

# PathScan® Phospho-Akt2 (Ser474) Sandwich ELISA Kit (Mouse Preferred)



✓ 1 Kit  
(96 assays)

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**For Research Use Only. Not For Use In Diagnostic Procedures.**

### Species Cross-Reactivity: M

**Description:** CST's PathScan® Phospho-Akt2 (Ser474) Sandwich ELISA Kit (Mouse Preferred) is a solid phase sandwich enzyme-linked immunosorbent assay (ELISA) that detects endogenous levels of Akt2 protein phosphorylated at Ser474. A phospho-Akt rabbit antibody has been coated onto the microwells. After incubation with cell lysates, phospho-Akt protein is captured by coated antibody. Following extensive washing, Akt2 mouse antibody is added to detect the captured phospho-Akt2 protein. Anti-mouse IgG, HRP-linked antibody\* is then used to recognize the bound detection antibody. HRP substrate TMB is added to develop color. The magnitude of the absorbance for this developed color is proportional to the quantity of Akt2 phosphorylated at Ser474.

\*Antibodies in kit are custom formulations specific to the kit.

**Specificity/Sensitivity:** CST's PathScan® Phospho-Akt2 (Ser474) Sandwich ELISA Kit (Mouse Preferred) detects endogenous levels of Akt2 protein phosphorylated at Ser474 in mouse (NIH/3T3) cells, as shown in Figure 1. Phospho-Akt protein specificity is demonstrated in Figure 2, while Akt2 kit sensitivity is shown in figure 3.

**Background:** Akt, also referred to as PKB or Rac, plays a critical role in controlling survival and apoptosis (1-3). This protein kinase is activated by insulin and various growth and survival factors to function in a wortmannin-sensitive pathway involving PI3 kinase (2,3). Akt is activated by phospholipid binding and activation loop phosphorylation at Thr308 by PDK1 (4) and by phosphorylation within the carboxy terminus at Ser473. The previously elusive PDK2 responsible for phosphorylation of Akt at Ser473 has been identified as mammalian target of rapamycin (mTOR) in a rapamycin-insensitive complex with rictor and Sin1 (5,6). Akt promotes cell survival by inhibiting apoptosis by phosphorylating and inactivating several targets, including Bad (7), forkhead transcription factors (8), c-Raf (9) and caspase-9. PTEN phosphatase is a major negative regulator of the PI3 kinase/Akt signaling pathway (10). LY294002 is a specific PI3 kinase inhibitor (11). Another essential Akt function is the regulation of glycogen synthesis through phosphorylation and inactivation of GSK-3 $\alpha$  and  $\beta$  (12,13). Akt may also play a role in insulin stimulation of glucose transport (12). In addition to its role in survival and glycogen synthesis, Akt is involved in cell cycle regulation by preventing GSK-3 $\beta$  mediated phosphorylation and degradation of cyclin D1 (14) and by negatively regulating the cyclin dependent kinase inhibitors p27 Kip (15) and p21 Waf1/CIP1 (16). Akt also plays a critical role in cell growth by directly phosphorylating mTOR in a rapamycin-sensitive complex containing raptor (17). More importantly, Akt

Product Includes	Item #	Kit Quantity	Color	Storage Temp
Phospho-Akt (Ser473) Rabbit mAb Coated Microwells*	47122	96 tests		4°C
Akt2 Mouse Detection mAb	13755	1 each	Green (Lyophilized)	4°C
Anti-mouse IgG, HRP-linked Antibody (ELISA Formulated)	13304	1 each	Red (Lyophilized)	4°C
Detection Antibody Diluent	13339	11 ml	Green	4°C
HRP Diluent	13515	11 ml	Red	4°C
TMB Substrate	7004	11 ml		4°C
STOP Solution	7002	11 ml		4°C
Sealing Tape	54503	2 sheets		4°C
ELISA Wash Buffer (20X)	9801	25 ml		4°C
ELISA Sample Diluent	11083	25 ml	Blue	4°C
Cell Lysis Buffer (10X)	9803	15 ml		-20°C

\*12 8-well modules – Each module is designed to break apart for 8 tests.

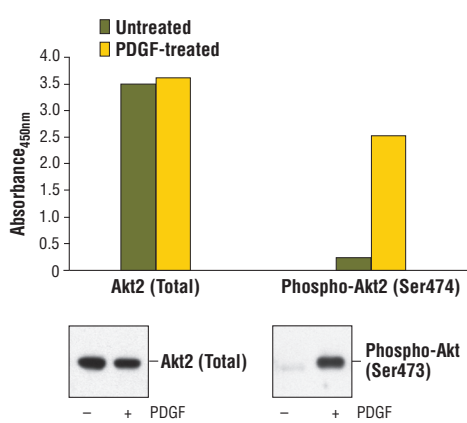


Figure 1: Treatment of NIH/3T3 cells with PDGF #9909 stimulates phosphorylation of Akt2 at Ser474, detected by PathScan® Phospho-Akt2 (Ser474) Sandwich ELISA Kit (Mouse Preferred) #7932, but does not affect levels of total Akt2 protein detected by PathScan® Total Akt2 Sandwich ELISA kit #7930. The absorbance readings at 450 nm are shown in the top figure, while the corresponding western blots using Akt2 (5B5) Rabbit mAb #2964 (left panel) and Phospho-Akt (Ser473) (193H12) Rabbit mAb #4058 (right panel) are shown in the bottom figure.

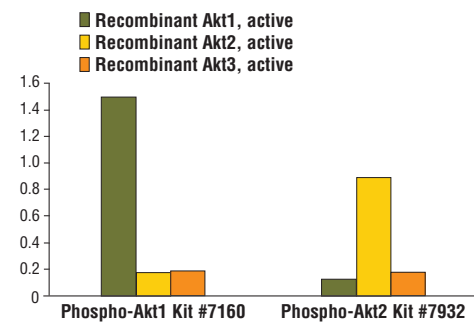


Figure 2: Comparison of specificity between two sandwich ELISA kits, #7160 for phospho-Akt1 (Ser473) and #7932 for phospho-Akt2 (Ser474), in the detection of recombinant Akt1 protein, recombinant Akt2 protein or recombinant Akt3 protein, which are active phosphorylated forms. 1.0 ng of recombinant protein (0.25 ng for Akt1) added in each microwell is assayed by using these two ELISA kits.

phosphorylates and inactivates tuberlin (TSC2), an inhibitor of mTOR within the mTOR-raptor complex (18). Inhibition of mTOR stops the protein synthesis machinery by inactivating p70 S6 kinase and activating the eukaryotic initiation factor 4E binding protein 1 (4E-BP1), an inhibitor of translation (18,19).

**Background References:**

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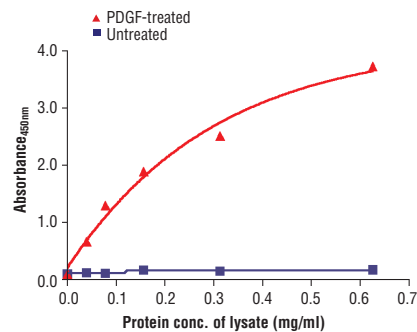


Figure 3: The relationship between protein concentration of lysates from untreated or PDGF-treated NIH/3T3 cells and the absorbance at 450 nm is shown. After starvation, NIH/3T3 cells (85% confluence) were treated with PDGF #9909 (50 ng/ml) for 10 min at 37°C and then lysed.

# PathScan® Sandwich ELISA Protocol (for kits with Lyophilized Antibodies)

## A Solutions and Reagents

**NOTE:** Prepare solutions with purified water.

- 1. Microwell strips:** Bring all to room temperature before use.
- 2. Detection Antibody:** Supplied lyophilized as a green colored cake or powder. Add 1.0 ml of Detection Antibody Diluent (green solution) to yield a concentrated stock solution. Incubate at room temperature for 5 min with occasional gentle mixing to fully reconstitute. To make the final working solution, add the full 1.0 ml volume of reconstituted Detection Antibody to 10.0 ml of Detection Antibody Diluent in a clean tube and gently mix. Unused working solution may be stored for 4 weeks at 4°C.
- 3. HRP-Linked Antibody\*:** Supplied lyophilized as a red colored cake or powder. Add 1.0 ml of HRP Diluent (red solution) to yield a concentrated stock solution. Incubate at room temperature for 5 min with occasional gentle mixing to fully reconstitute. To make the final working solution, add the full 1.0 ml volume of reconstituted HRP-Linked Antibody to 10.0 ml of HRP Diluent in a clean tube and gently mix. Unused working solution may be stored for 4 weeks at 4°C.
- 4. Detection Antibody Diluent:** Green colored diluent for reconstitution and dilution of the detection antibody (11 ml provided).
- 5. HRP Diluent:** Red colored diluent for reconstitution and dilution of the HRP-Linked Antibody (11 ml provided).
- 6. Sample Diluent:** Blue colored diluent provided for dilution of cell lysates.
- 7. 1X Wash Buffer:** Prepare by diluting 20X Wash Buffer (included in each PathScan® Sandwich ELISA Kit) in purified water.
- 8. Cell Lysis Buffer:** 10X Cell Lysis Buffer #9803: This buffer can be stored at 4°C for short-term use (1–2 weeks). Recommended: Add 1 mM phenylmethyl-sulfonyl fluoride (PMSF) immediately before use.
- 9. TMB Substrate (#7004).**
- 10. STOP Solution (#7002).**

*\*Note: Some PathScan® ELISA Kits may include HRP-Linked Streptavidin in place of HRP-Linked Antibody.*

## B Preparing Cell Lysates

**For adherent cells.**

1. Aspirate media when the culture reaches 80–90% confluence. Treat cells by adding fresh media containing regulator for desired time.
2. Remove media and rinse cells once with ice-cold 1X PBS.
3. Remove PBS and add 0.5 ml ice-cold 1X Cell Lysis Buffer plus 1 mM PMSF to each plate (10 cm diameter) and incubate the plate on ice for 5 min.
4. Scrape cells off the plate and transfer to an appropriate tube. Keep on ice.
5. Sonicate lysates on ice.
6. Microcentrifuge for 10 min (14,000 rpm) at 4°C and transfer the supernatant to a new tube. The supernatant is the cell lysate. Store at –80°C in single-use aliquots.

**For suspension cells**

1. Remove media by low speed centrifugation (~1200 rpm) when the culture reaches 0.5–1.0 x 10<sup>6</sup> viable cells/ml. Treat cells by adding fresh media containing regulator for desired time.
2. Collect cells by low speed centrifugation (~1200 rpm) and wash once with 5–10 ml ice-cold 1X PBS.
3. Cells harvested from 50 ml of growth media can be lysed in 2.0 ml of 1X Cell Lysis Buffer plus 1 mM PMSF.
4. Sonicate lysates on ice.
5. Microcentrifuge for 10 min (14,000 rpm) at 4°C and transfer the supernatant to a new tube. The supernatant is the cell lysate. Store at –80°C in single-use aliquots.

## C Test Procedure

1. After the microwell strips have reached room temperature, break off the required number of microwells. Place the microwells in the strip holder. Unused microwells must be resealed and stored at 4°C immediately.
2. Cell lysates can be undiluted or diluted with Sample Diluent (supplied in each PathScan® Sandwich ELISA Kit, blue color). Individual datasheets for each kit provide a sensitivity curve that serves as a reference for selection of an appropriate starting lysate concentration. The sensitivity curve shows typical kit assay results across a range of lysate concentration points.
3. Add 100 µl of each undiluted or diluted cell lysate to the appropriate well. Seal with tape and press firmly onto top of microwells. Incubate the plate for 2 hr at 37°C. Alternatively, the plate can be incubated overnight at 4°C.
4. Gently remove the tape and wash wells:
  - a. Discard plate contents into a receptacle.
  - b. Wash 4 times with 1X Wash Buffer, 200 µl each time for each well.
  - c. For each wash, strike plates on fresh towels hard enough to remove the residual solution in each well, but do not allow wells to completely dry at any time.
  - d. Clean the underside of all wells with a lint-free tissue.
5. Add 100 µl of reconstituted Detection Antibody (green color) to each well (refer to Section A, Step 2). Seal with tape and incubate the plate at 37°C for 1 hr.
6. Repeat wash procedure (Section C, Step 4).
7. Add 100 µl of reconstituted HRP-Linked secondary antibody (red color) to each well (refer to Section A, Step 3). Seal with tape and incubate the plate for 30 min at 37°C.
8. Repeat wash procedure (Section C, Step 4).
9. Add 100 µl of TMB Substrate to each well. Seal with tape and incubate the plate for 10 min at 37°C or 30 min at 25°C.
10. Add 100 µl of STOP Solution to each well. Shake gently for a few seconds.

**NOTE:** Initial color of positive reaction is blue, which changes to yellow upon addition of STOP Solution.

11. Read results.
  - a. **Visual Determination:** Read within 30 min after adding STOP Solution.
  - b. **Spectrophotometric Determination:** Wipe underside of wells with a lint-free tissue. Read absorbance at 450 nm within 30 min after adding STOP Solution.