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Phospho-AKT/GSK3ß/mTor ELISA Kit

Catalog #: PEL- AKT-SK

User Manual
Last Revised: June 12, 2024

Introduction

RayBio® Phospho-AKT/GSK3ß/mTor ELISA kit is an in vitro enzyme-linked immunosorbent assay for the measurement of human and mouse phospho-AKT, total AKT, phospho-GSK3β, total GSK3β, 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.

Storage / Stability

The entire kit may be stored at -20°C for up to 6 months from the date of shipment. Avoid repeated freeze-thaw cycles. For prepared reagent storage, see kit contents on the next page.

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Kit Components

Name	Catalog #	Size / Qty	Description	Storage / Stability After Preparation
Phospho-AKT/GSK3ß/mTor Microplate	PEL-AKT-SK-A	96 wells	Microplate coated with anti-pan AKT (column 1-4), GSK3β (column 5-8) and mTOR (column 9-12) antibodies.	1 month at -20°C*
Positive Control	3T3S001-1	1 vial	Lyophilized powder from treated NIH3T3 cell lysate.	1 week at -80°C
Anti-phospho AKT Detection Antibody	PEL-AKT-SK-C1	1 vial	Rabbit antiphospho-Akt (Ser473) (1 vial is enough to assay 3 strips)	5 days at 4°C
Anti-AKT Detection Antibody	PEL-AKT-SK-C2	1 vial	Rabbit anti-Akt (1 vial is enough to assay 3 strips)	5 days at 4°C
Anti-phospho GSK3β Detection Antibody	PEL-AKT-SK-C3	1 vial	Rabbit antiGSK3β (S9) (1 vial is enough to assay 3 strips)	5 days at 4°C
Anti-GSK3β Detection Antibody	PEL-AKT-SK-C4	1 vial	Rabbit anti-GSK3β (1 vial is enough to assay 3 strips)	5 days at 4°C
Anti-phospho mTOR Detection Antibody	PEL-AKT-SK-C5	1 vial	Rabbit anti-mTOR (S2448) (1 vial is enough to assay 3 strips)	5 days at 4°C
Anti-mTOR Detection Antibody	PEL-AKT-SK-C6	1 vial	Rabbit anti-mTOR (1 vial is enough to assay 3 strips)	5 days at 4°C
HRP-Conjugated anti-rabbit IgG	EL-ItemD1	25 µl	500x concentrated HRP-conjugated anti-rabbit IgG.	Do not store and reuse.
Wash Buffer	EL-ITEMB	25 ml	20X concentrated wash buffer	1 month at 4°C
Assay Diluent B	EL-ITEME	15 ml	5X concentrated assay diluent	1 month at 4°C
Lysis Buffer	EL-Lysis	5 ml	2X cell lysate buffer	1 month at 4°C
TMB One-Step Substrate Reagent	EL-TMB	12 ml	3,3,5,5'-tetramethylbenzidine (TMB) in buffer solution	N/A
Stop Solution	EL-STOP	8 ml	0.2 M sulfuric acid	N/A

*Return unused wells to the pouch containing desiccant pack, reseal along entire edge.

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Additional Materials Required

- Microplate reader capable of measuring absorbance at 450 nm
- Protease and Phosphatase inhibitors.
- Precision pipettes to deliver 2 µl to 1 ml volumes
- Adjustable 1-25 ml pipettes for reagent preparation
- 100 ml and 1 liter graduated cylinders
- Log-log graph paper or computer and software for ELISA data analysis.
- Absorbent paper
- Tubes to prepare standard or sample dilutions
- Distilled or deionized water
- Shaker

Sample Preparation

For the initial experiment, we recommend a serial dilution, such as a 5-fold to 50-fold dilution, for your cell lysates with prepared Assay Diluent (see Reagent Preparation step 2) 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.

Reagent Preparation

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

2. Assay Diluent B should be diluted 5-fold with deionized or distilled water before use.

3. Lysis Buffer should be diluted 2-fold with deionized or distilled water (for cell lysate and tissue lysate). We also recommend the addition of protease and phosphatase inhibitors (not included) to the lysis buffer prior to use.

4. Preparation of Positive Control: Briefly spin the Positive Control Vial. Add 450µl of prepared 1X Assay Diluent to positive control vial. Gently mix the powder to allow it to dissolve thoroughly. If a precipitate is seen in the solution after mixing, this can be removed by a quick centrifuge of the positive control vial, and then pipetting the supernatant only for the assay.

5. If the Wash Buffer (20X) concentrate 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.

6. Preparation of Detection Antibody: Briefly spin the vial of the detection antibody before use. Add 100 µl of 1X Assay Diluent into the vial to prepare a detection antibody

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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 30-fold with 1X Assay Diluent and used in step 5 of the Assay Procedure.

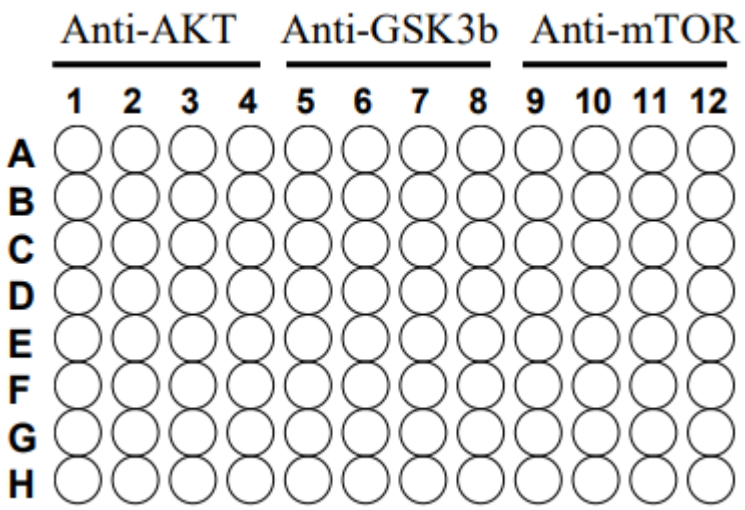
7. Preparation of HRP-conjugated anti-rabbit IgG: Briefly spin the vial of HRP-conjugated anti-rabbit IgG concentrate before use. HRP-conjugated anti-rabbit IgG should be diluted 500-fold with 1X Assay Diluent and used in step 7 of the Assay Procedure.

Assay Procedure

1. Bring all reagents and samples to room temperature (18 - 25ºC) before use. It is recommended to run all positive controls and samples in at least duplicate.

2. Label removable 8-well strips as appropriate for your experiment.

3. Add 100 µl of each sample or positive control (see Reagent Preparation step 4) and sample into appropriate wells. Cover the wells and incubate for 2.5 hours at room temperature or overnight at 4ºC with gentle shaking.



4. 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 auto-washer. Complete removal of liquid at each step is essential for 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.

5. Add 100 µl of prepared 1X detection antibody (see Reagent Preparation step 6) to each well. Incubate for 1 hour at room temperature with gentle shaking.

6. Discard the solution. Repeat the wash as in step 4.

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7. Add 100 µl of prepared HRP-conjugated anti-rabbit IgG solution against anti-AKT, anti-AKT(S473), anti-GSK3β, anti-GSK3β (S9), anti-mTOR and anti-mTOR(S2448) (see Reagent Preparation step 7) to corresponding wells. Incubate for 1 hour at room temperature with gentle shaking.
8. Discard the solution. Repeat the wash as in step 4.
9. Add 100 µl of TMB One-Step Substrate Reagent to each well. Incubate for 30 minutes at room temperature in the dark with gentle shaking.
10. Add 50 µl of Stop Solution to each well. Read at 450 nm immediately.

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Assay Procedure Summary

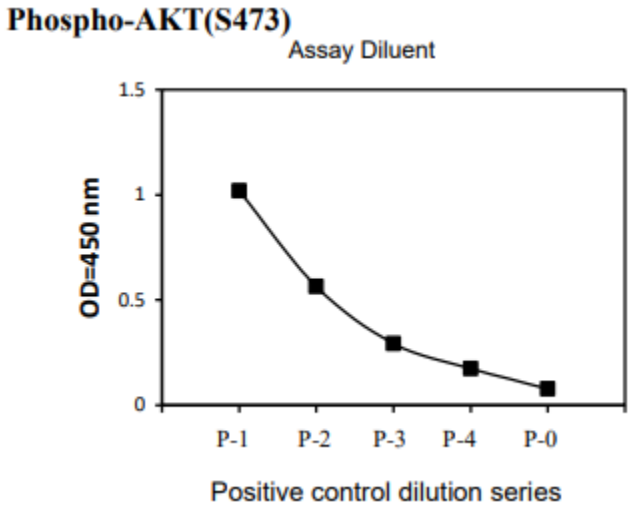
1. Prepare all reagents, samples and standards as instructed.
2. Add 100 µl of each positive control and sample to each well. Incubate 2.5 hours at room temperature or overnight at 4ºC with gentle shaking.
3. Add 100 µl prepared detection antibody to each well. Incubate for 1 hour at room temperature with gentle shaking.
4. Add 100 µl prepared HRP-conjugated anti-rabbit solution. Incubate for 1 hour at room temperature with gentle shaking.
5. Add 100 µl TMB One-Step Substrate Reagent to each well. Incubate 30 minutes at room temperature.
6. Add 50 µl Stop Solution to each well. Read at 450 nm immediately.

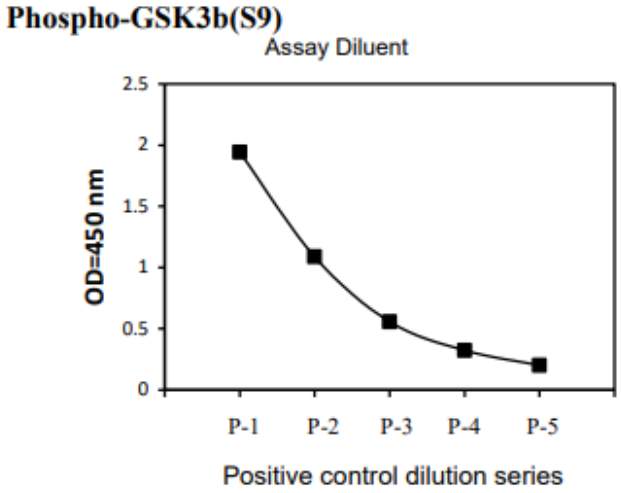
Typical Data

Calculate the mean absorbance for each set of duplicate positive controls and samples and subtract the average zero standard optical density.

A. Positive Control

NIH 3T3 cells were treated with recombinant human PDGFBB at 37oC for 10 min. Solubilize cells at 4 x 10^7 cells/ml in Cell Lysate Buffer. Serial dilutions of lysates were analyzed in this ELISA.





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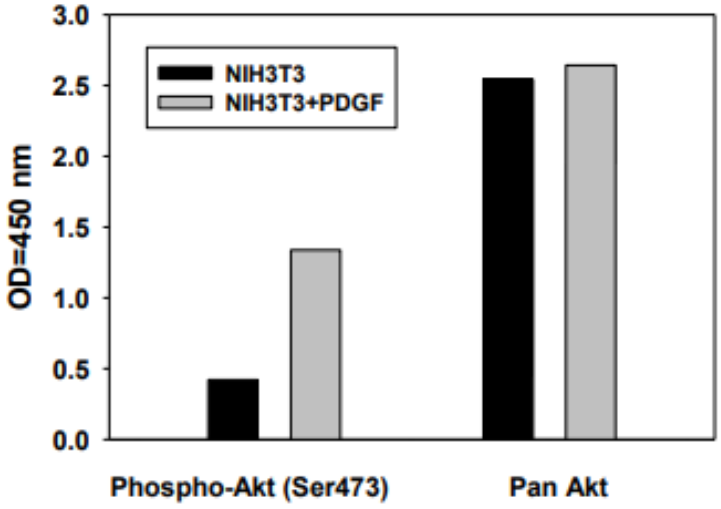
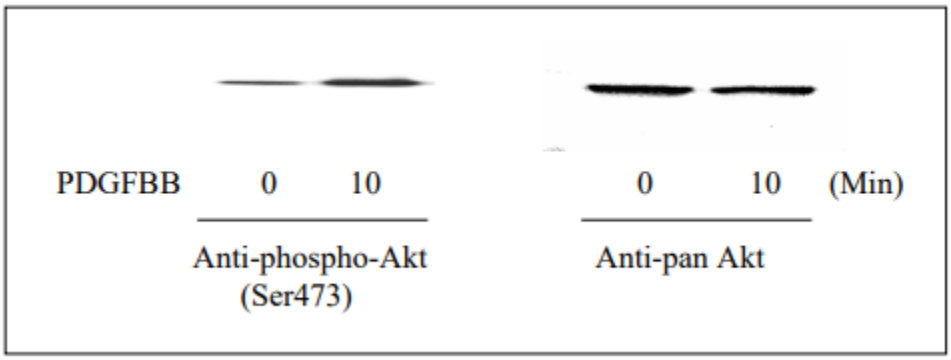
B. Recombinant Human PDGFBB Stimulation of NIH 3T3 Cell Lines

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.

i. ELISA Analysis of Phospho-AKT



ii. Western-Blot Analysis of Phospho-AKT



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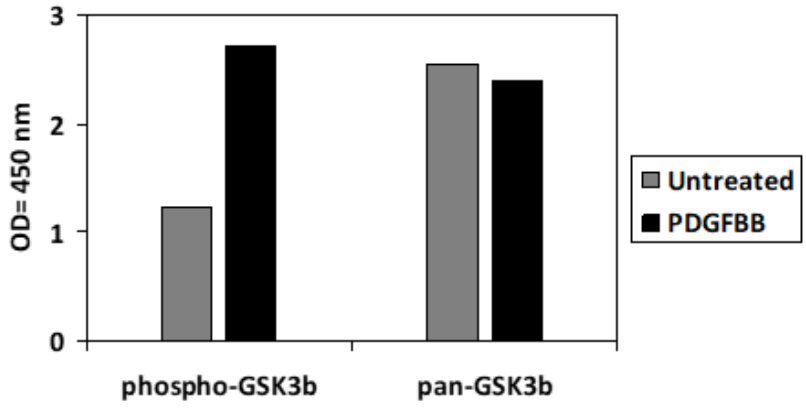
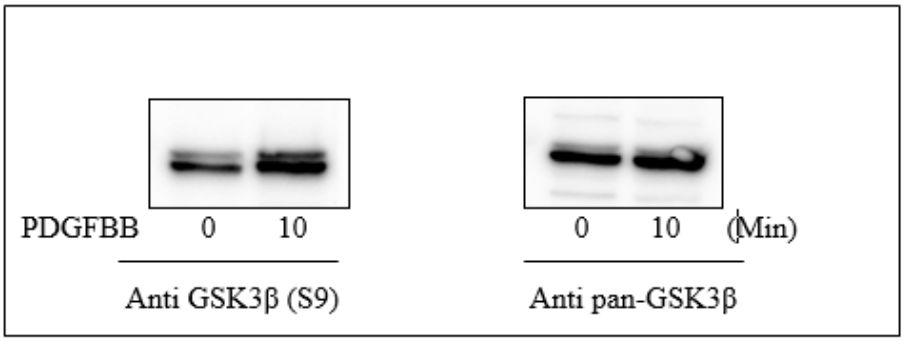
i. ELISA Analysis of Phospho-GSK3β



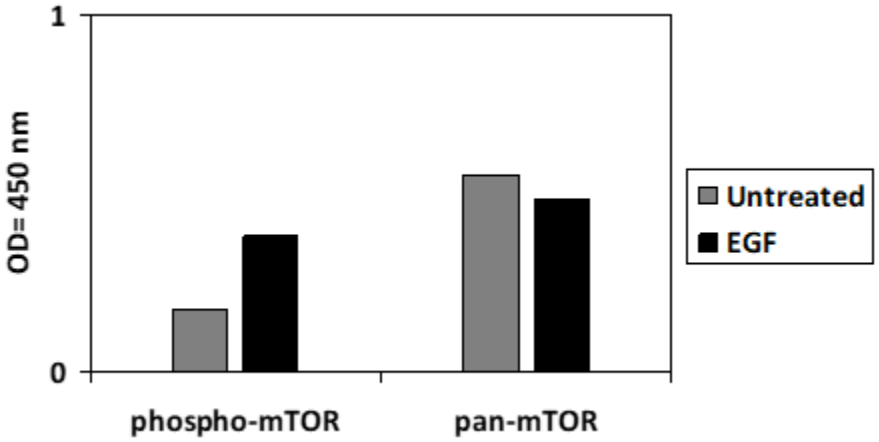
ii. Western-Blot Analysis of Phospho-GSK3β



C. 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.

i. ELISA Analysis of Phospho-mTOR



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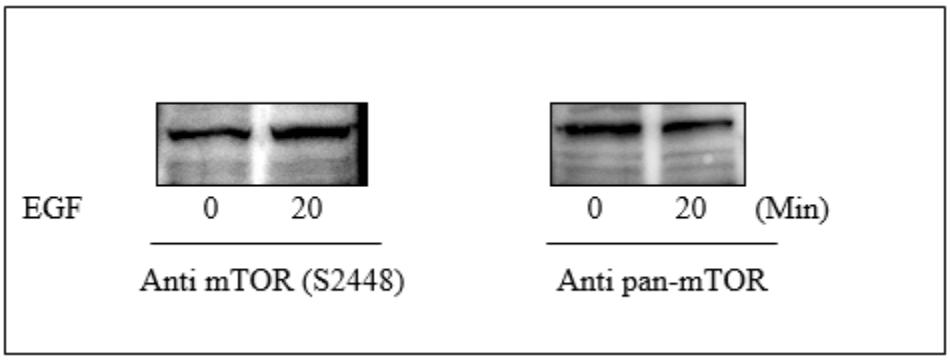
ii. Western-Blot Analysis of Phospho-mTOR



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Troubleshooting Guide

Problem	Cause	Solution
Low signal in samples	• Sample concentration is too low • Improper preparation of detection antibody • Too brief incubation times • Inadequate reagent volumes or improper dilution	• Increase sample concentration Briefly spin down vials before opening. Dissolve the powder thoroughly. • Ensure sufficient incubation time; assay procedure step 3 may be done overnight Check pipettes and ensure correct preparation
High signal in samples	• Sample concentration is too high	• Reduce sample concentration
Large CV	• Inaccurate pipetting Air bubbles in wells	• Check pipettes • Remove bubbles in wells
High background	• Plate is insufficiently washed • Contaminated wash buffer	• Review the manual for proper wash. If using a plate washer, ensure that all ports are unobstructed. • Make fresh wash buffer
Low sensitivity	• Improper storage of the ELISA kit • Stop solution • Improper primary or secondary antibody dilution	• Store your standard at <- 70ºC after reconstitution, others at 4ºC. Keep substrate solution protected from light. • Add stop solution to each well before reading plate • Ensure correct dilution