

RayBio®
Cell-Based Phosphotyrosine
ELISA kit

User Manual

RayBio® Cell-Based
Phosphotyrosine ELISA Kit Protocol

(Cat#: CBEL-TYROSINE)



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 Phosphotyrosine
ELISA Kit Protocol**

TABLE OF CONTENTS

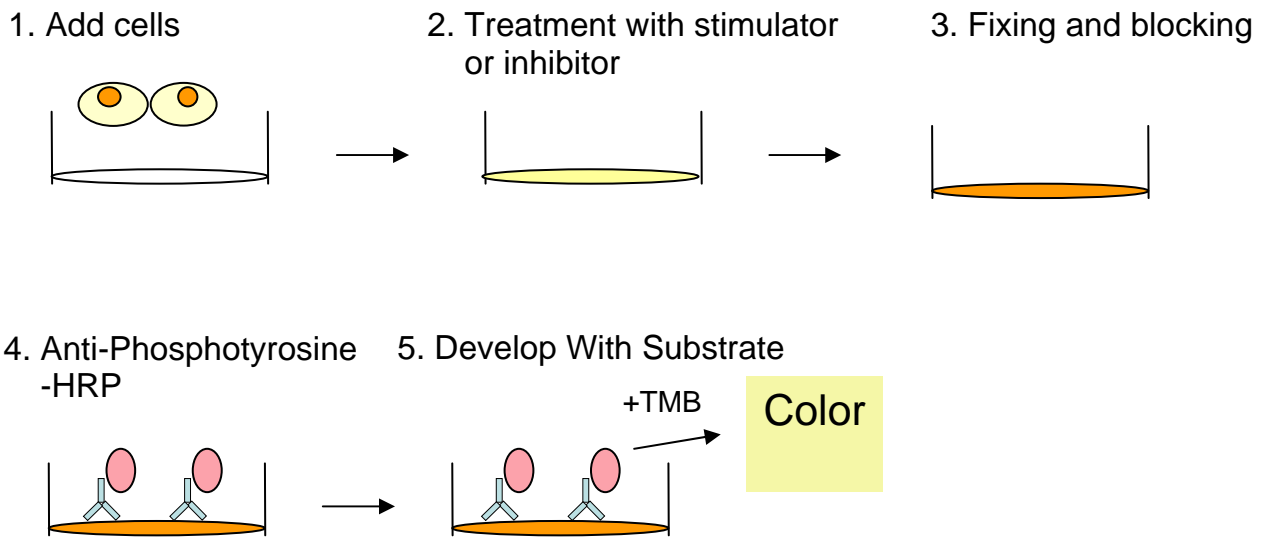
I.	Introduction.....	2
II.	Reagents.....	3
III.	Storage.....	4
IV.	Additional Materials Required.....	4
V.	Reagent Preparation.....	4
VI.	Assay Procedure.....	5
VII.	Assay Procedure Summary.....	7
VIII.	Quality control data.....	8
IX.	References.....	9
X.	Troubleshooting Guide.....	10

I. INTRODUCTION

Protein phosphorylation is instrumental in the regulation of protein activity within a cell. It plays important roles in the control of cell proliferation and differentiation. 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 Phosphotyrosin ELISA kit is a very rapid, convenient and sensitive assay kit which can monitor the activation or function of important biological pathways in cells. It can be used for measuring the relative amount of Phosphotyrosin and screen the effect of various treatment, inhibitors (such as siRNA or chemicals), or activators **in cultured human, mouse and rat cell lines**. By determining Phosphotyrosin protein in your experimental model system, you can verify pathway activation in your cell line without spending excess time and effort in preparing cell lysate and performing an analysis of Western Blot. In the Cell-Based EGFR (activated) ELISA kit, cells are seeded into a 96 well tissue culture plate. The cells are fixed after various treatment, inhibitors or activators. After blocking, HRP-Anti-Phosphotyrosin is pipetted into the wells and incubated. The wells are washed, 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.

Cell Based Phosphotyrosine procedure



II. REAGENTS

1. Microplate (Item A): two 96 well tissue culture plates (12 x 8 wells) for cell culture.
2. Wash Buffer A (20x) (Item B): 25 ml of 20x concentrated.
3. Wash Buffer B (20x) (Item C): 25 ml of 20x concentrated solution.
4. Fixing Solution (Item D): 30 ml of fixing solution.
5. Anti-Phosphotyrosine-HRP Concentrate (Item E): 14 μ l of 2000x concentrated HRP-conjugated anti-Phosphotyrosine.
6. Blocking Solution (5x) (Item F): 15 ml of 5x concentrated solution.
7. TMB One-Step Substrate Reagent (Item G): 24 ml of 3,3',5,5'-tetramethylbenzidine (TMB) in buffered solution.
8. Stop Solution (Item H): 14 ml of sulfuric acid.
9. Quenching Buffer Concentrate (Item I): 2 ml of 30x concentrated solution.

III. STORAGE

May be stored for up to 6 months at 2° to 8°C (Anti-Phosphotyrosine-HRP Concentrate should be stored at -20°C) from the date of shipment.

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°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.
2. If the Wash Buffer A (20x) or B (20x) 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.
3. Anti-Phosphotyrosine-HRP (2000x) (Item E) should be diluted 2000-fold with 1x Blocking Solution.
4. Blocking Solution (5x) (Item F): should be diluted 5-fold with deionized or distilled water.

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

VI. ASSAY PROCEDURE:

1. Seed 100 μ l of 20,000 cells into each well in a 96 well plate and incubate for overnight at 37°C, 5% CO₂.

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). Coat the 96 well microplate (Item A) by adding 100 μ l 0.1% Gelatin (sigma, Cat#: G-2500) into each well and incubate for 10 minutes at room temperature if seeding HUVECs or HMEC-1. Aspirate each well and then seed your cells into each well.

2. Apply the treatment of growth factors or cytokines according to manufacture's instructions. Discard the cell culture medium and wash 4 times with 1x Wash Buffer A (200 μ l each). 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). Please don't directly face the cells with tips when adding any reagents or wash buffers, because the cells may drop off the wells.

3. Add 100 μ l of Fixing Solution (Item D) into each well and incubate for 20 minutes at room temperature with shaking.

4. Wash the plate 3 times with 1x Wash Buffer A, then let the plate upside down to remove all of wash buffer.
5. Add 200 μ l of prepared 1x Quenching Buffer (Item I) and incubate 20 min at room temperature.
6. Wash the plate 4 times with 1x Wash Buffer A, then let the plate upside down to remove all of wash buffer.
7. Add 200 μ l of prepared 1x Blocking Solution (Item F) and incubate for 1 hour at 37°C.
8. Wash 3 times with 1x Wash Buffer B (200 μ l each), then tap the plate upside down to remove all of excess wash buffer.
9. Add 100 μ l of prepared anti-Phosphotyrosine-HRP to each well and incubate for 1 hour at room temperature with shaking.
10. Wash 4 times with 1x Wash Buffer B (200 μ l each), then tap the plate upside down to remove all of excess Wash Buffer.
10. Add 100ul of TMB to each well and incubate for 30 minutes with shaking at room temperature in the dark.
11. Add 50ul of stop solution to each well and read at 450 nm, measure immediately.

VII. ASSAY PROCEDURE SUMMARY

1. Seed 20,000 cells into each well and incubate overnight.



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



3. Add 100 μ l of fixing solution into each well and incubate for 10 minutes at room temperature.



4. Add 200 μ l of prepared 1x Quenching Buffer and incubate 20 min at RT.



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



6. Add 100 μ l of Anti-Phosphotyrosine-HRP and incubate for 1 h at room temperature.



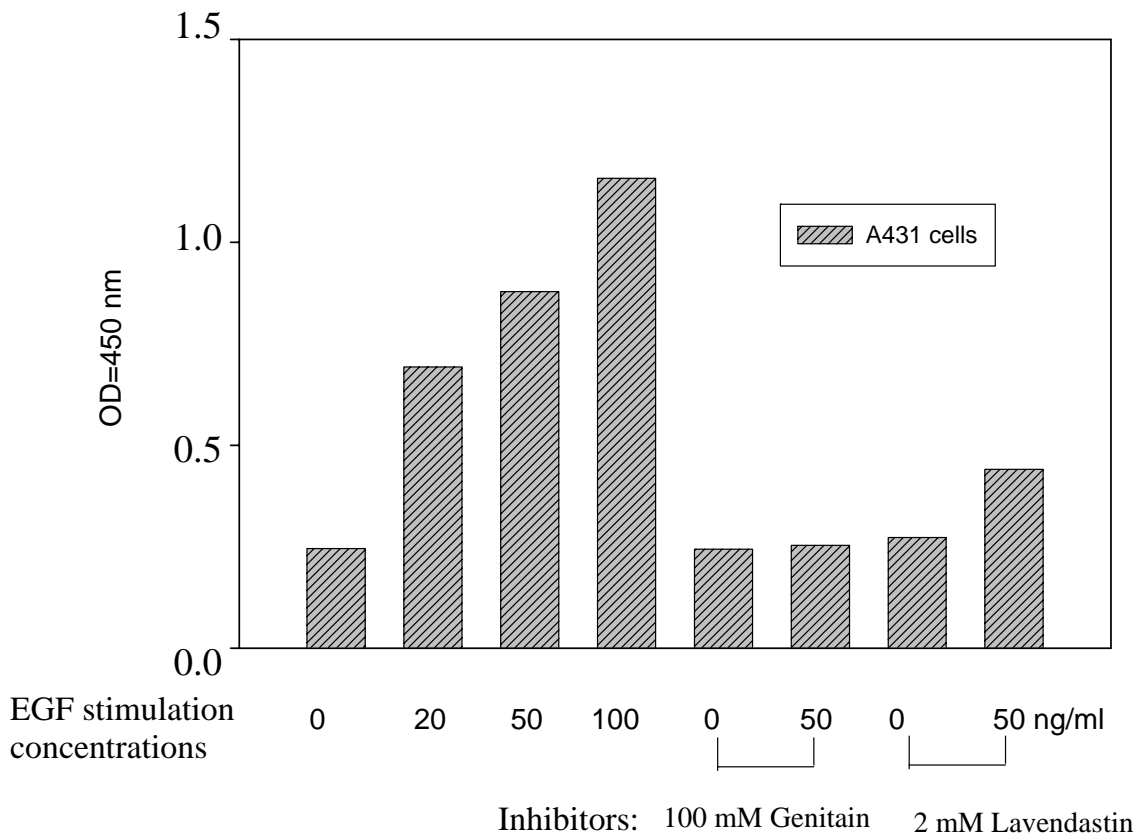
7. Add 100 μ l TMB One-Step Substrate Reagent to each well.
Incubate 30 minutes at room temperature.



8. Add 50 μ l Stop Solution to each well.
Read at 450 nm immediately.

VIII. QUALITY CONTROL DATA

Representative results of cellular phosphotyrosine are shown below:



1. Seed 30,000 A431 cells into appropriate well in microplate. Cells were incubated at 37°C in 5% CO₂ over night.

2. The cells were treated for 30 min with 50 μ l of 100 mM Genistein or 2 mM Lavendustin in appropriate wells at room temperature prior to EGF stimulation.
3. Added 50 μ l different concentrations of rhEGF (0, 20 or 100 ng/ml, in serum free DMEM) to appropriate wells (shown below). Then incubated for 10 min at 37°C .
4. Discarded the solution and wash 3 times with 1x Wash Buffer A (200 μ l each) immediately. Then tap the plate upside down to remove all of excess wash buffer. And then follow on part 3 to 14 in VI. Assay Procedure.

IX. REFERENCES

1. Gingras, M.-C., et al. 2002 *Mol. Cell Biol.* 22:41-56.
2. Ruff-Jamison, S., et al. 1991 *J. Biol. Chem.* 266:6607.
3. Maile, L. A., and Clemmons, D. R. 2002 *J. Biol. Chem.* 277:8955-8960.

X: TROUBLESHOOTING GUIDE

Problem	Cause	Solution
1. Low signal	1. Improper storage of the ELISA kit	1. Item E should be store at -20 °C, other at 4°C. Keep 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 produce cells drop off from the wells. Reducing 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 buiffer 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 addin reagents or wash buffer.

Over 100 ELISA kits, custom ELISA kit choose from over 300 list visit www.raybiotech.com for details.

Notes:

This product is for research use only.



©2004 RayBiotech, Inc.