to remove any excess/remaining buffer.

   Note: To avoid cell loss, do not pipette directly onto the cells. Instead, gently dispense the liquid down the wall of cell culture wells. Also avoid the use of vacuum suction or too forcefully tapping the microplate when discarding any solution.

6. Add 100 µL of Fixing Solution (Item D) into each well and incubate for 20 minutes at room temperature. The fixing solution is used to permeabilize the cells.

7. Wash the plate 3 times with 1× Wash Buffer A (200 µL each), then tap the plate upside down to remove all of wash buffer.

8. Add 200 µL of the prepared 1× Quenching Buffer (Item E) into each well and incubate 20 minutes at room temperature. The quenching buffer is used to minimize the background response.

9. Wash 4 times with 1× Wash Buffer A, then tap the plate upside down to remove all of excess wash buffer.

10. Add 200 µL of the prepared 1× Blocking Buffer (Item F) into each well and incubate for 1 hour at 37 °C.

11. Wash 3 times with prepared 1× Wash Buffer B (Item C).

    Note: If needed, the microplate may be stored at –80 °C for several days after this wash.

12. Add 50 µL of the prepared 1× primary antibody (Item G or H) into each corresponding well and incubate for 2 hours at room temperature.

    Note: Item I2 is the secondary antibody for Item H (primary antibody).

13. Wash 4 times with 1× Wash Buffer B, then tap the plate upside down to remove all of excess wash buffer.

14. Add 50 µL of 1× HRP Conjugated secondary antibody (Item I2) into each well and incubate for 1 hour at room temperature.

15. Wash 4 times with 1× Wash Buffer B.

16. Add 100 µL of the TMB Substrate (Item J) into each well and incubate for 30 minutes at room temperature in the dark.

17. Add 50 µL of the Stop Solution (Item K) into each well. Read at 450 nm immediately.

**Results**

Representative results of Cell-Based ERK1/2 ($Thr^{202}/Tyr^{204}$) are shown below:

1. Seeded 30,000 A431 cells 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 wash 3 times with 1× Wash Buffer A (200 µL each) immediately. Then flipped the plate upside down and tapped to remove all of excess wash buffer. The protocol was then followed as stated.

**Figure 3.**

A431 cells were stimulated by different concentrations of EGF for 10 minutes at 37 °C.

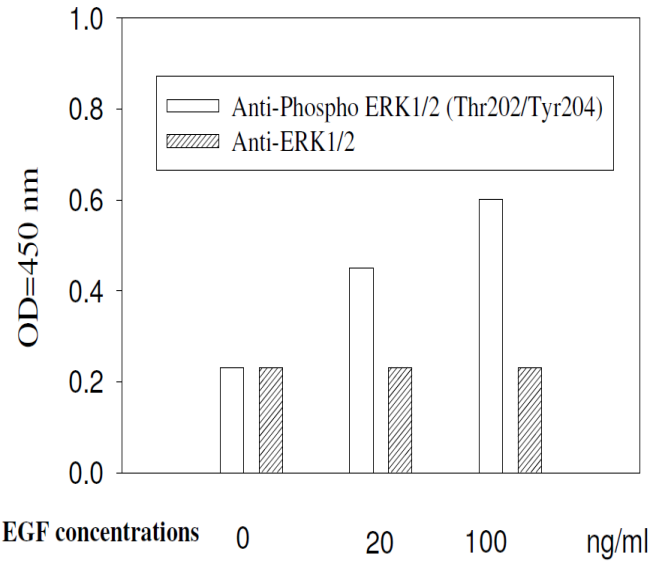
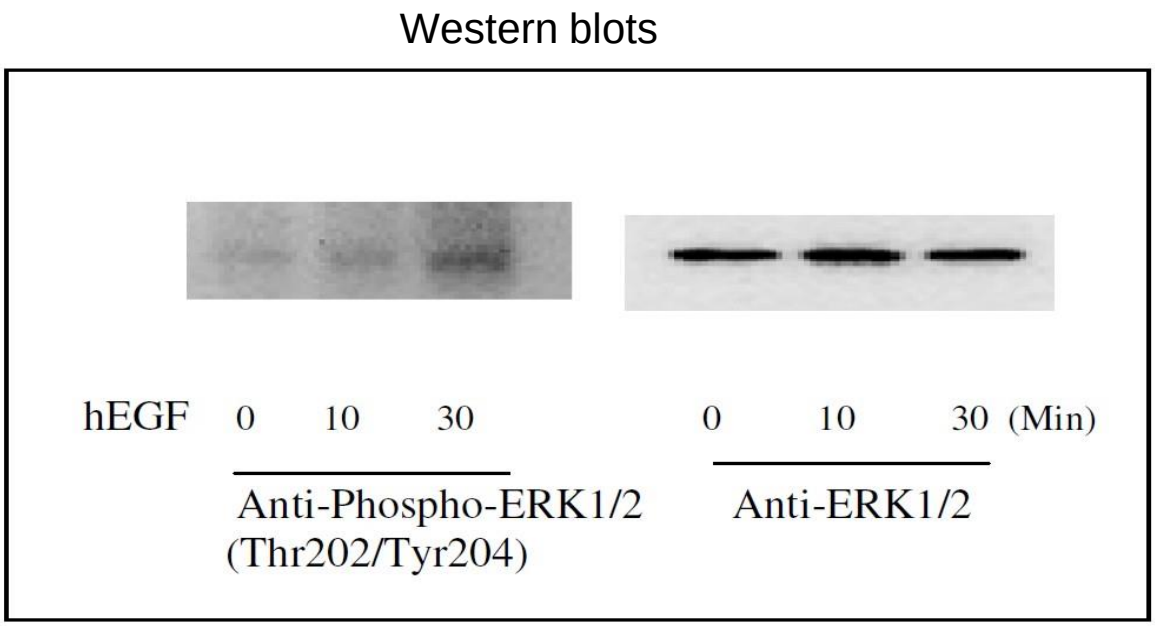


**Figure 4.**
Western blot analysis of extracts from 100 ng/mL hEGF treated A431 cells. Phospho-ERK1/2 ($Thr^{202}/Tyr^{204}$) and ERK1/2 antibodies were used in both detection assays.




**References**

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2. Michael J. Clemens and Michael C. 1997. Protein Phosphorylation in Cell Growth Regulation. 1 Edition.
3. Clark, E.A., and Hynes, R.O., J. Biol. Chem., **271**, 14814 (1996).
4. Hunter, T., Cell, **80**, 225-236 (1995).
5. Meng, J., and Casey, P.J., J. Biol. Chem., **277**, 43417-43424 (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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