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Product Information

Cell-Based ELISA Kit for detecting phospho-STAT5 (pTyr^{694}) in cultured cell lines
adequate for 96 assays (96 well plate)

Catalog Number **RAB0450**
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-STAT5 (pTyr^{694}) 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 STAT5 (Tyr^{694}) phosphorylation and screening the effects of various treatments, inhibitors (such as siRNA or chemicals), or activators in cultured human cell lines.

By determining STAT5 protein phosphorylation 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-STAT5 (pTyr^{694}) 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-STAT5 (pTyr^{694}) or anti-STAT5 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.

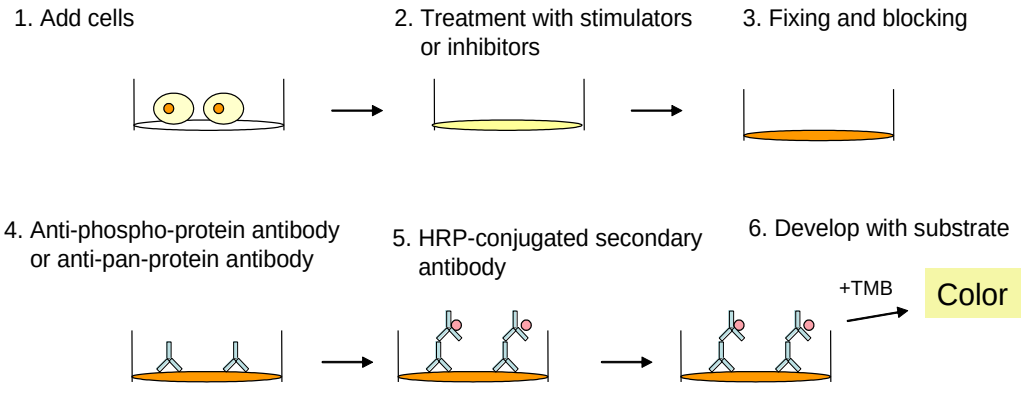


Fig.1. Cell-Based protein phosphorylation procedure

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)	2–8 °C (1 month)
G	2,000x Mouse Anti-phospho-STAT5 ($pTyr^{694}$) Concentrate – RABS694G	1 vial (7 µL)	–20 °C
H	500x Mouse Anti-STAT5 Concentrate – RABSTAT5H	1 vial (10 µL)	
I-2	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

For R&D 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 x *g*) Items G, H, and I before opening to ensure maximum recovery.

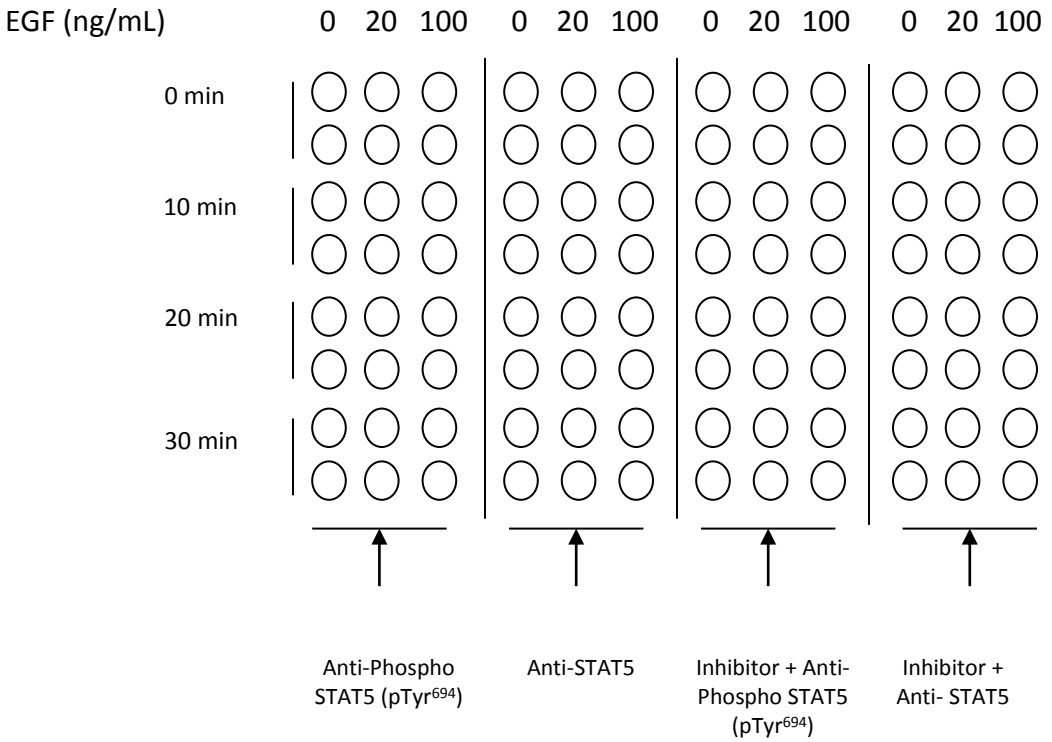
Table 2.

	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 ANTIBODY	G	2,000x Mouse Anti-phospho-STAT5 ($pTyr^{694}$) Concentrate	Dilute 2,000-fold with 1x Blocking Solution	5 μL of reconstituted stock plus 9,995 μL of 1x Blocking Buffer yields 10 mL of 1x working solution
PRIMARY ANTIBODY	H	500x Mouse Anti-STAT5 Concentrate	Dilute 500-fold with 1x Blocking Solution	10 μL of concentrate plus 4,990 μL of 1x Blocking Buffer yields 5 mL of 1x working solution
SECONDARY ANTIBODY	I-2	HRP-conjugated Anti-Mouse IgG Concentrate	Dilute 1,000-fold with 1x Blocking Buffer if Item G is primary antibody	10 μL of concentrate plus 9,990 μL of 1x Blocking Buffer yields 10 mL of 1x working solution
SECONDARY ANTIBODY	I-2	HRP-conjugated Anti-Mouse IgG Concentrate	Dilute 500-fold with 1x Blocking Buffer if Item H is primary antibody	10 μL of concentrate plus 4,990 μ L of 1x Blocking Buffer yields 5 mL of 1x working solution
	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 20,000 cells into each well of the uncoated 96 Well Microplate (Item A) provided and incubate overnight at 37 °C with 5% CO_2.

   Notes: 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.

   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 for the desired time points.

   Note: It is recommended to dissolve the inhibitors or activators into serum free cell culture medium and then treat 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 1X 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 with shaking. The fixing solution is used to permeabilize the cells.

7. 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.

8. 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.

9. Wash the plate 4 times with 1x Wash Buffer A.

10. Add 200 μL of prepared 1x Blocking Solution (Item F) and incubate for 1 hour at 37 °C.

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

    Note: If needed, the microplate may be stored at –20 °C for several days after this wash.

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

    Note: Item I-2 is the secondary antibody for Item H (primary antibody).

13. Wash 4 times with 1x Wash Buffer B.

14. Add 50 μL of the prepared 1x HRP-conjugated secondary antibody (Item I-2) into each well and incubate for 1 hour at room temperature.

    Note: The Item I-2 dilution factor will depend on the primary antibody.

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

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

17. Add 50 μL of Stop Solution (Item K) into each well and read at 450 nm, measure OD immediately.

Results

Representative results of Cell-Based phospho-STAT5 ($pTyr^{694}$) Elisa Kit are shown below:

Notes:

- 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.

- 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.

- Discarded the solution and wash 3 times with 1x Wash Buffer A (200 μL each) immediately. Then flipped the plate upside down and tapped 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.

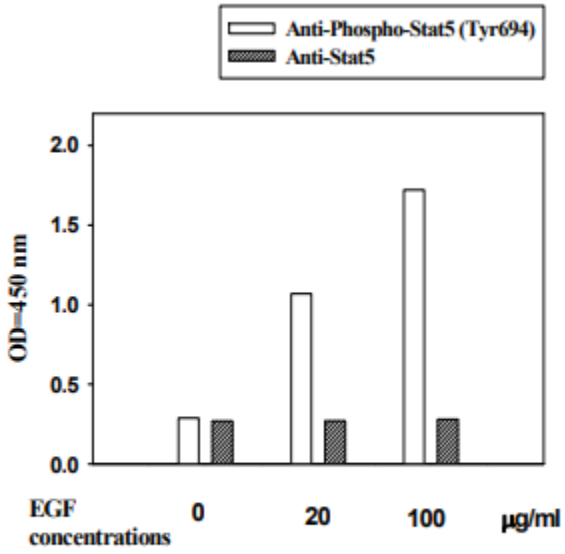


Fig. 3A. A431 cells were stimulated by different concentrations of EGF for 10 minutes at 37°C

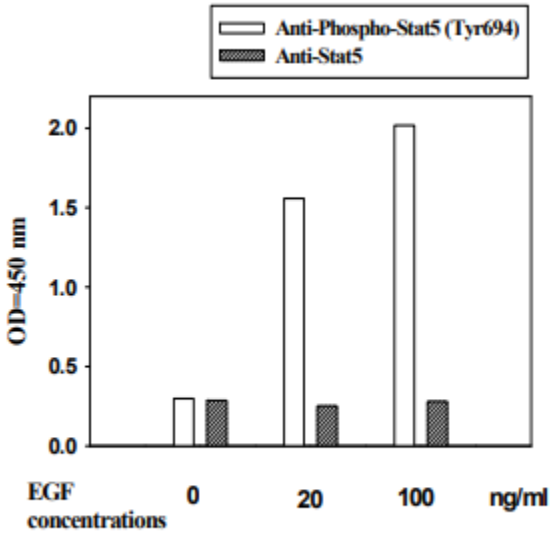
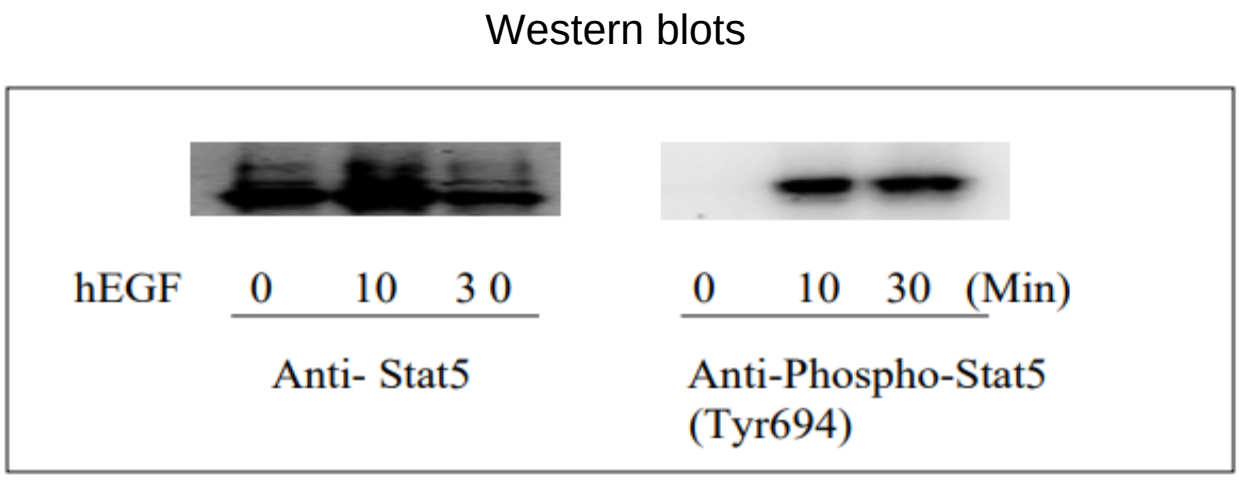


Fig. 3B. A431 cells were stimulated by different concentrations of EGF for 30 minutes at 37°C

Figure 4.
Western blot analysis of extracts from 100 ng/mL hEGF treated A431 cells. Phospho-Stat5 ($pTyr^{694}$) and Anti-Stat5 antibodies were used in both detection assays.



References

1. Wakao, H. et al., EMBO J., **12**, 2182 (1994).
2. Gouilleus, F. et al., EMBO J., **13**, 4361 (1994).
3. Michael J. Clemens and Michael C. 1997. Protein Phosphorylation in Cell Growth Regulation. 1 Edition.
4. Park, D. S. et al., Mol. Biol. Cell, **13**, 3416-3430 (2002).

Appendix

Troubleshooting Guide

Problem	Cause	Solution
Low signal	Improper storage of the ELISA kit	Store all of components according to bulletin 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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