

## Product Information

### Cell-Based ELISA Kit for detecting phospho-STAT3 (pTyr<sup>705</sup>) in cultured cell lines

adequate for 96 assays (1 × 96 well plate)

Catalog Number **RAB0444**

Storage Temperature -20 °C

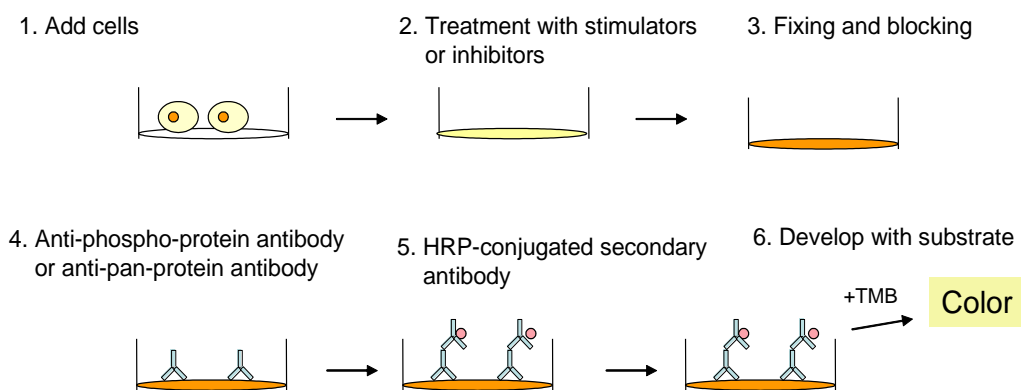
## TECHNICAL BULLETIN

### 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 Stat3 (Tyr<sup>705</sup>) 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 Stat3 (Tyr<sup>705</sup>) 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 Stat3 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 Stat3 (Tyr<sup>705</sup>) 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-Stat3 (Tyr<sup>705</sup>) or Anti-Stat3 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

**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) |                               |
| G    | Phospho-specific Antibody Concentrate – RABS705G          | 1 vial (10 µL) | –20 °C                        |
| H    | Pan-Stat3 Antibody Concentrate - RABSTAT3H                | 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 factor or cytokine.
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.

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     |
| <b>PRIMARY</b>            | G   | Phospho-specific Antibody Concentrate            | Dilute 500-fold with 1x Blocking Solution   |
|                           | H   | Pan-Stat3 Antibody Concentrate                   |   |
| <b>SECONDARY ANTIBODY</b> | I1  | HRP-conjugated Anti-Rabbit IgG Concentrate       | Dilute 1,000-fold with 1x Blocking Solution                                       |
|                           | I2  | HRP-conjugated Anti-Mouse IgG Concentrate        |   |
| J                         | TMB Substrate Reagent                         | No preparation                                   | N/A   |
| K                         | Stop Solution, contains 0.2 M sulfuric acid   |  |   |

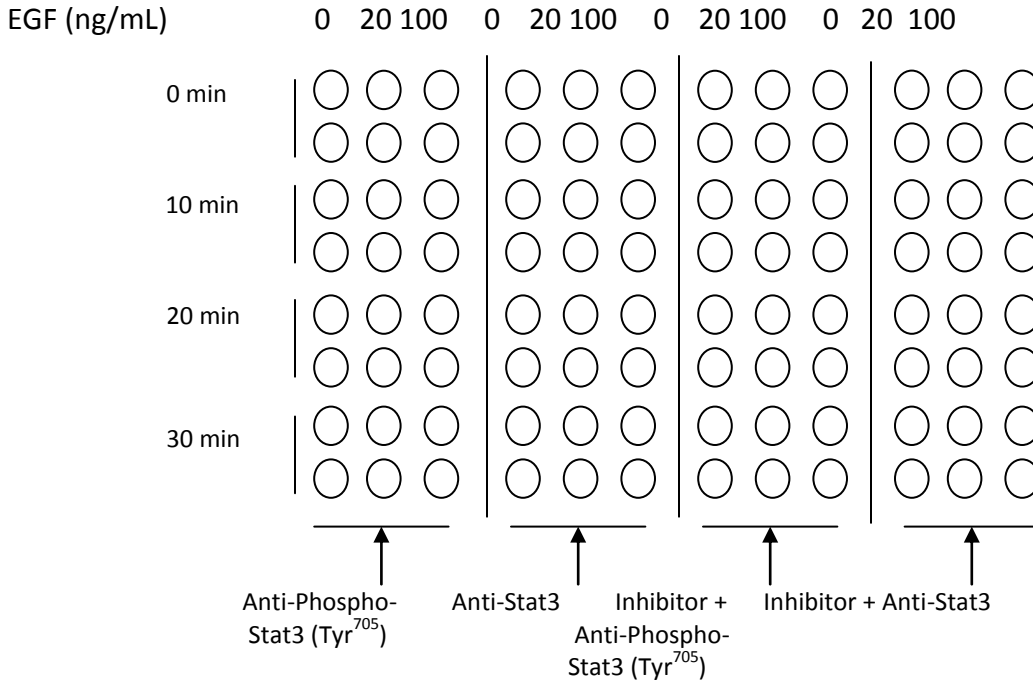
## Procedure

**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  $\mu$ L of 10,000–30,000 cells into each well of a 96 well plate and incubate overnight at 37 °C and 5% CO<sub>2</sub>.

**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  $\mu$ L 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<sup>®</sup> 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  $\mu$ L 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  $\mu\text{L}$  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  $\mu\text{L}$  each), then tap the plate upside down to remove all of wash buffer.
6. Add 200  $\mu\text{L}$  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  $\mu\text{L}$  each), then tap the plate upside down to remove all of wash buffer.
8. Add 200  $\mu\text{L}$  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  $\mu\text{L}$  each), then tap the plate upside down to remove all of excess wash buffer.

**Note:** The plate may be stored at  $-70\text{ }^{\circ}\text{C}$  for several days.

10. Add 50  $\mu\text{L}$  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  $\mu\text{L}$  each), then tap the plate upside down to remove all of excess wash buffer.
12. Add 50  $\mu\text{L}$  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  $\mu\text{L}$  each), then tap the plate upside down to remove all of excess wash buffer.
14. Add 100  $\mu\text{L}$  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  $\mu\text{L}$  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%  $\text{CO}_2$  overnight.
2. Added 50  $\mu\text{L}$  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 3A & 3B). Then incubated for 10 or 30 minutes at 37 °C.
3. Discarded the solution and washed 3 times with 1x Wash Buffer A (200  $\mu\text{L}$  each) immediately. Then tapped the plate upside down to remove all of excess wash buffer and followed with Procedure, steps 4–15.

### Figures 3A and 3B.

A431 cells were stimulated by different concentrations of EGF.

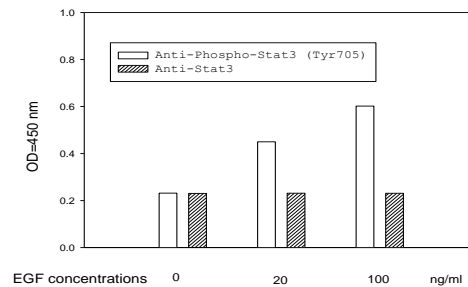


Fig. 3A. A431 cells were stimulated by different concentrations of EGF for 10 min at 37°C

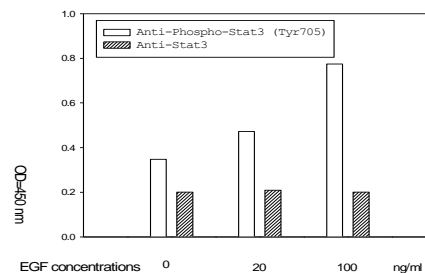
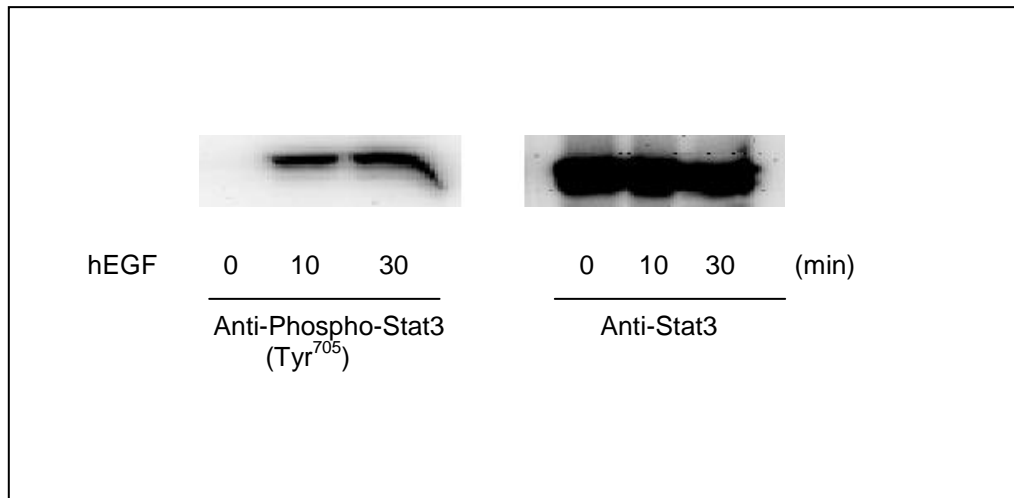


Fig. 3B. A431 cells were stimulated by different concentration EGF for 30 min at 37°C

## Western blots

**Figure 4.**

Western blot analysis of extracts from 100 ng/mL hEGF treated A431 cells. Phospho-stat3 (Tyr<sup>705</sup>) and stat3 antibodies were used in both detection assays.

**References**

1. Kanai, M., et al., *Oncogene*, **22**, 548-554 (2003).
2. Fu, X.Y. et al., *Cell*, **74**, 1135 (1993.).
3. Smith, P.D., and Crompton, M.R., *Biochem. J.*, **331**, 381 (1998).

**Appendix**  
Troubleshooting Guide

| <b>Problem</b>  | <b>Cause</b>                      | <b>Solution</b>   |
|-----------------|-----------------------------------|---|
| 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.                  |

SA,SG,KCP,KJ,MAM 08/18-1