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Product Information

Phospho-Akt (pSer^{473}) ELISA Kit
for detection of human, mouse, or rat phospho-Akt (pSer^{473}) in cell and tissue lysates

Catalog Number **RAB0011**
Storage Temperature –20 °C

TECHNICAL BULLETIN

Product Description

The Phospho-Akt (pSer^{473}) ELISA kit is an *in vitro* enzyme-linked immunosorbent assay for the measurement of human, mouse, and rat phospho-Akt (pSer^{473}). An anti-pan-Akt (pSer^{473}) 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-Akt (pSer^{473}) antibody is used to detect phosphorylated Akt (pSer^{473}). 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 (pSer^{473}) 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) and HRP-conjugated Anti-Rabbit IgG Concentrate (Item D1).
4. Phospho-Akt (pSer^{473})-specific Antibody Concentrate (Item C1) - RABA473C1: 2 vials of rabbit anti-Akt (pSer^{473}) (each vial is enough to assay half microplate).
5. HRP-conjugated Anti-Rabbit IgG Concentrate (Item D1) - RABHRP4: 25 µL of 500x HRP-conjugated Anti-rabbit IgG concentrate.
6. TMB One-Step Substrate Reagent (Item H) - RABTMB4: 12 mL of 3,3′,5,5′-tetramethylbenzidine (TMB) in buffered solution.
7. Phosphorylation ELISA Stop Solution (Item I) – RABSTOP3: 8 mL of 0.2 M sulfuric acid.
8. 2x Cell Lysate Buffer (Item J) - RABCLB1: 5 mL of 2x cell lysis buffer (not including protease and phosphatase inhibitors).
9. Phopho-Akt (pSer^{473}) 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 for the cell lysates with 1x 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.

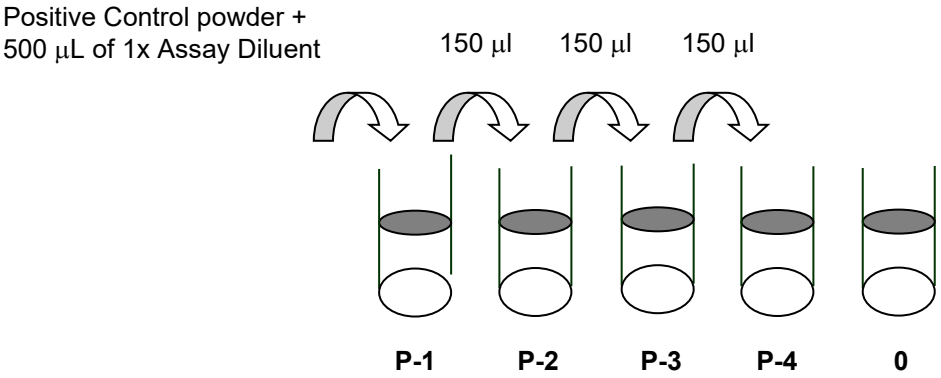
Reagent Preparation

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 µL 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 a 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 µL of 1x Assay Diluent into each tube. Use the Positive Control (P-1) 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) before use. Add 100 µL 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 –70 °C for one month). The anti-phospho-Akt (pSer^{473}) antibody should be diluted 55-fold with 1x Assay Diluent and used in Procedure, step 4.

6. Briefly spin the HRP-conjugated anti-rabbit IgG (Item D1) 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 D1) and pipette up to mix gently. Add 10 µL of HRP-conjugated anti-rabbit IgG concentrate into a tube with 5 mL 1x of Assay Diluent to prepare a 500-fold diluted HRP-conjugated anti-rabbit IgG solution.

Storage/Stability
Store the kit at –20 °C Please use within 1 year from the date of shipment. Avoid repeated freeze-thaw cycles.

After initial use, Wash Buffer Concentrate (Item B), Assay Diluent (Item E), TMB One-Step Substrate Reagent (Item H), Stop Solution (Item I), and Cell Lysate Buffer (Item J) should be stored at 2–8 °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.

Store Item D-1 at 2–8 °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.

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 or positive control into appropriate wells. Cover well with plate holder and incubate for 2.5 hours at room temperature or overnight at 4 °C with shaking.

3. Discard the solution and wash 4 times with 1x Wash Solution. Wash by filling each well with Wash Buffer (300 µL) 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 µL of prepared 1x detection anti-phospho-Akt ($pSer^{473}$) antibody (see Preparation, step 5) to each well. 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 (see Preparation, step 6) to each well. Incubate for 1 hour at room temperature with shaking.

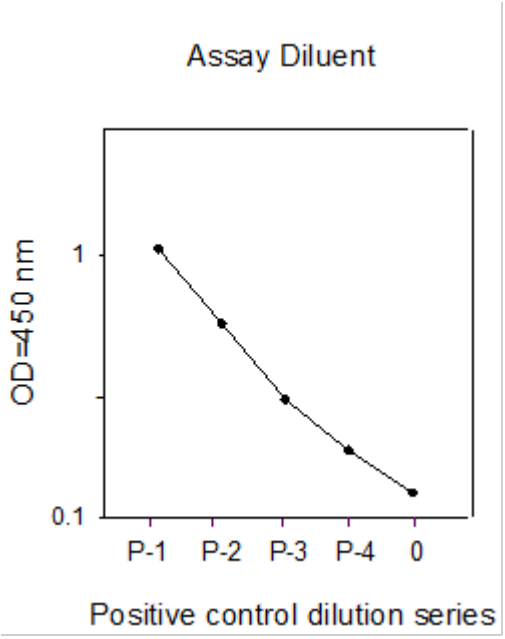
7. Discard the solution. Repeat the wash as in step 3.

8. Add 100 µL 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 µL of Stop Solution (Item I) to each well. Read at 450 nm immediately.

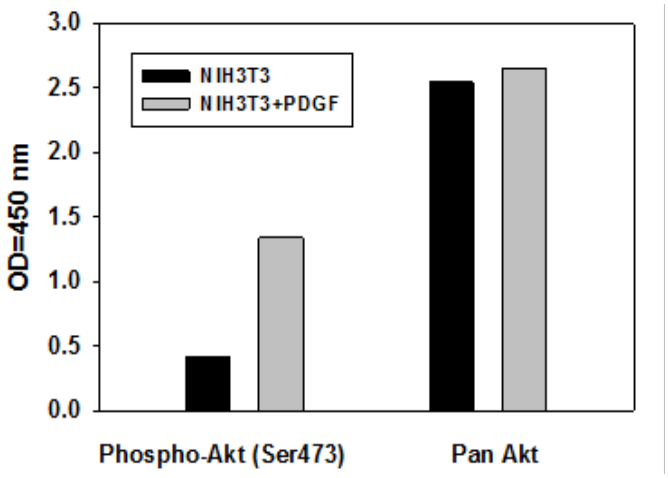
Results
Typical Data
ELISA data analysis: Average the duplicate readings for each sample or positive.

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

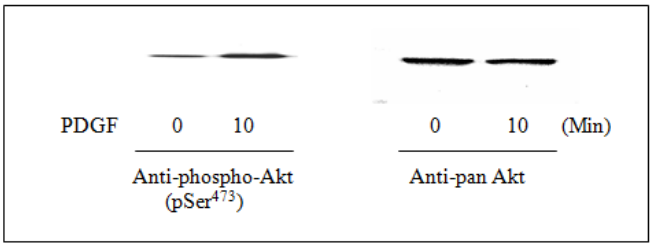


PDGF Stimulation of NIH3T3 Cell Lines: NIH3T3 cells were treated or untreated with PDGF for 10 minutes. Cell lysates were analyzed using this phospho ELISA kit and Western blot.

ELISA



Western blot



References

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

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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