

Product Information

Phospho-Akt (pSer⁴⁷³)/pan-Akt ELISA Kit
for detection of human, mouse, or rat phospho-Akt (pSer⁴⁷³) and pan-Akt in cell and tissue lysates

Catalog Number **RAB0012**
Storage Temperature -20 °C

TECHNICAL BULLETIN

Product Description

The Phospho-Akt (pSer⁴⁷³)/pan-Akt ELISA is an *in vitro* enzyme-linked immunosorbent assay for the measurement of phospho-Akt (pSer⁴⁷³) and pan Akt 1 in human, mouse, and rat cell lysates (to help normalize the results of phospho-Akt from different cell lysate being compared). A pan-Akt antibody has been coated onto a 96-well plate. Samples are pipetted into the wells and Akt present in a sample is bound to the wells by the immobilized antibody. The wells are washed and anti-phospho-Akt (pSer⁴⁷³) or anti-pan-Akt is used to detect phosphorylated or pan-Akt, respectively. After washing away unbound antibody, HRP-conjugated anti-rabbit IgG 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 Akt (pSer⁴⁷³) or pan-Akt bound. The Stop Solution changes the color from blue to yellow, and the intensity of the color is measured at 450 nm.

Components

1. Capture Antibody-Coated Microplate (Item A) - RABASA: 96 wells (12 strips × 8 wells) coated with anti-pan-Akt antibody.
2. 20x Wash Buffer Concentrate (Item B) - RABWASH5: 25 mL of 20x concentrated solution.
3. 5x Assay Diluent (Item E) - RABDIL11: 15 mL of 5x concentrated buffer. For diluting cell lysate sample, detection antibody (Item C-1 and Item C-2), and secondary antibody (Item D-1) concentrate.
4. Phospho-Akt (pSer⁴⁷³)-specific Antibody Concentrate (Item C1) - RABA473C1: 1 vial of rabbit anti-phospho-Akt (pSer⁴⁷³) (1 vial is enough to assay half microplate).
5. anti-pan-Akt Antibody Concentrate (Item C2) - RAB0011D: 1 vial of rabbit anti-pan-Akt (1 vial is enough to assay half microplate).
6. HRP-conjugated Anti-Rabbit IgG Concentrate (Item D1) - RABHRP4: 25 µL of 500x HRP-conjugated Anti-rabbit IgG concentrate.

7. ELISA Colorimetric TMB Reagent (HRP Substrate, Item H) - RABTMB3: 12 mL of 3,3',5,5'-tetra-methylbenzidine (TMB) in buffered solution.
8. Phosphorylation ELISA Stop Solution (Item I) - RABSTOP3: 8 mL of 0.2 M sulfuric acid.
9. 2x Cell Lysate Buffer (Item J) - RABCLB1: 5 mL of 2x cell lysis buffer (not including protease and phosphatase inhibitors).
10. Phospho-Akt (pSer⁴⁷³) Lyophilized Positive Control Sample (Item K) - RABA473K: 1 vial of lyophilized powder from A431 cell lysate.

Reagents and Equipment Required but Not Provided.

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.

Precautions and Disclaimer

For R&D use only. Not for drug, household, or other uses. Please consult the Safety Data Sheet for information regarding hazards and safe handling practices

Preparation Instructions

Sample Preparation

2x Cell Lysate Buffer should be diluted 2-fold with deionized or distilled water to yield 1x Cell Lysate Buffer (addition of protease and phosphatase inhibitors to 1x Cell Lysate Buffer is recommended prior to sample preparation).

Cell lysates - Rinse cells with PBS, making sure to remove any remaining PBS before adding the Cell Lysate Buffer. Solubilize cells at 4×10^7 cells/mL in 1x Cell Lysate Buffer. 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 supernatants 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.

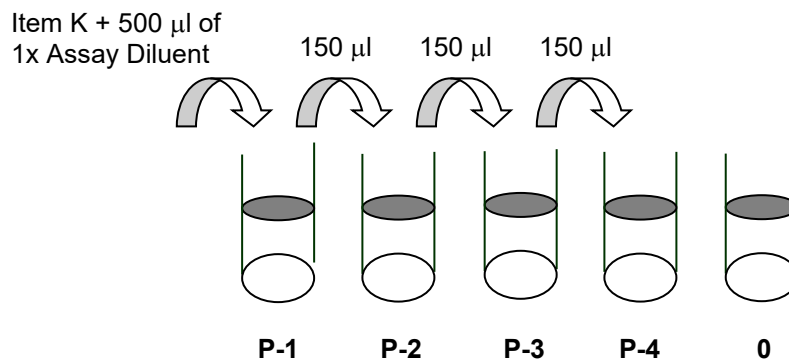
For the initial experiment, it is recommend to perform serial dilution testing such as 5-fold and 50-fold dilution of the cell lysates with Assay Diluent (Item E) before use.

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 (18–25 °C) before use.
2. Item E, 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 of Item K. Add 500 μ L of 1x Assay Diluent (Item E, Assay Diluent should be diluted 5-fold with deionized or distilled water before use) into Item K vial to prepare Positive Control (P-1) Solution. Dissolve the powder thoroughly by a gentle mix (if any precipitate in the solution is found, remove by centrifugation). Pipette 300 μ L of 1x Assay Diluent into each tube. Use the Positive Control (P-1) Solution to produce a dilution series (see Figure 1). Mix each tube thoroughly before the next transfer. 1x Assay Diluent serves as the background.

Figure 1.
Dilution Series for Positive Control



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-1 or Item C-2) 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 anti-phospho-Akt (pSer⁴⁷³) or anti-pan-Akt antibody should be diluted 55-fold with 1x Assay Diluent Procedure, step 4.
 6. Briefly spin the HRP-conjugated anti-rabbit IgG (Item D-1) before use. Pipette up and down to mix gently. HRP-conjugated anti-rabbit IgG concentrate should be diluted 500-fold with 1x Assay Diluent.
- For example: Briefly spin the vial (Item D-1) and pipette up and down to mix gently. Add 10 μ L of HRP-conjugated anti-rabbit IgG concentrate into a tube with 5 mL of 1x Assay Diluent to prepare a 500-fold diluted HRP-conjugated anti-rabbit IgG solution.

Storage/Stability

Store the kit at $-20\text{ }^{\circ}\text{C}$. It remains active for up to 1 year. Avoid repeated freeze-thaw cycles.

The reconstituted standard should be stored at $-20\text{ }^{\circ}\text{C}$ or $-70\text{ }^{\circ}\text{C}$ ($-70\text{ }^{\circ}\text{C}$ is recommended). Opened microplate strips or reagents may be stored for up to 1 month at $2\text{--}8\text{ }^{\circ}\text{C}$. Return unused wells to the pouch containing desiccant pack and reseal along entire edge.

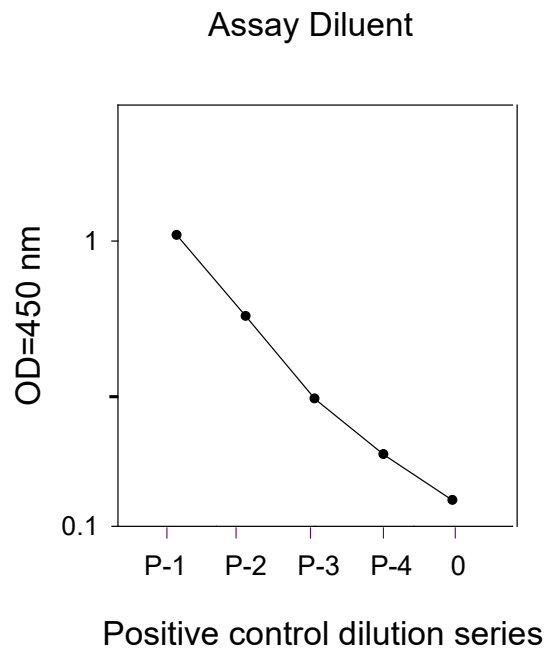
Procedure

1. Bring all reagents to room temperature ($18\text{--}25\text{ }^{\circ}\text{C}$) before use. It is recommended that all samples or Positive Control should be run at least in duplicate.
2. Add $100\text{ }\mu\text{L}$ of each sample or positive control into appropriate wells. Cover well with plate holder and incubate for 2.5 hours at room temperature or overnight at $4\text{ }^{\circ}\text{C}$ with shaking.
3. Discard the solution and wash 4 times with 1x Wash Solution. Wash by filling each well with Wash Buffer ($300\text{ }\mu\text{L}$) using a multichannel 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 $100\text{ }\mu\text{L}$ of prepared 1x rabbit anti-phospho-Akt (pSer⁴⁷³) antibody or 1x rabbit anti-pan-Akt (see 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 $100\text{ }\mu\text{L}$ of prepared 1x HRP-conjugated anti-rabbit IgG to corresponding well. Incubate for 1 hour at room temperature with shaking.
7. Discard the solution. Repeat the wash as in step 3.
8. Add $100\text{ }\mu\text{L}$ 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 $50\text{ }\mu\text{L}$ of Stop Solution (Item I) to each well. Read at 450 nm immediately.

Typical Data

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

Positive Control: A431 cells were treated with recombinant human EGF at $37\text{ }^{\circ}\text{C}$ for 20 minutes. Solubilize cells at 4×10^7 cells/mL in Cell Lysate Buffer. Serial dilutions of lysates were analyzed with this ELISA kit. Please see Reagent Preparation, step 3 for detail.



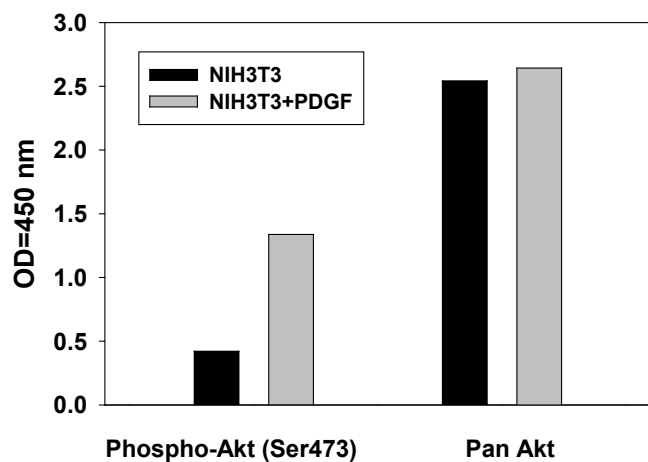
Recombinant Human PDGF Stimulation of NIH3T3 Cell

Line: NIH3T3 cells were treated or untreated with recombinant human PDGF for 10 minutes. Cell lysates were analyzed using this phospho ELISA kit and Western Blot.

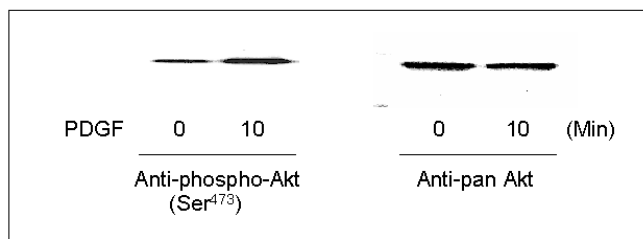
References

1. Hajduch, E. et al., FEBS Lett, **492**, 199-203 (2001).
2. Burgering, B.M., and Coffey, P.J., Nature, **376**, 599-602 (1995).
3. Franke, T.F. et al., Cell, **88**, 435-7 (1997).

ELISA



Western blot



Appendix
Troubleshooting Guide

Problem	Cause	Solution
Poor standard curve	Inaccurate pipetting	Check pipettes
	Improper standard dilution	Ensure a brief spin of Item C and dissolve the powder thoroughly with gentle mixing.
Low signal	Too brief incubation times	Ensure sufficient incubation time; Procedure, step 2 may change to over night
	Inadequate reagent volumes or improper dilution	Check pipettes and ensure correct preparation
Large CV	Inaccurate pipetting	Check pipettes
High background	Plate is insufficiently washed	Review the manual for proper wash. If using a plate washer, check that all ports are unobstructed.
	Contaminated wash buffer	Make fresh wash buffer
Low sensitivity	Improper storage of the ELISA kit	Store the standard at ≤ -20 °C after reconstitution, others at 4 °C. Keep substrate solution protected from light
	Stop solution	Stop solution should be added to each well before measurement.

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