ab126455 - STAT1 (pS727) + total STAT1 ELISA Kit

Instructions for Use

For the quantitative measurement of human and mouse phosphorylated STAT1 (pS727) and total STAT1 concentrations in cell lysates.

This product is for research use only and is not intended for diagnostic use.

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1. Introduction

ab126455 is a very rapid, convenient and sensitive assay kit that can monitor the activation or function of important biological pathways in cell lysates. By determining phosphorylated STAT1 protein in your experimental model system, you can verify pathway activation in your cell lysates. You can simultaneously measure numerous different cell lysates without spending excess time and effort in performing a Western Blot analysis.

This Sandwich ELISA kit is an in vitro enzyme-linked immunosorbent assay for the measurement of human phospho-STAT1 (Ser727) and total STAT1 (help normalize the results of phospho-STAT1 from different cell lysate being compared). An anti-pan STAT1 antibody has been coated onto a 96-well plate. Samples are pipetted into the wells and STAT1 present in a sample is bound to the wells by the immobilized antibody and the wells are washed. In select wells, rabbit anti-phospho-Stat1 (Ser727) antibody is added to detect phosphorylated STAT1. In the remaining wells, biotinylated anti-Stat1 antibody is used to detect pan STAT1. After washing away unbound antibody, HRP-conjugated Streptavidin is pipetted to the wells. The wells are again washed, a TMB substrate solution is added to the wells and color develops in proportion to the amount of STAT1 (Ser727) or total STAT1 bound. The Stop Solution changes the color from blue to yellow, and the intensity of the color is measured at 450 nm.

2. Assay Summary

Prepare all reagents, samples and positive controls as instructed.

Add 100 µl sample or positive control to each well. Incubate 2.5 hours at room temperature or overnight at 4°C.

Add 100 µl prepared detection antibody to half the wells. Incubate 1 hour at room temperature.

Add 100 µl HRP-conjugated anti-rabbit IgG solution or 100 µl of prepared HRP-Streptavidin solution. Incubate 1 hour at room temperature.

Add 100 µl TMB One-Step Substrate Reagent to each well. Incubate 30 minutes at room temperature.

Add 50 µl Stop Solution to each well. Read at 450 nm immediately.

3. Kit Contents

- STAT1 Microplate (Item A): 96 wells (12 strips x 8 wells) coated with anti-pan STAT1 antibody

- Wash Buffer Concentrate (20x) (Item B): 25 ml of 20x concentrated solution.

- Positive Control (Item K): 1 vial of lyophilized powder from A431 cell lysate.

- Detection Antibody anti-phospho STAT1 (Ser727) (Item C-1): 1 vial of rabbit anti-phospho-Stat1 (Ser727) (1 vial is enough to assay half of the microplate).

- Detection Antibody anti-pan STAT1 (Item C-2): 1 vials of biotinylated anti-pan STAT1.

- HRP-conjugated anti-rabbit IgG concentrate (Item D-1): 200 µl of 500X concentrated HRP-conjugated anti-rabbit IgG.

- HRP-Streptavidin Concentrate (Item G), 1 vials (200 µl) HRP-conjugated streptavidin concentrate.

- TMB One-Step Substrate Reagent (Item H): 12 ml of 3,3,5,5'-tetramethylbenzidine (TMB) in buffered solution.

- Stop Solution (Item I): 8 ml of 0.2 M sulfuric acid.

- Assay Diluent (Item E): 15 ml of 5x concentrated buffer. For diluting cell lysate sample, detection antibodies (Items C-1 and C-2), HRP-conjugated anti-rabbit IgG concentrate (item D-1) and HRP-Streptavidin Concentrate (Item G).

- Cell Lysis Buffer (Item J): 10 ml 2X cell lysis buffer (not including protease and phosphatase inhibitors).

4. Storage and Handling

Upon receipt, the kit should be stored at –20 °C. After initial use, Wash Buffer Concentrate (Item B), Assay Diluent (Item E), TMB One-Step Substrate Reagent (Item H), Stop Solution (Item I) and Cell Lysis Buffer (Item J) should be stored at 4 °C to avoid repeated freeze-thaw cycles. Return unused wells to the pouch containing desiccant pack, reseal along entire edge and store at –20 °C. Reconstituted Positive Control (Item K) should be stored at -80 °C.

5. Additional Materials Required

- Microplate reader capable of measuring absorbance at 450 nm.
- Protease and Phosphatase inhibitors.
- Shaker.
- Precision pipettes to deliver 2 µl to 1 ml volumes.
- Adjustable 1-25 ml pipettes for reagent preparation.
- 100 ml and 1 L graduated cylinders.
- Distilled or deionized water.
- Tubes to prepare sample dilutions.

6. Preparation of Samples

Cell lysates - Rinse cells with PBS, making sure to remove any remaining PBS before adding the Cell Lysis Buffer. Solubilize cells at 4 x 107 cells/ml in 1x Cell Lysis Buffer (we recommend adding protease and phosphatase inhibitors to Cell Lysis Buffer prior to sample preparation). Pipette up and down to resuspend and incubate the lysates with shaking at 2 - 8°C for 30 minutes. Microcentrifuge at 13,000 rpm for 10 minutes at 2 - 8°C, and transfer the supernates into a clean test tube. Lysates should be used immediately or aliquoted and stored at –70°C. Avoid repeated freeze-thaw cycles. Thawed lysates should be kept on ice prior to use.

For the initial experiment, we recommend to do a serial dilution testing such as 5-fold and 100-fold dilution for your cell lysates with 1x Assay Diluent (Item E) before use.

Note: The fold dilution of sample used depends on the abundance of phosphorylated proteins and should be determined empirically. More of the sample can be used if signals are too weak. If signals are too strong, the sample can be diluted further.

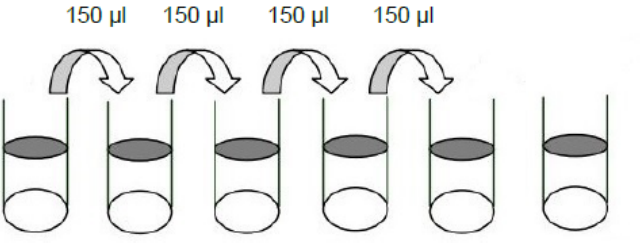
Cell Lysis Buffer should be diluted 2-fold with deionized or distilled water before use (recommend to add protease and phosphatase inhibitors).

7. Preparation of Reagents

Bring all reagents and samples to room temperature (18 - 25°C) before use.

Item E, Assay Diluent should be diluted 5-fold with deionized or distilled water before use to make 1X Assay Diluent.

Preparation of Positive Control: Briefly spin the Positive Control vial of Item K. Add 400 µl 1X Assay Diluent into Item K vial to prepare Positive Control (P-1) Solution (See i. Positive Control of Data Analysis for a typical result). Dissolve the powder thoroughly by a gentle mix. Pipette 300 µl 1X Assay Diluent into each tube. Use the P-1 to produce a dilution series (shown below). Mix each tube thoroughly before the next transfer. 1X Assay Diluent serves as the background.



	P-1	P-2	P-3	P-4	P-5		Blank (P-0)
Diluent volume	Item K + 400 µl	300 µl	300 µl	300 µl	300 µl		300 µl

If the Wash Concentrate (20x) (Item B) contains visible crystals, warm to room temperature and mix gently until dissolved. Dilute 20 ml of Wash Buffer Concentrate into deionized or distilled water to yield 400 ml of 1x Wash Buffer.

Briefly spin the vial of rabbit anti-phospho-Stat1 (Ser727) (Item C-1). Add 100 µl of 1X Assay Diluent into the vial to prepare a phospho detection antibody concentrate. Pipette up and down to mix gently (the concentrate can be stored at 4°C for 5 days or at -80°C for one month). The concentrate should then be diluted 55-fold with 1X Assay Diluent.

Briefly spin the biotinylated antibody (Item C-2) before use. Add 100 µl of 1x Assay Diluent into the vial to prepare a biotinylated antibody concentrate. Pipette up and down to mix gently (the concentrate can be stored at 4°C for 5 days or at –80°C for one month). The biotinylated STAT1 antibody should be diluted 55-fold with 1x Assay Diluent.

Preparation of HRP-conjugated anti-rabbit IgG: Briefly spin the vial of HRP-conjugated anti-rabbit IgG concentrate (Item D-1) before use. HRP-conjugated anti-rabbit IgG should be diluted 500-fold with 1X Assay Diluent.

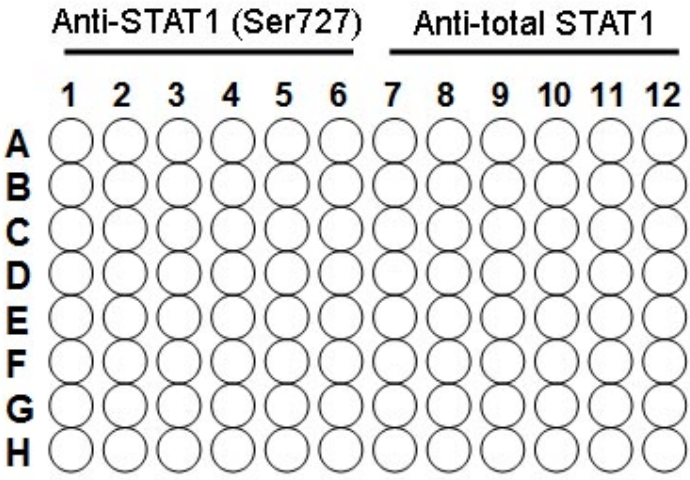
Briefly spin the HRP-Streptavidin concentrate vial (Item G) and pipette up and down to mix gently before use. HRP-Streptavidin concentrate should be diluted 100 fold with 1x Assay Diluent.

For example: Briefly spin the vial. Add 10 µl of HRP-conjugated anti-rabbit IgG concentrate into a tube with 5.0 mL 1x Assay Diluent, pipette up and down to mix gently to prepare a 500-fold diluted HRP-conjugated anti-rabbit IgG solution. Mix well.

8. Assay Method

1. Bring all reagents to room temperature (18 - 25°C) before use. It is recommended that all samples or Positive Control should be run at least in duplicate. It is also recommended to run the positive controls in singlet for each of the pan and phospho-specific antibodies.
2. Add 100 µl of each sample or positive control into appropriate wells. Cover well with plate holder and incubate for 2.5 hours at room temperature or overnight at 4°C with shaking.

   96 well microplate coated with anti-pan STAT1 antibody:



3. Discard the solution and wash 4 times with 1x Wash Solution. Wash by filling each well with Wash Buffer (300 µl) using a multi-channel pipette or autowasher. Complete removal of liquid at each step is essential to good performance. After the last wash, remove any remaining Wash Buffer by aspirating or decanting. Invert the plate and blot it against clean paper towels.
4. Add 100 µl of prepared 1X rabbit anti-phospho-Stat1 (Ser727) antibody (see Reagent Preparation) into the wells designated to detect phosphorylated protein. Add 100 µl of prepared 1X biotinylated anti-Stat1 antibody (see Reagent Preparation) to the remaining wells to detect pan protein. Incubate for 1 hour at room temperature with gentle shaking.
5. Discard the solution. Repeat the wash as in step 3.
6. Add 100 µl of prepared HRP-conjugated anti-rabbit IgG solution (see Reagent Preparation) to the wells corresponding with rabbit anti-phospho-Stat1 (Ser727) in order to detect phosphorylated protein. To the remaining wells (corresponding with biotinylated anti-Stat1), add 100 µl of prepared HRP-Streptavidin solution (see Reagent Preparation) in order to detect pan protein. Incubate for 1 hour at room temperature with gentle shaking.
7. Discard the solution. Repeat the wash as in step 3.
8. Add 100 µl of TMB One-Step Substrate Reagent (Item H) to each well. Incubate for 30 minutes at room temperature in the dark with shaking.

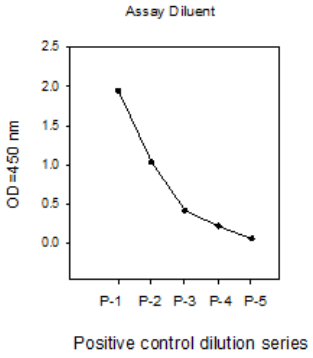
9. Add 50 µl of Stop Solution (Item I) to each well. Read at 450 nm immediately.

9. Data Analysis

ELISA data analysis: Average the duplicate readings for each sample or positive.

i. Positive Control

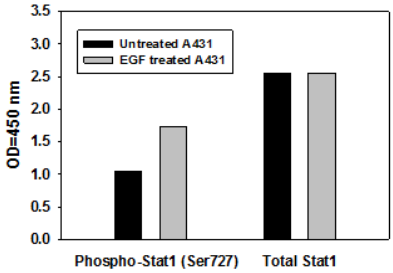
A431 cells were treated with recombinant human EGF at 37°C for 20 min. Solubilize cells at 4 x 107 cells/ml in Cell Lysis Buffer. Serial dilutions of lysates were analyzed in this ELISA. Please see step 3 of Preparation of Reagents for detail.



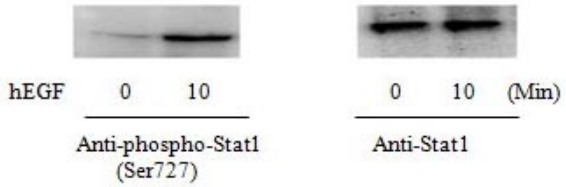
ii. Recombinant Human EGF Stimulation of A431 Cell Lines

A431 cells were treated or untreated with 100 ng/ml recombinant human EGF for 10 min. Cell lysates were analyzed using this phosphoELISA and Western Blot.

a) ELISA



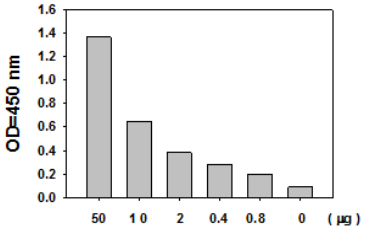
b) Western Blot Analysis



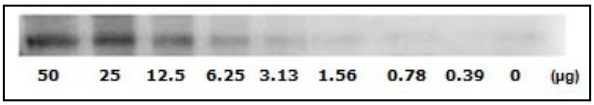
iii. Sensitivity

The A431 cells were treated with 100 ng/ml recombinant human EGF for 20 minutes. Serial dilutions of lysates were analyzed in this ELISA and by Western blot. Immunoblots were incubated with anti-phospho-STAT1 (Ser727).

a) ELISA



b) Western Blot Analysis



10. Troubleshooting

Problem	Cause	Solution
Sample signals	Too low: Sample concentration is too low.	Increasing sample concentration.
	Too high: Sample concentration is too high.	Reduce sample concentration.
Large CV	Inaccurate pipetting	Check pipettes
High background	Plate is insufficiently washed.	Review the manual for proper washing. If using an automated plate washer, check that all ports are unobstructed.
	Contaminated wash buffer.	Make fresh wash buffer.

Positive Control: Low signal	Improper storage of the ELISA kit.	Upon receipt, the kit should be stored at -20°C. Store the positive control at -70°C after reconstitution.
	Stop solution	Stop solution should be added to each well before measurement and read OD immediately.
	Improper primary or secondary antibody dilution.	Ensure correct dilution.

Technical Support

For all technical or commercial enquiries please go to:
www.abcam.com/contactus
www.abcam.cn/contactus (China)
www.abcam.co.jp/contactus (Japan)