RayBio® Cell-Based Stat1 (Tyr701) ELISA kit

User Manual

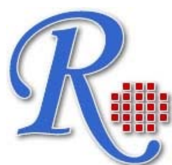
RayBio® Cell-Based Stat1 (Tyr701) ELISA Kit Protocol

Cat#: CBEL-STAT1-1 (96 Assays)
Cat#: CBEL-STAT1-2 (192 Assays)

RayBiotech, Inc.

We Provide You With Excellent Protein Array System And Service

Tel:(Toll Free)1-888-494-8555 or 770-729-2992; Fax:770-206-2393; web: www.raybiotech.com Email: info@raybiotech.com



RayBiotech, Inc.

RayBio® Cell-Based Stat1 (Tyr701)

ELISA Kit Protocol

TABLE OF CONTENTS

I. Introduction…….………………………………..2

II. Reagents…………………………..…………...3

III. Storage.………………………………………..4

IV. Additional Materials Required…………………...4

V. Reagent Preparation……………………………...4

VI. Assay Procedure……………………………........5

VII. Assay Procedure Summary……………………....7

VIII. Quality control data……………………………....8

IX. References………………………………………11

X. Troubleshooting Guide.………………………...12

I. INTRODUCTION

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 RayBio® Cell-Based Stat1 (Tyr701) 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 Stat1 (Tyr701) 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 Stat1protein phosphorylation in your experimental model system, you can verify pathway activation in your cell lines without spending excess time and effort in preparing cell lysate and performing an analysis of Western Blot. In the Cell-Based Stat1 (Tyr701) 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-Stat1 (Tyr701) or Anti-Stat1(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.

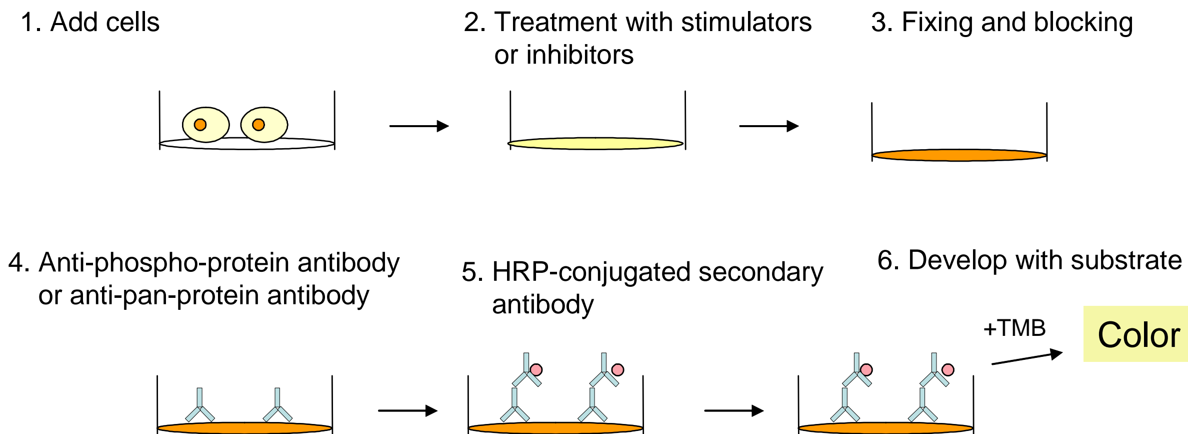


Fig.1. Cell-Based protein phosphorylation procedure

II. REAGENTS (96/192 assays per kit)

1. Microplate (Item A): one (96 assays)/two (192 assays) of 96 well tissue culture plate(s) (12 x 8 wells) for cell culture.
2. Wash Buffer Concentrate A (20x) (Item B): 30 ml of 20x concentrated buffer.
3. Wash Buffer Concentrate B (20x) (Item C): 30 ml of 20x concentrated buffer.
4. Fixing Solution (Item D): 30 ml of fixing solution.
5. Quenching Buffer Concentrate (Item E): 2 ml of 30x concentrated solution.
6. Blocking Solution (5x) (Item F): 20 ml of 5x concentrated solution.
7. Mouse Anti-Phospho-Stat1 (Tyr701) Concentrate (Item G): one (96 assays)/two (192 assays) tube(s) 7µl of 1000x concentrated anti-phospho-Stat1 (Tyr701).
8. Mouse Anti-Stat1Concentrate (Item H): one (96 assays)/two (192 assays) tube(s) 7 µl of 1000x Concentrated anti-Stat1.
9. Anti-Mouse IgG Concentrate (Item I): one (96 assays)/two (192 assays) of tube(s)10 µl 1000x concentrated HRP-conjugated anti-mouse IgG.

10. TMB One-Step Substrate Reagent (Item J): one (96 assays)/two (192 assays) of bottle(s) 12 ml of 3,3',5,5'-tetramethylbenzidine (TMB) in buffered solution.
11. Stop Solution (Item K): 14 ml of sulfuric acid.

III. STORAGE

Upon receipt, the kit should be stored at -20 ^{o}C. Please use within 6 months from the date of shipment. Item B, D, E, F, J and I should be stored at 4 ^{o}C to avoid repeated freeze-thaw cycles after initial use. Item G and H should be stored at -20 ^{o}C after use. Item I store at 2-8 ^{o}C for up to one month (store at -20 ^{o}C for up to 6 months, avoid repeated freeze-thaw cycles).

IV. ADDITIONAL MATERIALS REQUIRED

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 ^{o}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.

V. 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. Mouse Anti-Phospho-Stat1 (Tyr701) Concentrate (Item G): should be diluted 1000-fold with 1x Blocking Solution (Briefly spin the tube, Item G before use).
5. Mouse Anti-Stat1 Concentrate (Item H): should be diluted 1000-fold with 1x Blocking Solution (Briefly spin the tube, Item H before use).
6. Anti-Mouse IgG Concentrate (Item I): should be diluted 1000-fold with 1x Blocking Solution (Briefly spin the tube, Item I before use).

VI. ASSAY PROCEDURE:

1. Design your experiment. For example, see Fig2 and 3.

2. Seed 100 µl of 30,000 cells into each well in a 96 well plate and incubate for overnight at 37°C, 5% CO_2.

Notice: 1). The cell number used is dependent on cell lines and the relative amount of protein phosphorylation . More or less cells may be used.

2). Pre-coat the 96 well microplate (Item A) by adding 100 µl poly-L-Lysine (sigma, Cat#: P4832) into each well and then follow manufacturer's instructions if seeding HUVECs, HMEC-1 or other loosely attached cells. Or use CellBind or poly-lysine treated tissue culture plates from other brand.

3). The cells can be starved 4~24 hours dependent on cell lines prior to treatment (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.

1). Dissolve your inhibitors or activators into serum free cell culture medium and then treat the cells or according to manufacturer's instructions.

2).To avoid cell loss, do not dispense liquid directly onto the cell surface. Instead, gently touch the pipet tips to and gently dispense the liquid down the wall of cell culture wells. Flip the plate over a sink to remove wash buffer 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 min 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 -80°C for several days.

10. Add 50 µl of 1x Anti-Phospho-Stat1 (Tyr701) (Item G) or 1x

Anti-Stat1 (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 Anti-Mouse IgG (Item I) and incubate for 1 hour at room temperature.

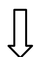
13. Wash 4 times with 1x Wash Buffer B (200 µl each), then tap the plate upside down to remove all of excess wash buffer.

14. Add 100 µl of TMB 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.

VII. ASSAY PROCEDURE SUMMARY

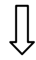
1. Seed 30,000 cells into each well and incubate overnight.



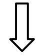
2. Apply various treatment, inhibitors or activators according to manufacturer's instructions.

3. Add 100 µl of Fixing Solution into each well and incubate for 20 minutes at room temperature with shaking.

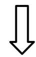
4. Add 200 µl of prepared 1x Quenching Buffer and incubate 20 minutes at room temperature.



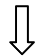
5. Add 200 µl of Blocking Solution and incubate for 1 hour at 37°C.



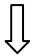
6. Add 50 µl of prepared 1x Anti-Phospho-Stat1 (Tyr701) or 1x Anti-Stat1 to each well and incubate for 2 hours at room temperature.



7. Add 50 µl of prepared 1x HRP-Anti-Mouse IgG and incubate for 1 hour.



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



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

VIII. QUALITY CONTROL DATA

Representative results of Cell-Based Stat1 (Tyr701) are shown below:

Note:

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

2. Added 50 µl different concentrations of rhEGF (0, 20 or 100 ng/ml, in serum free DMEM) to appropriate wells (shown below). Then incubated for 10, or 30 min 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 then followed on step 3 to

14 in part VI Assay Procedure.

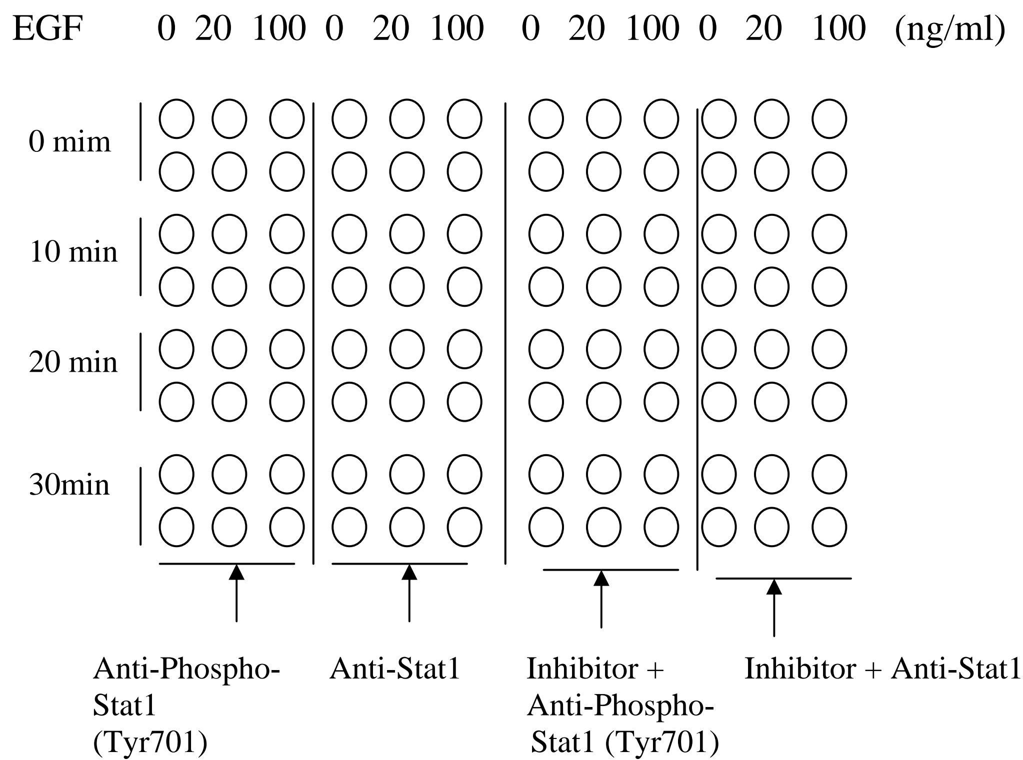


Fig. 2 Example of how to seed cells for RayBio® cell-based assay

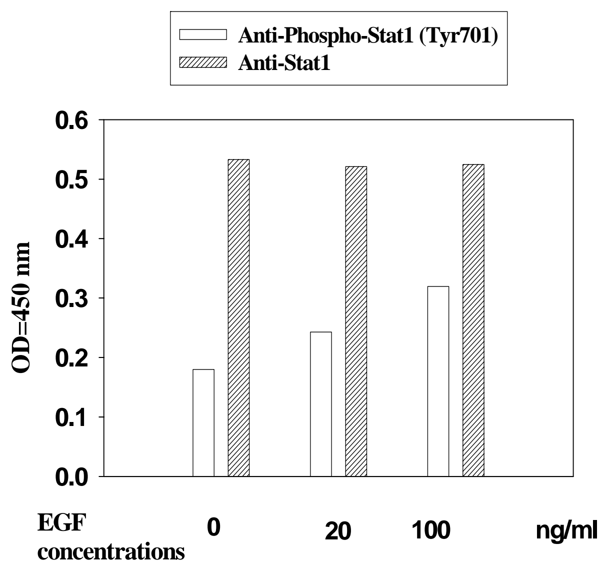


Fig. 3A. A431 cells were stimulated by different concentrations of EGF for 10 minutes at 37°C

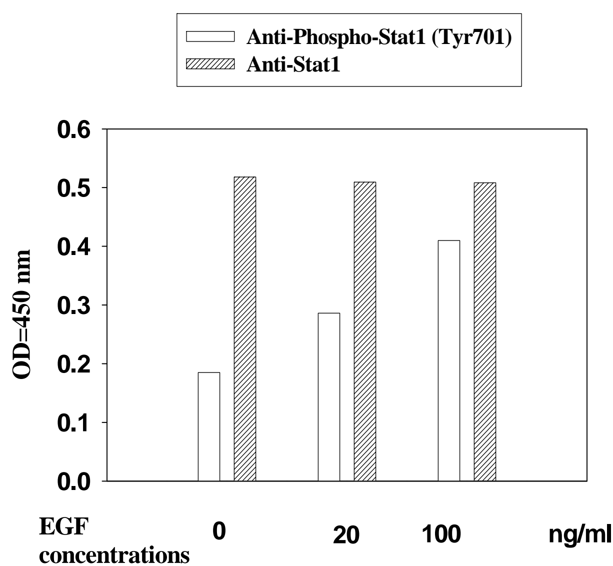


Fig. 3B. A431 cells were stimulated by different concentrations of EGF for 30 minutes at 37°C.

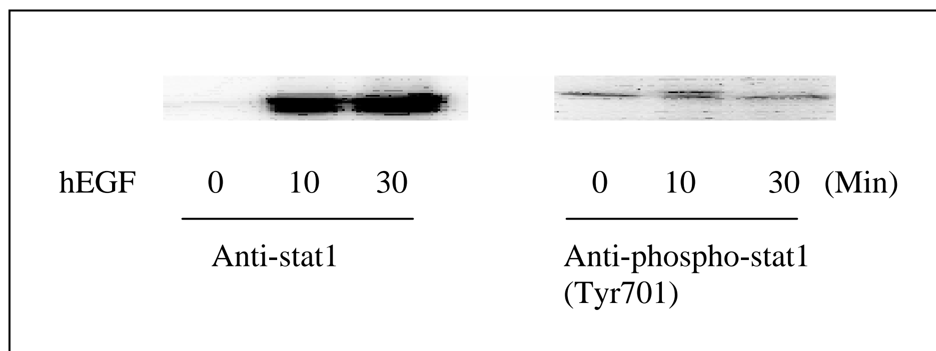


Fig. 4. Western blot analysis of extracts from 100 ng/ml hEGF treated A431 cells. Phospho-stat1 (Tyr701) and stat1 antibodies were used in both detection assays.

IX. REFERENCES:

1. Michael J. Clemens and Michael C. 1997. *Protein Phosphorylation in Cell Growth Regulation*. 1 Edition.

2. Fu, X.Y., et al. 1993 *Cell* 74:1135.

3. Darnell, J.E. 1997 *Science* 277:1630.

X: TROUBLESHOOTING GUIDE

Problem	Cause	Solution
1. Low signal	1. Improper storage of the ELISA kit	1. Store all of components according to manual instructions. Keep TMB substrate solution in dark
	2. Improper dilution	2. Ensure correct preparation of antibody and reagents
	3. Cells drop off from the wells	3. Some of treatments may make cells drop off from the wells. Reduce inhibitor or activator concentration.
2. High background	1. Inadequate washing	1. Be sure to remove all of washing solution and follow the recommendation for washing
	2. Too much cells	2. Reduce the cell number
3. Large CV	1. Inaccurate pipetting	1. Check pipette
	2. Remaining wash buffer in the well	2. Remove all of wash buffer
	3. Cells drop off from the wells	3. Please don't directly face the cells with tips when adding reagents or wash buffer.

Over 200 ELISA kits, custom ELISA kit choose from over 300 list visit www.raybiotech.com for details.

This product is for research use only.

©2004 RayBiotech, Inc.