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Product Information

Cell-Based ELISA for detecting phospho-STAT6 (pTyr^{641}) in cultured cell lines
adequate for 96 assays (1 × 96 well plate)

Catalog Number **RAB0452**
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-STAT6 (pTyr^{641}) 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 STAT6 (pTyr^{641}) phosphorylation and screening the effects of various treatments, inhibitors (such as siRNA or chemicals), or activators **in cultured human and mouse cell lines**.

By determining the phosphorylation of the STAT6 protein 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-STAT6 (pTyr^{641}) ELISA 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-phospho-STAT6 (Tyr^{641}) or anti-STAT6 (primary antibody) is pipetted into the wells and incubated. The wells are washed, and HRP-conjugated 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.

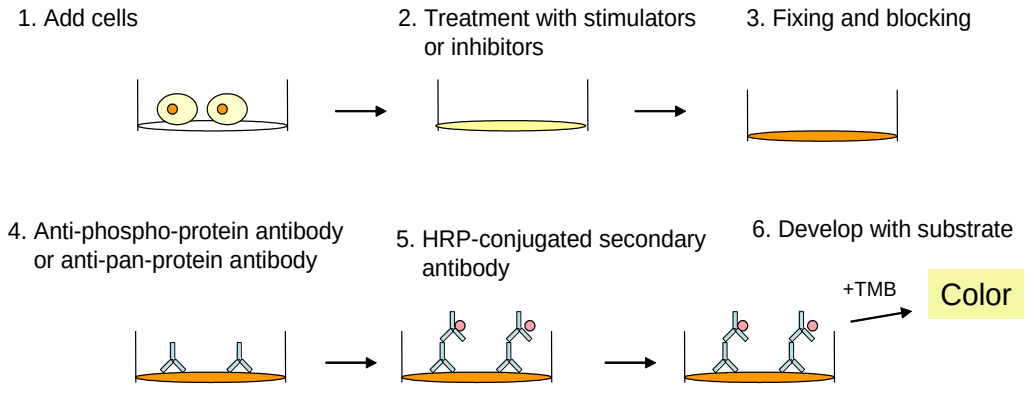


Fig.1. Cell-Based protein phosphorylation procedure

Components

1. Uncoated Microplate (Item A) - RABPLATE1: 96 well tissue culture plate (12 × 8 wells).
2. 20x Wash Buffer Concentrate A (Item B) - RABWASH1: 30 mL of 20x concentrated buffer.
3. 20x Wash Buffer Concentrate B (Item C) - RABWASH2: 30 mL of 20x concentrated buffer.
4. Fixing Solution (Item D) - RABFIX1: 30 mL of fixing solution.
5. Quenching Solution for Cell-based ELISA Assay (Item E) - RABQUENCH: 2 mL of 30x concentrated solution.
6. 5x Blocking Solution (Item F) - RABBLOCK: 20 mL of 5x concentrated solution.
7. Phospho-specific Antibody Concentrate (Item G) - RABS641G: one tube (96 assays) of 10 μL of 500x concentrated anti-phospho-STAT6 ($pTyr^{641}$) raised in rabbit.
8. Pan STAT6 Antibody Concentrate (Item H) - RABSTAT6H: one tube (96 assays) of 5 μL of 5,000x Concentrated anti-STAT6 raised in mouse.
9. HRP-conjugated Anti-Rabbit IgG Concentrate (Item I2) - RABHRP2: one tube (96 assays) of 10 μL of 1,000x concentrated HRP-conjugated anti-rabbit IgG.
10. HRP-conjugated Anti-Mouse IgG Concentrate (Item I1) - RABHRP1: one tube (96 assays) of 10 μl of 1,000x concentrated HRP-conjugated anti-mouse IgG.
11. TMB Substrate Reagent (Item J) - RABTMB1: one bottle with 12 mL of 3,3′,5,5′-tetramethylbenzidine (TMB) in buffered solution.
12. Stop Solution (Item K) - RABSTOP1: 14 mL of sulfuric acid.

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. 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

1. 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.

2. Quenching Buffer Concentrate (Item E): should be diluted 30-fold with 1x Wash Buffer A before use.

3. Blocking Solution (5x) (Item F): should be diluted 5-fold with deionized or distilled water.

4. Rabbit Anti-Phospho-STAT6 (Tyr^{641}) Concentrate (Item G): should be diluted 500-fold with 1x Blocking Solution (Briefly spin Item G before use).

5. Mouse Anti-STAT6 Concentrate (Item H): should be diluted 1,000-fold with 1x Blocking Solution (Briefly spin Item H before use).

6. HRP-Anti-mouse IgG Concentrate (Item I1) and HRP-Anti-rabbit IgG Concentrate (Item I2): should be diluted 1,000-fold with 1x Blocking Solution (Briefly spin the tube before use).

Storage/Stability

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

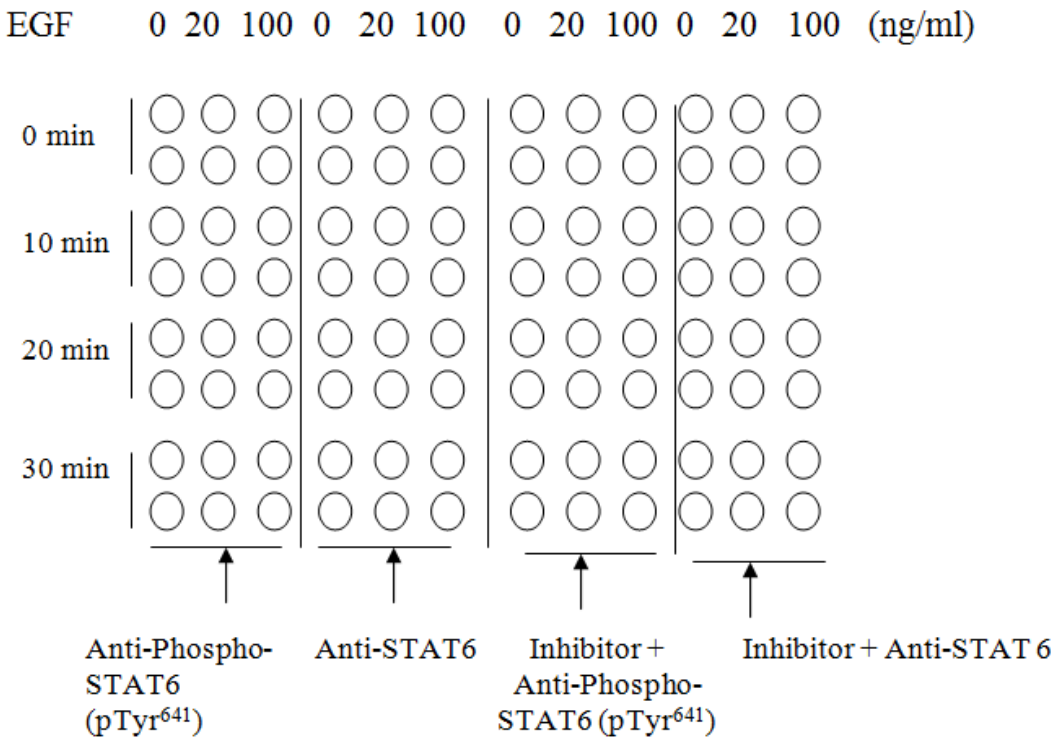
Items B, D, E, F, J, I1, and I2 should be stored at 2–8 °C to avoid repeated freeze-thaw cycles after initial use.

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

Items G and H 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 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 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 °C for several days.

10. Add 50 µL of 1x Anti-Phospho-STAT6 ($pTyr^{641}$) (Item G) or 1x Anti-STAT6 (Item H) to 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 Anti-Rabbit IgG (Item I2) or HRP-conjugated Anti-Mouse IgG (Item I1) to corresponding wells and incubate for 1 hour at room temperature.

    For example, add 50 µL of HRP-conjugated Anti-Rabbit IgG (Item I2) to the wells treated with rabbit Anti-Phospho-STAT6 ($pTyr^{641}$) or add 50 µL of HRP-conjugated Anti-Mouse IgG (Item I1) to the wells treated with mouse Anti-STAT6.

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, seed 30,000 A431 cells into appropriate well in microplate. Cells were incubated at 37 °C in 5% CO_2 over night.

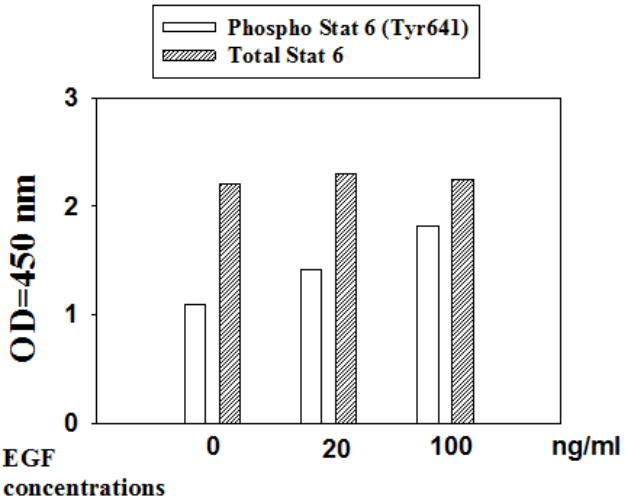
2. The cells were starved overnight before treatment (inhibitor or activator).

3. Added 50 µL of different concentrations of rhEGF (0, 20, or 100 ng/mL in serum free DMEM) to appropriate wells. Then incubated for 10 minutes at 37 °C.

4. 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.

Figure 3.

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



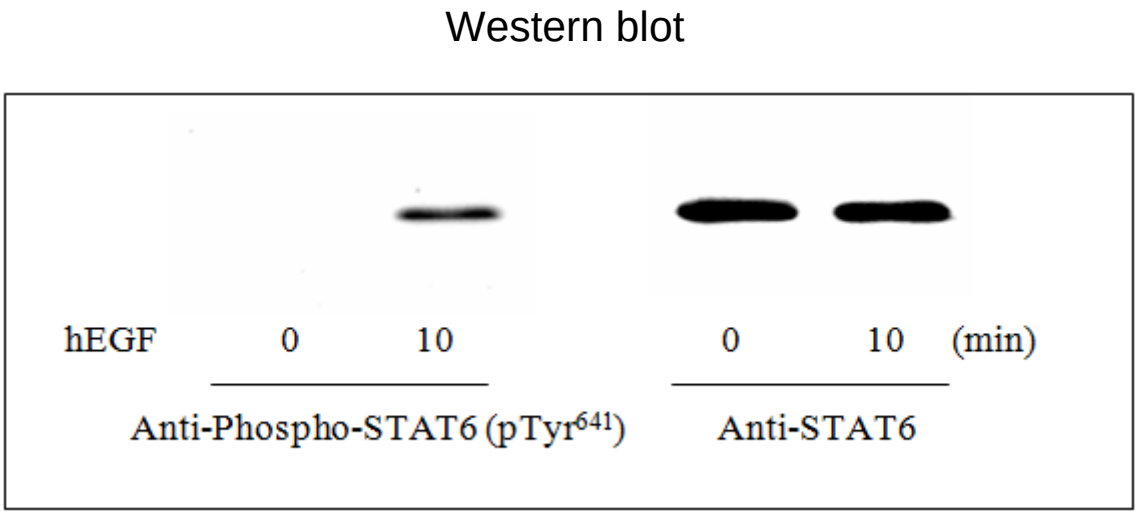


Figure 4.
Western blot analysis of extracts from 100 ng/mL hEGF treated A431 cells.
Phospho-STAT6 ($pTyr^{641}$) and STAT6 antibodies were used in both detection assays.

References

1. Quelle, F.W. *et. al*., Mol. Cell. Biol., **15**, 3336-3343 (1995).
2. Patel, K.R., *et al*., J. Biol.Chem., **271**, 22175-22182 (1996).

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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