

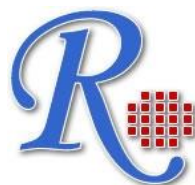
RayBio® Phospho-AKT/GSK3β/mTor ELISA Kit

**For measuring phosphorylated AKT (S473), GSK3β (S9),
mTOR (S2448) and Total AKT, GSK3β, mTOR
in human and mouse cell lysates.**

**User Manual
(Revised Nov. 20th, 2018)**

**RayBio® Phospho-AKT/GSK3β/mTor ELISA Kit
Protocol**

(Cat#: PEL-AKT-SK)

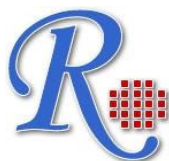


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I. INTRODUCTION

RayBio® Phospho-AKT/GSK3 β /mTor ELISA Kit is a very rapid, convenient and sensitive assay kit that can monitor the activation or function of important biological pathways in human and mouse cell lysates. By determining phosphorylated AKT, GSK3 β and mTOR 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 Blotting analysis.

This Sandwich ELISA kit is an in vitro enzyme-linked immunosorbent assay for the measurement of human and mouse phospho-AKT, total AKT, phospho-GSK3 β , total GSK3 β , phospho-mTOR and total mTOR. For each target, a capture antibody has been coated onto microwells. Samples are pipetted into the wells and target protein present in a sample is bound to the wells by the immobilized antibody. The wells are washed and a detection antibody is used to detect the captured target protein. After washing away unbound antibody, an HRP-conjugated secondary antibody 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 target protein bound. The Stop Solution changes the color from blue to yellow, and the intensity of the color is measured at 450 nm.

II. MATERIAL PROVIDED

1. 1 Microplates (Item A): 96 wells (12 strips x 8 wells) coated with anti-pan AKT (column 1-4), GSK3 β (column 5-8) and mTOR (column 9-12) antibodies.
2. Wash Buffer Concentrate (20x) (Item B): 25 ml of 20x concentrated solution.
3. Assay Diluent (Item E2): 15 ml of 5x concentrated buffer. For diluting cell lysate sample, detection antibody (Item C) and HRP-conjugated IgG Concentrate (Item D).
4. Detection Antibody Akt (Ser473) (Item C-1): 1 vial of rabbit anti-phospho-Akt (Ser473) (1 vial is enough to assay 3 strips).
5. Detection Antibody Akt (Item C-2): 1 vial of rabbit anti-Akt (1 vial is enough to assay 3 strips).
6. Detection Antibody GSK3 β (S9) (Item C-3): 1 vial of rabbit anti-GSK3b (S9) (1 vial is enough to assay 3 strips).
7. Detection Antibody GSK3 β (Item C-4): 1 vial of rabbit anti-GSK3 β (1 vial is enough to assay 3 strips).
8. Detection Antibody mTOR (S2448) (Item C-5): 1 vial of rabbit anti-mTOR (S2448) (1 vial is enough to assay 3 strips).
9. Detection Antibody mTOR (Item C-6): 1 vial of **biotinylated** anti-mTOR (1 vial is enough to assay 3 strips).
10. HRP-conjugated Anti-rabbit IgG (Item D-1), 25 μ l of 1000x concentrated HRP-conjugated anti-rabbit IgG.
11. HRP-conjugated Streptavidin (Item G), 200 μ l of 100x concentrated HRP-conjugated Streptavidin.
12. TMB One-Step Substrate Reagent (Item H): 12 ml of 3,3',5,5'-tetramethylbenzidine (TMB) in buffered solution.
13. Stop Solution (Item I): 8 ml of 0.2 M sulfuric acid.
14. Cell Lysate Buffer (Item J): 5 ml 2x cell lysis buffer (not including protease and phosphatase inhibitors).
15. Positive Control 3T3S001-1 (Item K): 1 vial of lyophilized powder from NIH3T3 cell lysate.

III. STORAGE

Upon receipt, the kit should be stored at -20°C . Please use within 6 months from the date of shipment. After initial use, Wash Buffer Concentrate (Item B), Assay Diluent (Item E2), TMB One-Step Substrate Reagent (Item H), Stop Solution (Item I) and Cell Lysate 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 . Item D store at $2-8^{\circ}\text{C}$ for up to one month (store at -20°C for up to 6 months, avoid repeated freeze-thaw cycles). Reconstituted Positive Control (Item K) should be stored at -70°C .

IV. ADDITIONAL MATERIALS REQUIRED

1. Microplate reader capable of measuring absorbance at 450 nm.
2. Protease and Phosphatase inhibitors.
3. Shaker.
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. Distilled or deionized water.
8. Tubes to prepare sample dilutions.

V. SAMPLE PREPARATION

Cell lysates - Rinse cells with PBS, making sure to remove any remaining PBS before adding the lysis buffer. Solubilize cells at 4×10^7 cells/ml in 1x Lysis Buffer (we recommend adding protease and phosphatase inhibitors to lysis buffer prior to sample preparation). Pipette up and down to resuspend and incubate the lysates with shaking at $2 - 8^{\circ}\text{C}$ for 30 minutes.

Microcentrifuge at 13,000 rpm for 10 minutes at $2 - 8^{\circ}\text{C}$, 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.

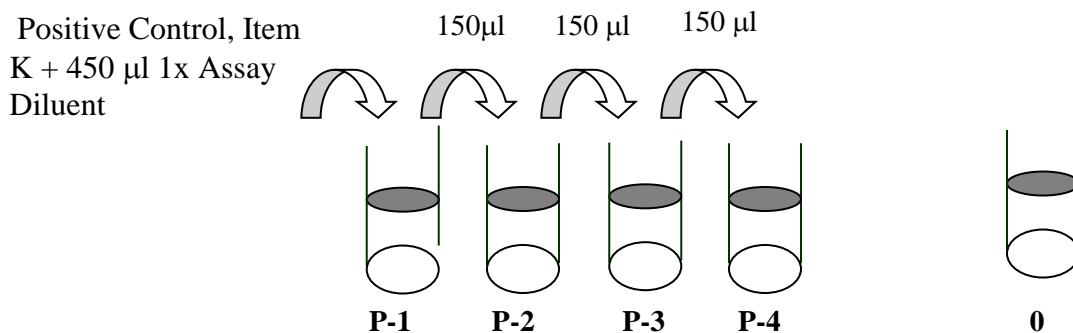
For the initial experiment, we recommend a serial dilution, such as 5-fold to 50-fold, for your cell lysates with Assay Diluent (Item E2) before use.

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

VI. REAGENT PREPARATION

1. Bring all reagents and samples to room temperature (18 - 25°C) before use.
2. Assay Diluent (Item E2) should be diluted 5-fold with deionized or distilled water before use.
3. Preparation of Positive Control: Briefly spin the Positive Control vial of Item K. Add 450 μ l 1x Assay Diluent (Item E2) into Item K to prepare Positive Control (P-1) solution. Dissolve the powder thoroughly by a gentle mix (it can be removed by centrifuge if any precipitate in the solution is found). Pipette 300 μ l 1x Assay Diluent into each tube. Use the Positive Control (P-1) solution to produce a dilution series (shown below). Mix each tube thoroughly before the next transfer. 1x Assay Diluent serves as the background. (See i. Positive Control of part IX. TYPICAL DATA for a typical result on page 10).



4. 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.
5. Briefly spin the detection antibody (Item C) before use. Add 100 μ l of 1x Assay Diluent into the vial to prepare a 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 detection antibody concentrate should be diluted 30-fold with 1x Assay Diluent and used in step 4 of Part VII Assay Procedure.
6. Briefly spin the HRP-conjugated anti-rabbit or Streptavidin (Item D-1 or G) before use. HRP-conjugated anti-rabbit IgG concentrate should be diluted 1000-fold with 1x Assay Diluent or HRP-conjugated Streptavidin concentrate should be diluted 100-fold with 1x Assay Diluent.

For example: Briefly spin the vial. Add 5 μ l 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 1000-fold diluted HRP-conjugated anti-rabbit IgG solution. Mix well.

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

VII. ASSAY PROCEDURE:

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.
2. Add 100 µl of each sample 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 pan antibodies:

	<u>Anti-AKT</u>				<u>Anti-GSK3b</u>				<u>Anti-mTOR</u>			
	1	2	3	4	5	6	7	8	9	10	11	12
A	○	○	○	○	○	○	○	○	○	○	○	○
B	○	○	○	○	○	○	○	○	○	○	○	○
C	○	○	○	○	○	○	○	○	○	○	○	○
D	○	○	○	○	○	○	○	○	○	○	○	○
E	○	○	○	○	○	○	○	○	○	○	○	○
F	○	○	○	○	○	○	○	○	○	○	○	○
G	○	○	○	○	○	○	○	○	○	○	○	○
H	○	○	○	○	○	○	○	○	○	○	○	○

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 detection antibody (Reagent Preparation step 5) to appropriate wells. Incubate for 1 hour at room temperature with shaking.

5. Discard the solution. Repeat the wash as in step 3.
6. Add 100 μ l of prepared 1x HRP-conjugated anti-rabbit IgG against anti-AKT, anti-AKT(S473), anti-GSK3 β , anti-GSK3 β (S9) and anti-mTOR(S2448)

or 1x HRP-conjugated **Streptavidin against biotinylated anti-mTOR** (see Reagent Preparation step 6) to corresponding wells. Incubate for 1 hour at room temperature with shaking.
7. Discard the solution. Repeat the wash as in step 3.
8. Add 100 μ l 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 μ l of Stop Solution (Item I) to each well. Read at 450 nm immediately.

VIII. ASSAY PROCEDURE SUMMARY

1. Prepare all reagents, samples and standards as instructed.



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



3. Add 100 μ l prepared primary antibody to each well.
Incubate 1.0 hours at room temperature.



4. Add 100 μ l prepared 1X HRP-Conjugated antibody solution.
Incubate 1 hour at room temperature.



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



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

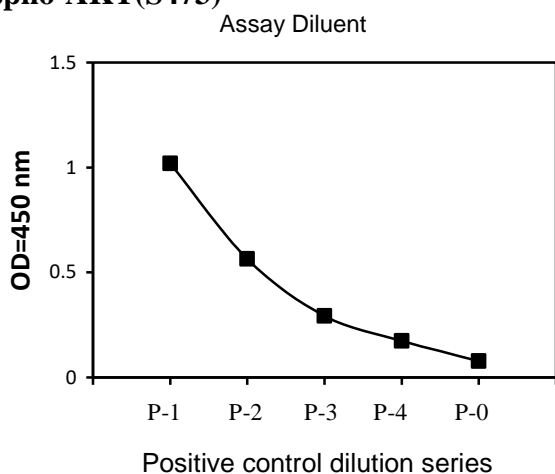
IX. TYPICAL DATA

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

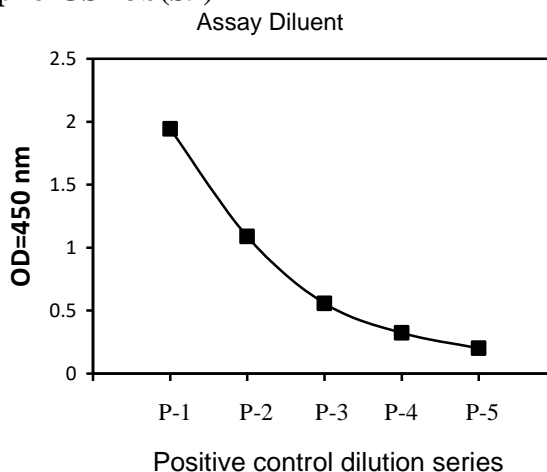
i. Positive Control

NIH 3T3 cells were treated with recombinant human PDGFBB at 37°C for 10 min. Solubilize cells at 4×10^7 cells/ml in Cell Lysate Buffer. Serial dilutions of lysates were analyzed in this ELISA. Please see step 3 of Part VI Reagent Preparation for detail.

Phospho-AKT(S473)



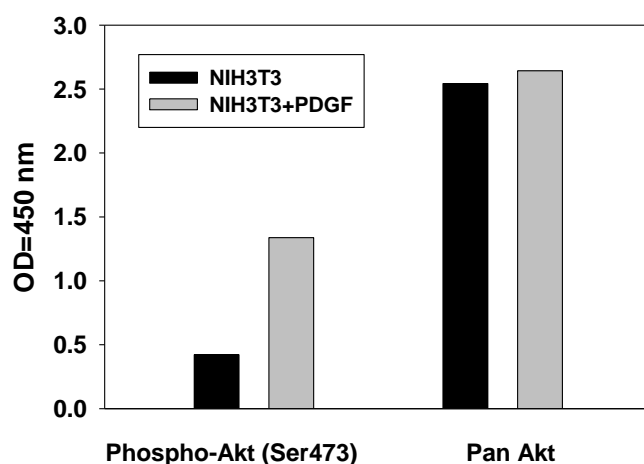
Phospho-GSK3b(S9)



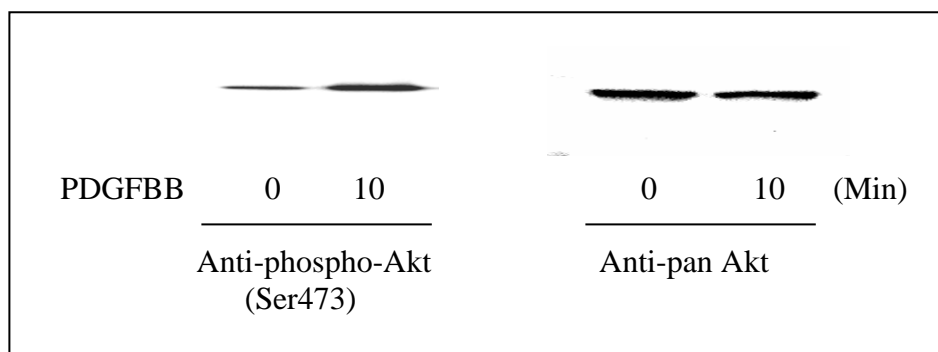
ii. Recombinant Human PDGFBB Stimulation of NIH 3T3 Cell Lines

NIH 3T3 cells were treated or untreated with 50 ng/ml recombinant human PDGFBB for 10 min. Cell lysates were analyzed using this phosphoELISA and Western Blot.

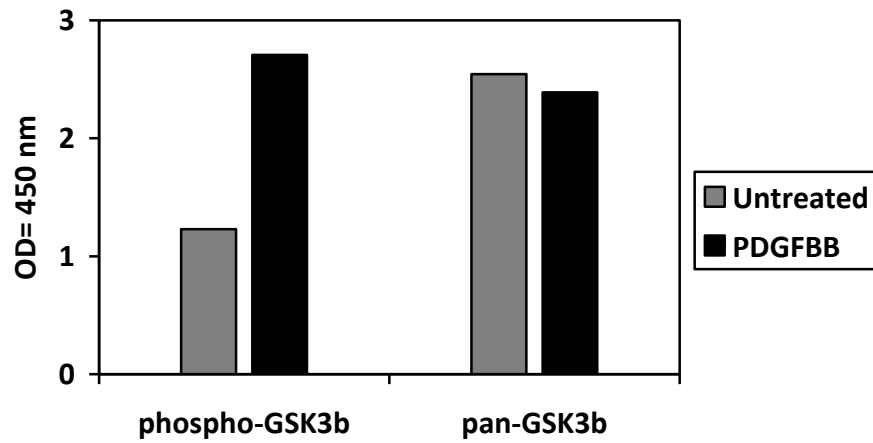
A). ELISA Analysis of Phospho-AKT



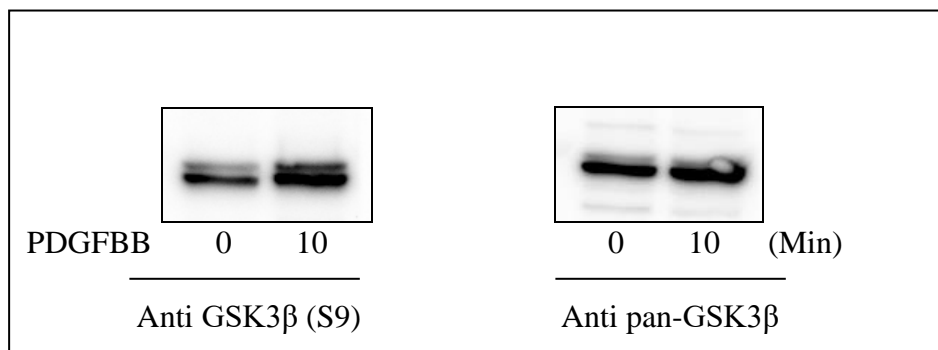
B). Western-Blot Analysis of Phospho-AKT



A). ELISA Analysis of Phospho-GSK3 β



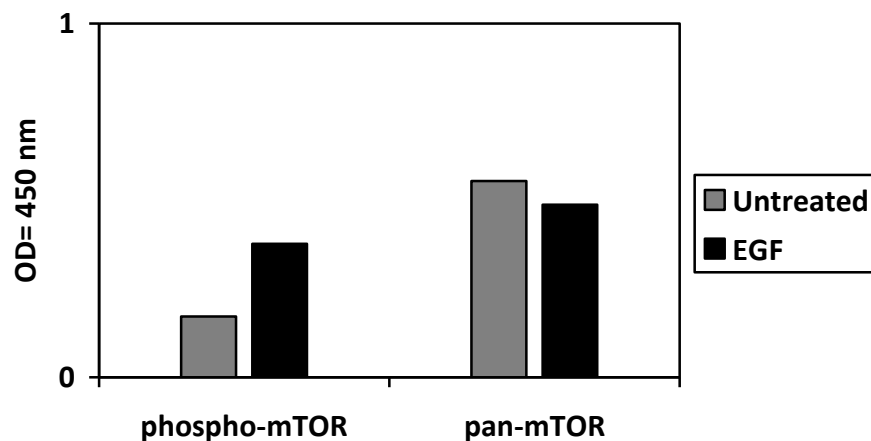
B). Western-Blot Analysis of Phospho-GSK3 β



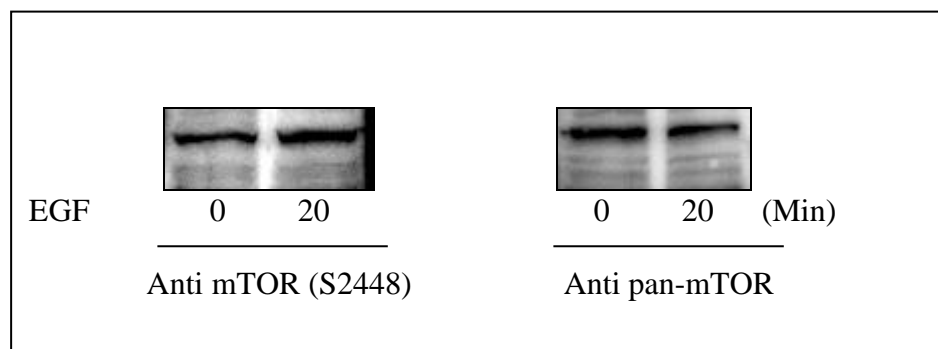
iii. Recombinant Human EGF Stimulation of A431 Cell Lines

A431 cells were treated or untreated with 100 ng/ml recombinant human EGF for 20 min. Cell lysates were analyzed using this phosphoELISA and Western Blot.

A). ELISA Analysis of Phospho-mTOR



B). Western-Blot Analysis of Phospho-mTOR



X. TROUBLESHOOTING GUIDE

Problem	Cause	Solution
1. Sample signals: a. Too low b. Too high	a. Sample concentration is too low b. Sample concentration is too high	a. Increasing sample concentration b. Reducing sample concentration
2. Large CV	a. Inaccurate pipetting	a. Check pipettes
3. High background	a. Plate is insufficiently washed b. Contaminated wash buffer	a. Review the manual for proper washing. If using an automated plate washer, check that all ports are unobstructed. b. Make fresh wash buffer
4. Low positive control signal	a. Improper storage of the ELISA kit b. Stop solution c. Improper primary or secondary antibody dilution	a. Upon receipt, the kit should be stored at -20°C. Store the positive control at -70°C after reconstitution. b. Stop solution should be added to each well before measurement and read OD immediately. c. Ensure correct dilution

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