



Technical Manual

Phospho-AKT/GSKbeta/mTor PharmaGenie ELISA Kit

- **Catalogue Code: SBR1746**
- **Sandwich Principle**
- **Research Use Only**

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Introduction

Assay Genies' 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.

How do our ELISA kits work?

This Sandwich ELISA kit is an in vitro enzyme-linked immunosorbent assay for the measurement of human and mouse phosphoAKT, 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.

Storage & Expiry

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, Assay Diluent, TMB One-Step Substrate Reagent, Stop Solution and Cell Lysate Buffer 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 should be stored at -70°C .

Kit Contents

Each kit contains reagents for 96 assays including:

No.	Component	96-Well Kit	Storage
1	Microplate coated with coated with anti-pan AKT (column 1-4), GSK3 β (column 5-8) and mTOR (column 9-12) antibodies.	8 x 12	-20°C*
2	Wash Buffer Concentrate (20X)	25ml	4°C
3	Assay Diluent (5X)	15ml	4°C
4	Detection Antibody Akt (Ser473) (1 vial is enough to assay 3 strips)	1 vial rabbit anti-phospho-Akt (Ser473)	-20°C
5	Detection Antibody Akt (1 vial is enough to assay 3 strips)	1 vial of rabbit anti-Akt	-20°C
6	Detection Antibody GSK3 β (S9) (1 vial is enough to assay 3 strips)	1 vial (rabbit antiGSK3b (S9))	-20°C
7	Detection Antibody GSK3 β (1 vial is enough to assay 3 strips)	1 vial (rabbit anti-GSK3 β)	-20°C
8	Detection Antibody mTOR (S2448) (1 vial is enough to assay 3 strips)	1 vial (rabbit antimTOR (S2448))	-20°C
9	Detection Antibody mTOR (1 vial is enough to assay 3 strips)	1 vial of biotinylated antimTOR	-20°C
10	HRP-conjugated Anti-rabbit IgG (1000x)	25 μ l	1 month at 2-8°C
11	HRP-conjugated Streptavidin (100x)	200 μ l	4°C
12	TMB One-Step Substrate Reagent	12ml	4°C
13	Stop Solution	8ml	4°C
14	Cell Lysate Buffer (2x)	5ml	4°C
15	Positive Control 3T3S001-1	1 vial (Lyophilized)	-70°C

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

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 μ l 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.

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°C for 30 minutes.

Microcentrifuge at 13,000 rpm for 10 minutes at 2 - 8°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 before use.

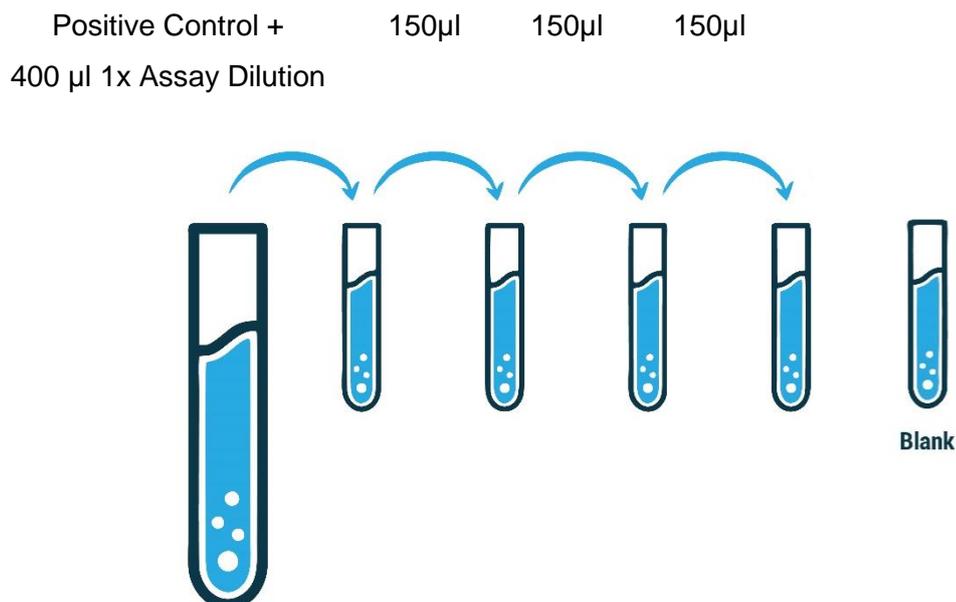
Note: *The fold dilution of sample used depends on the abundance of phosphorylated proteins and should be determined empirically. More of the sample can be used if signals are too weak. If signals are too strong, the sample can be diluted further.*

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

Reagent Preparation

1. Bring all reagents and samples to room temperature (18 - 25°C) before use.
2. Assay Diluent should be diluted 5-fold with deionized or distilled water before use.
3. Preparation of Positive Control: Briefly spin the Positive Control vial. Add 450 µl 1x Assay Diluent into Positive Control 3T3S001-1 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 µl 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 Positive Control of part TYPICAL DATA for a typical result on page 9).

DILUTION SERIES



4. If the Wash Concentrate (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.

5. Briefly spin the detection antibody vial before use. Add 100 μ l of 1X Assay Diluent B into each 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 -80°C for one month). The detection antibody concentrate should then be diluted 30-fold with 1X Assay Diluent and used in step 4 of the Assay Procedure.

6. Briefly spin the HRP-conjugated anti-rabbit or Streptavidin 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 antirabbit 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).

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:

	Anti-AKT				Anti-GSK3b				Anti-mTOR			
	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 auto washer. 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 µl of prepared 1x HRP-conjugated anti-rabbit IgG against anti-AKT, anti-AKT(S473), anti-GSK3β, anti-GSK3β (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.

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7. Discard the solution. Repeat the wash as in step 3.
 8. Add 100 μ l of TMB One-Step Substrate Reagent to each well. Incubate for 30 minutes at room temperature in the dark with shaking.
 9. Add 50 μ l of Stop Solution to each well. Read at 450 nm immediately.

Assay Procedure Summary

1. Prepare all reagents, samples and standards as instructed.
2. Add 100 μ l positive control, or sample to each well. Incubate 2.5 hour 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.

Typical Data

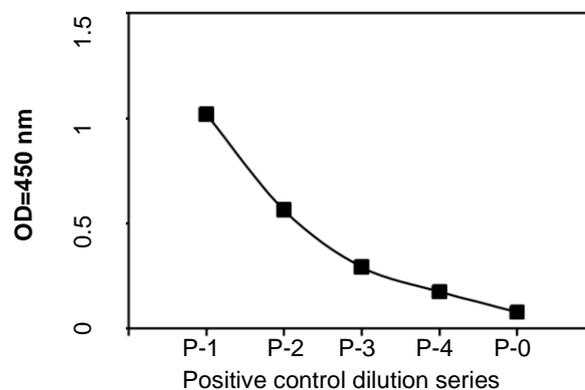
ELISA data analysis: Average the duplicate readings for each sample or positive.

A. 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 Reagent Preparation for detail.

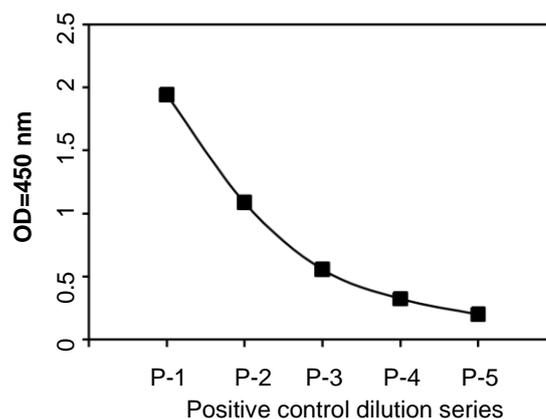
Phospho-AKT(S473)

Assay Diluent



Phospho-GSK3b(S9)

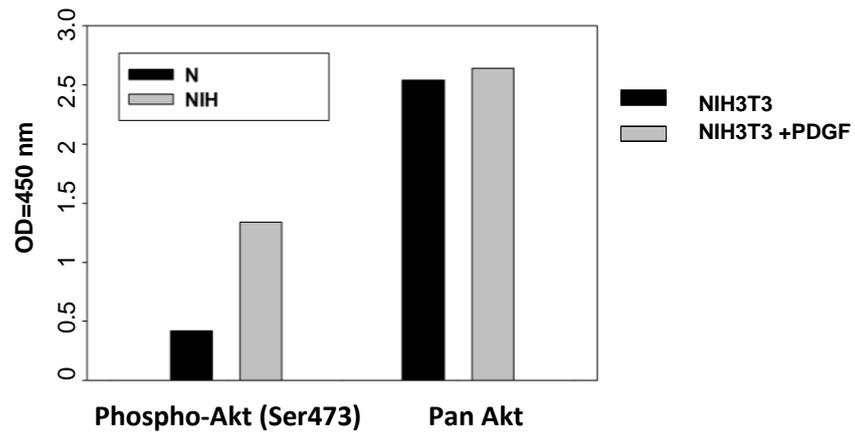
Assay Diluent



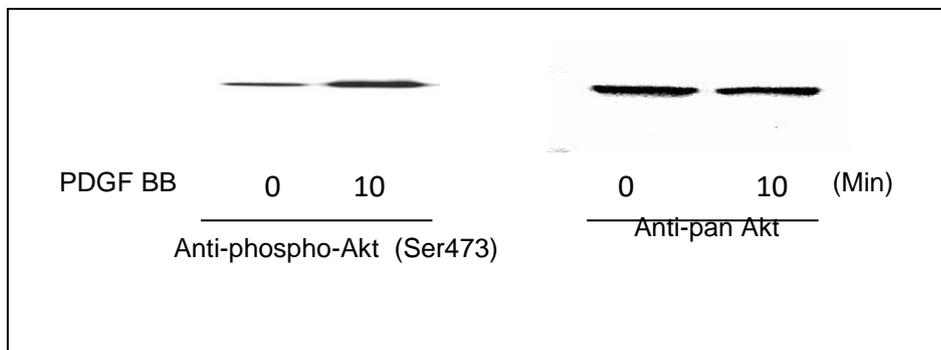
B. Recombinant Human PDGFBB Stimulation of NIH 3T3 Cell Line

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

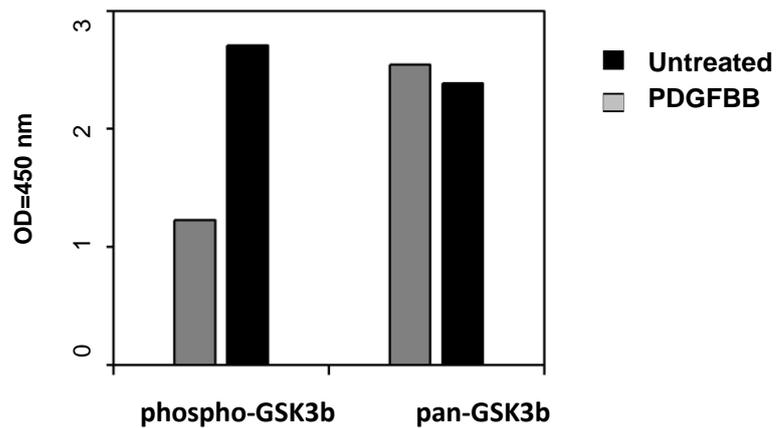
i. ELISA Analysis of Phospho-AKT



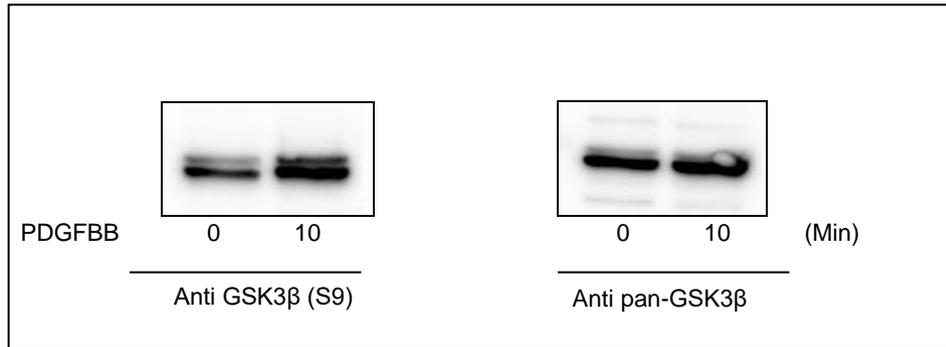
ii. Western-Blot Analysis of Phospho-AKT



i. ELISA Analysis of Phospho-GSK3 β



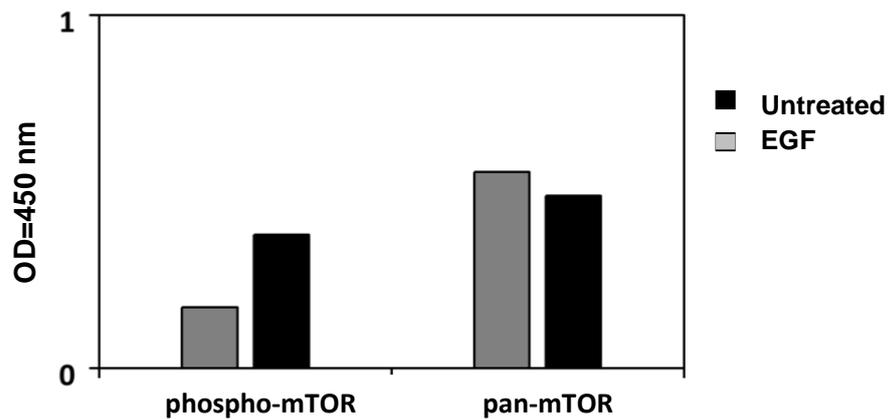
ii. Western-Blot Analysis of Phospho-GSK3 β



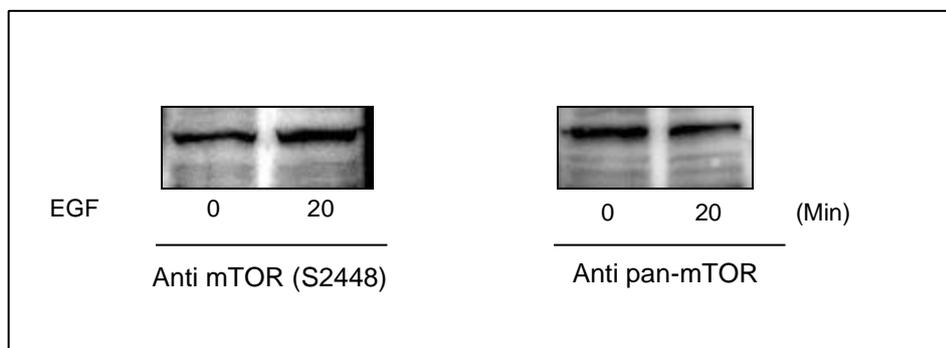
C. Recombinant Human EGF Stimulation of A431 Cell Lines

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

i. ELISA Analysis of Phospho-mTOR



ii. Western-Blot Analysis of Phospho-mTOR



Troubleshooting

Problem	Causes	Solutions
Low signal	<ul style="list-style-type: none">• Sample concentration is too low	<ul style="list-style-type: none">• Increase sample concentration
High Signal	<ul style="list-style-type: none">• Sample concentration is too high	<ul style="list-style-type: none">• Reduce sample concentration
Large CV	<ul style="list-style-type: none">• Inaccurate pipetting	<ul style="list-style-type: none">• Check pipettes
High background	<ul style="list-style-type: none">• Plate is insufficiently washed• Contaminated wash buffer	<ul style="list-style-type: none">• Review the manual for proper wash. If using a plate washer, ensure that all ports are unobstructed.• Make fresh wash buffer
Low positive control signal	<ul style="list-style-type: none">• Improper storage of the ELISA kit• Stop Solution• Improper primary or secondary antibody dilution	<ul style="list-style-type: none">• Upon receipt, the kit should be stored at -20°C. Store the positive control at -70°C after reconstitution• Stop solution should be added to each well before measurement and read OD immediately.• Ensure correct dilution

Assay Genie 100% money-back guarantee!

If you are not satisfied with the quality of our products and our technical team cannot resolve your problem, we will give you 100% of your money back.

Contact Details



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