

Product Information

Cell-Based phospho-STAT ELISA Sampler Kit for detecting phospho-STAT1 (pTyr⁷⁰¹), phospho-STAT3 (pTyr⁷⁰⁵), and phospho-STAT5 (pTyr⁶⁹⁴) in cultured cell lines

adequate for 192 assays (2 × 96 well plate)

Catalog Number **RAB0454**

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 phospho-STAT 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 (see Figure 1). It can be used for measuring the relative amount of STAT1 (pTyr⁷⁰¹), STAT3 (pTyr⁷⁰⁵), and STAT5 (pTyr⁶⁹⁴) phosphorylation and screening the effect of various treatments, inhibitors (such as siRNA or chemicals), or activators **in cultured human, mouse, and rat cell lines**.

By determining the phosphorylation of the STAT proteins in the experimental model system, pathway activation can be verified in the cell lines without spending time and effort in preparing a cell lysate and performing Western blot analysis. In the Cell-Based phospho-STAT 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, 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.

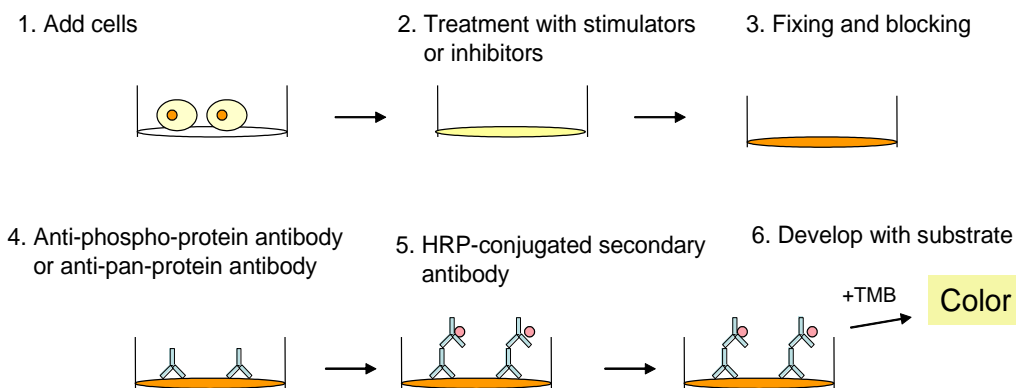


Fig.1. Cell-Based protein phosphorylation procedure

Components

- 2 Uncoated Microplate (Item A) - RABPLATE1: two 96 well tissue culture plates (12 × 8 wells) for cell culture.
- 20x Wash Buffer Concentrate A (Item B) - RABWASH1: 30 mL of 20x concentrated buffer.
- 20x Wash Buffer Concentrate B (Item C) - RABWASH2: 30 mL of 20x concentrated buffer.
- Fixing Solution (Item D) - RABFIX1: 30 mL of fixing solution.
- Quenching Solution for Cell-based ELISA Assay (Item E) - RABQUENCH: 2 mL of 30x concentrated solution.
- 5x Blocking Solution (Item F) - RABBLOCK: 20 mL of 5x concentrated solution.
- Phospho-specific Antibody Concentrate (Item G1) – RABS701G: one tube (30 assays) of anti-phospho-STAT1 (pTyr⁷⁰¹).
- Pan STAT1 Antibody Concentrate (Item H1) - RABSTAT1H: one tube (30 assays) of anti-STAT1.
- Phospho-specific Antibody Concentrate (Item G2) - RABS705G (Item G2): one tube (30 assays) of anti-phospho-STAT3 (pTyr⁷⁰⁵).
- Pan STAT3 Antibody Concentrate (Item H2) - RABSTAT3H: one tube (30 assays) of anti-STAT3.
- Phospho-specific Antibody Concentrate (Item G3) - RABS694G: one tube (30 assays) of anti-phospho-STAT5 (pTyr⁶⁹⁴).
- Pan Stat5Antibody Concentrate (Item H3) - RABSTAT5H: one tube (30 assays) of anti-STAT5.
- HRP-conjugated Anti-Mouse IgG Concentrate (Item I) - RABHRP1: two tubes (192 assays) 10 µL of concentrated HRP-conjugated anti-mouse IgG.
- TMB Substrate Reagent (Item J) - RABTMB1: two bottles (192 assays) 12 mL of 3,3',5,5'-tetramethylbenzidine (TMB) in buffered solution.
- Stop Solution (Item K): 14 mL of sulfuric acid.

Reagents and Equipment Required but Not Provided.

- A model cell line, Protein tyrosine kinase inhibitors, growth factor or cytokine.
- Microplate reader capable of measuring absorbance at 450 nm.
- 37 °C incubator.
- Precision pipettes to deliver 2 µL to 1 mL volumes.
- Adjustable 1-25 mL pipettes for reagent preparation.
- 100 mL and 1 liter graduated cylinders.
- Absorbent paper.
- Distilled or deionized water.
- 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.

Preparation Instructions

Reagent Preparation

- Wash Buffer A (20x) (Item B) or B (20x) (Item C) should be diluted 20-fold with deionized or distilled water. If the Wash Buffer A (20x) or B (20x) contains visible crystals, warm to room temperature and mix gently until dissolved. Dilute 25 mL of Wash Buffer Concentrate into deionized or distilled water to yield 500 mL of 1x Wash Buffer.
- Quenching Buffer Concentrate (Item E): should be diluted 30-fold with 1x Wash Buffer A before use.
- Blocking Solution (5x) (Item F): should be diluted 5-fold with deionized or distilled water.
- Preparation of antibody: Briefly spin the antibody tubes (Items G1, G2, G3, H1 H2 and H3) before use. Add 100 mL of 1x Blocking Solution into each tube to prepare each primary antibody concentrate. Pipette up and down to mix gently (the concentrate can be stored at –20 °C for 1 month, avoid repeated freeze-thaw cycles). Each antibody concentrate should be diluted 15-fold with 1x Blocking Solution before use.
- HRP-conjugated Anti-Mouse IgG Concentrate (Item I): should be diluted 1,000-fold with 1x Blocking Solution before use (except for against anti-Stat5, Item H3). It should be diluted 500-fold with 1x Blocking Solution for anti-Stat5 (Item H3).

Storage/Stability

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

Items B, D, E, F, J, and I should be stored at 2–8 °C to avoid repeated freeze-thaw cycles after initial use.

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

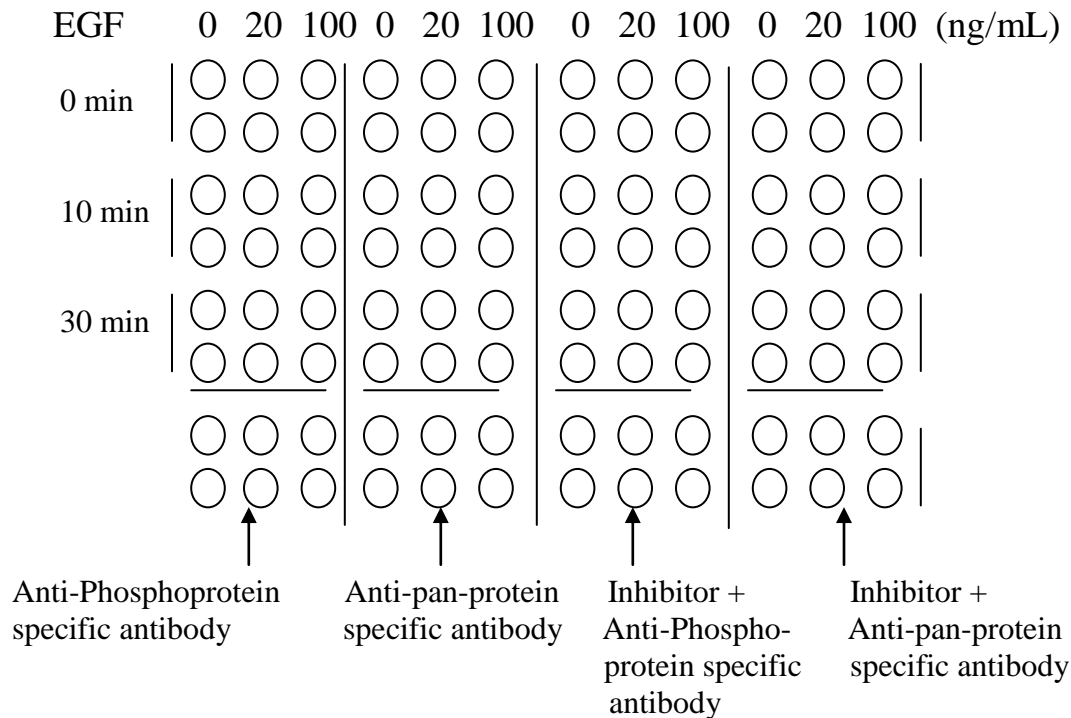
Items G1, G2, G3, H1 H2 and H3 should be stored at –20 °C after use.

Procedure

1. Design the experiment, see Figure 2.

Figure 2.

Example of Seeding Cells for Cell-Based Assay



2. Seed 100 μL of 30,000 (for STAT1 and STAT3) or 20,000 (for STAT5) cells into each well of a 96 well plate and incubate for overnight at 37 °C and 5% CO_2 .

Note: The 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 from other brand 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. Discard the cell culture medium and wash 3 times with 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.
5. Wash the plate 3 times with 1x Wash Buffer A, 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.
7. Wash the plate 4 times with 1x Wash Buffer A, 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 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 $-70\text{ }^{\circ}\text{C}$ for several days.

10. Add 50 μL of 1x anti-phosphoprotein specific antibody (Item G1, G2 or G3) or anti-pan-protein specific antibody (Item H1, H2, or H3) to the corresponding well and incubate for 2 hours at room temperature with shaking (overnight at 4 °C for Item G2 and H2 of STAT3).
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 Anti-Mouse IgG (Item I) and incubate for 1 hour at room temperature.
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 to each well and incubate for 30 minutes with shaking at room temperature in the dark.
15. Add 50 μL of stop solution to each well and read at 450 nm, measure OD immediately.

Results

Representative results are shown:

Note:

1. In Procedure, step 2, 100 μL of 30,000 (for STAT1 and STAT3) or 20,000 (for STAT5) A431 cells into each appropriate well in microplate. Cells were incubated at 37 °C in 5% CO_2 overnight.
2. In Procedure, step 3, added 50 μL of stimulator solutions with different concentrations: 0, 20, or 100 ng/mL of rhEGF in serum free DMEM. Then incubated for 10 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.

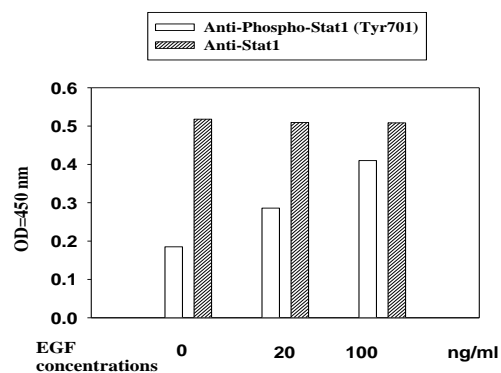


Fig. 3.1. A431 cells were stimulated by different concentrations of EGF for 30 minutes at 37°C.

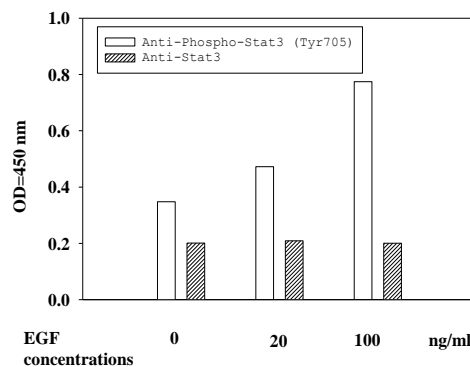


Fig. 3.2. A431 cells were stimulated by different concentration EGF for 30 min at 37°C

Western blots

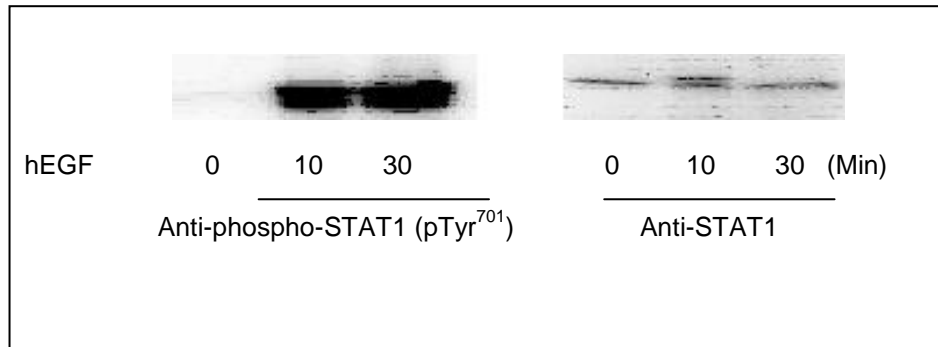


Fig. 4.1. Western blot analysis of extracts from 100 ng/mL hEGF treated A431 cells. Phospho-STAT1 (pTyr⁷⁰¹) and STAT1 antibodies were used in both detection assays.

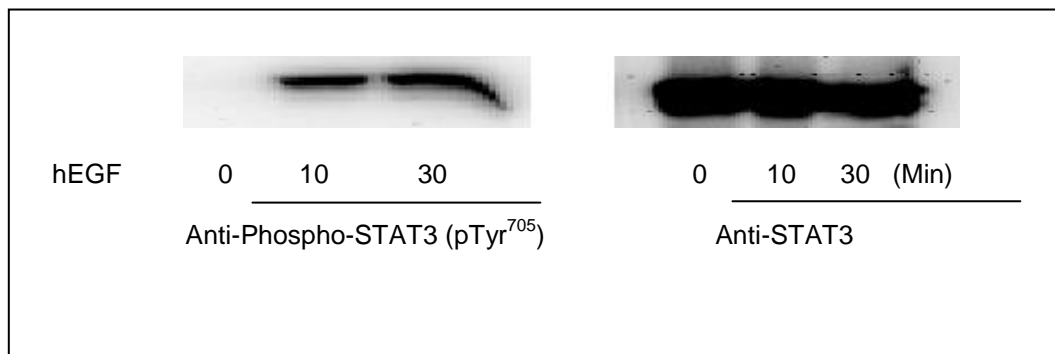


Fig. 4.2. Western blot analysis of extracts from 100 ng/mL hEGF treated A431 cells. Phospho-STAT3 (pTyr⁷⁰⁵) and STAT3 antibodies were used in both detection assays.

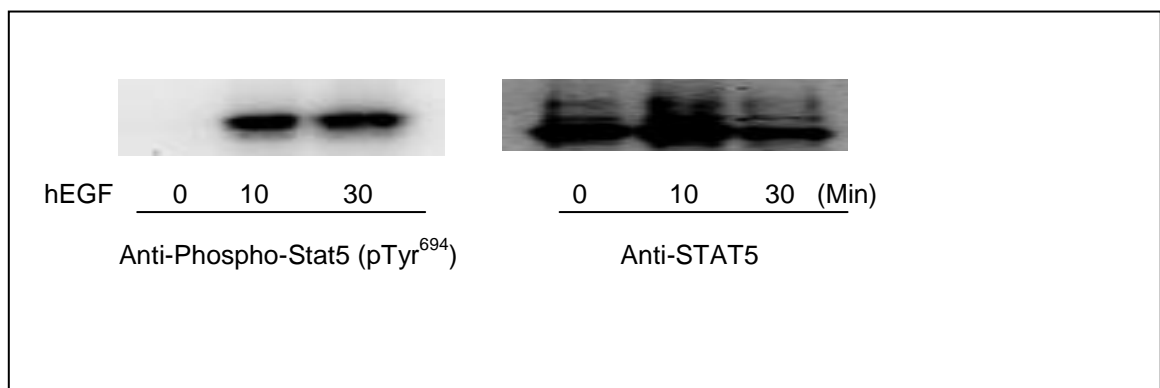


Fig. 4.3. Western blot analysis of extracts from 100 ng/mL hEGF treated A431 cells. Phospho-STAT5 (pTyr⁶⁹⁴) and Anti-STAT5 antibodies were used in both detection assays.

References

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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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