RayBio®
Cell-Based Phosphotyrosine ELISA kit

User Manual

RayBio® Cell-Based Phosphotyrosine ELISA Kit Protocol

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

RayBiotech, Inc.

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I. INTRODUCTION

Protein phosphorylation is instrumental in the regulation of protein activity within a cell. It plays important roles in the living cell 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 Phosphotyrosine 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 Phosphotyrosin and screen the effects of various treatments, 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 Phosphotyrosin ELISA kit, cells are seeded into a 96 well tissue culture plate. The cells are fixed after various treatments, 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.

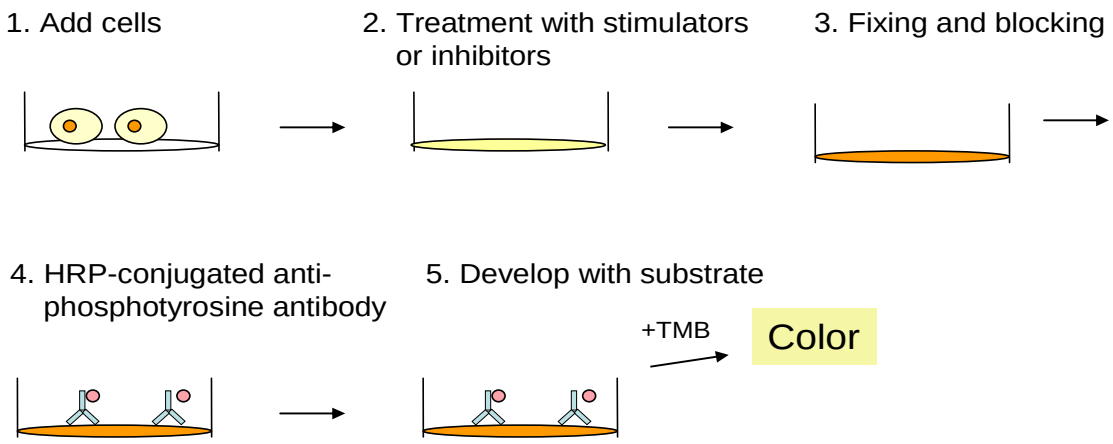

Fig.1. Cell-Based protein phosphorylation procedure

II. REAGENTS (96/192 Assays per kit)

1. Microplate (Item A): one (96 assays)/two (192 assays) 96 well tissue culture plate(s) (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): one (96 assays)/two (192 assays) tube(s) 7 µl 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): one (96 assays)/ two (192 assays) bottle(s) of 12 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

Upon receipt, the kit should be stored at -20 °C. Please use within 6 months from the date of shipment. Item B, C, D, F, G, H and I should be stored at 4 °C to avoid repeated freeze-thaw cycles after initial use (Item E should be stored -20 °C).

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 (Briefly spin the tube, Item E before use).

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 µl of 10,000~30,000 cells into each well in a 96 well plate and incubate for overnight at 37^{o}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).

2. Apply the treatments 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 µl each). Then tap the plate upside down to remove all of excess Wash Buffer A.

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.

3. Add 100 µl 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 tap the plate upside down to remove all of wash buffer.

5. Add 200 µl 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 tap the plate upside down to remove all of wash buffer.

7. Add 200 µl of prepared 1x Blocking Solution (Item F) and incubate for 1 hour at 37^{o}C.

8. Wash 3 times with 1x Wash Buffer B (200 µl each), then tap the plate upside down to remove all of excess wash buffer.

9. Add 100 µl of prepared anti-Phosphotyrosine-HRP to each well and incubate for 1 hour at room temperature with shaking.

   Note: The plate may be stored at -80^{o}C for several days.

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

11. Add 100ul of TMB to each well and incubate for 30 minutes with

shaking at room temperature in the dark.

12. Add 50ul of stop solution to each well and read at 450 nm, measure immediately.

VII. ASSAY PROCEDURE SUMMARY

1. Seed 10,000~30,000 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.

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

5. Add 200 µl of Blocking Solution and incubate for 1 hour at 37^{o}C.

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

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

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

VIII. QUALITY CONTROL DATA

Representative results of cellular phosphotyrosine are shown below:

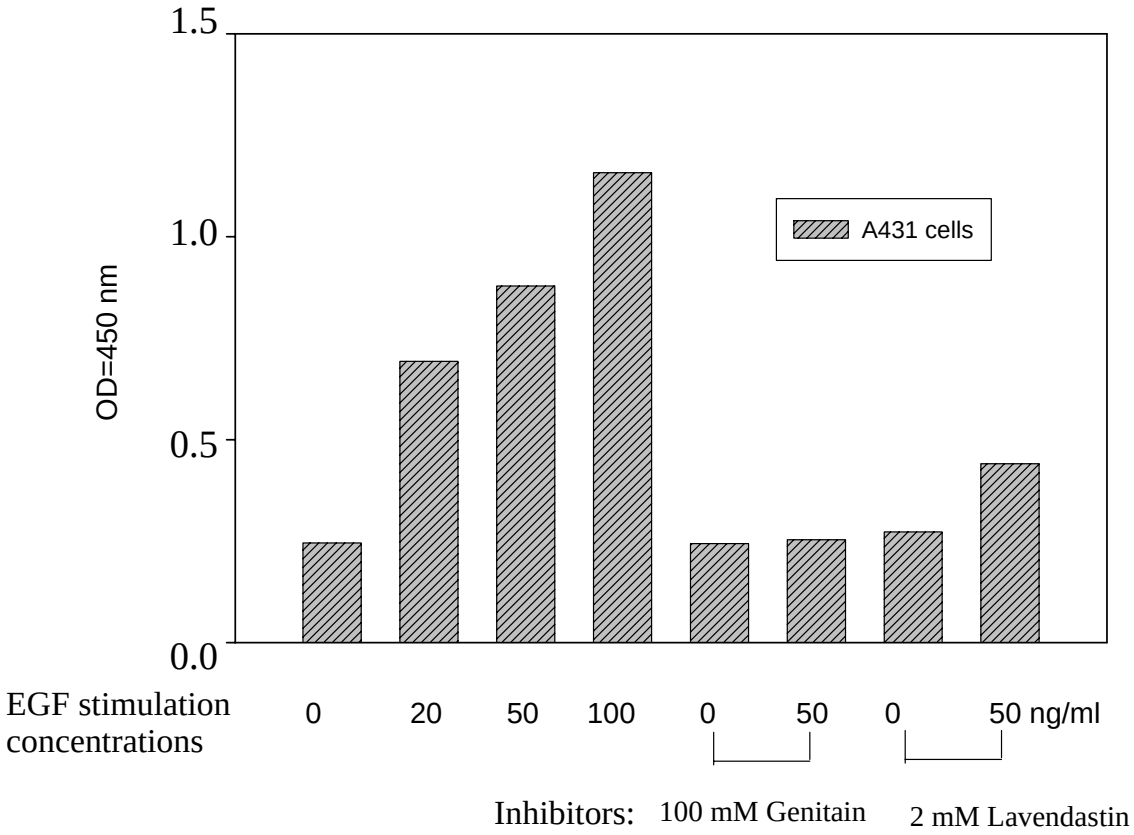

Fig 2. A431 cells were treated for 30 min with 50 µl of 100 mM Genistein or 2 mM Lavendustin in appropriate wells at room temperature prior to EGF stimulation. Added 50 µl different concentrations of rhEGF (0, 20 or 100 ng/ml in serum free DMEM) to appropriate wells. Then incubated for 10 min at 37^{o}C .

Note:

1. Seeded 10,000 A431 cells into appropriate well in microplate. Cells were incubated at 37°C in 5% CO_2 over night.

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

3. The cells were treated for 30 min with 50 µl of 100 mM Genistein or 2 mM Lavendustin in appropriate wells at room temperature prior to EGF stimulation.

4. 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 min at 37°C .

5. Discarded the solution and wash 3 times with 1x Wash Buffer A (200 µl 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. 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.

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Notes:

This product is for research use only.