

Phospho-Akt Pathway Sampler Kit

✓ 1 Kit
(7 x 40 microliters)

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This product is for *in vitro* research use only and is not intended for use in humans or animals.
This product is not intended for use as a therapeutic or in diagnostic procedures.

Products Included	Product #	Quantity	Mol. Wt.	Source
Phospho-Akt (Ser473) (D9E) Rabbit mAb	4060	40 µl	60 kDa	Rabbit IgG
Phospho-Akt (Thr308) (C31E5) Rabbit mAb	2965	40 µl	60 kDa	Rabbit IgG
Akt (pan) (C67E7) Rabbit mAb	4691	40 µl	60 kDa	Rabbit IgG
Phospho-c-Raf (Ser259) Antibody	9421	40 µl	74 kDa	Rabbit IgG
Phospho-GSK-3β (Ser9) (5B3) Rabbit mAb	9323	40 µl	46 kDa	Rabbit IgG
Phospho-PTEN (Ser380) Antibody	9551	40 µl	54 kDa	Rabbit IgG
Phospho-PDK1 (Ser241) Antibody	3061	40 µl	58 to 68 kDa	Rabbit IgG
Anti-rabbit IgG, HRP-linked Antibody	7074	100 µl		Goat
LY294002 (PI3 Kinase Inhibitor)	9901	.3 mg	307 kDa	

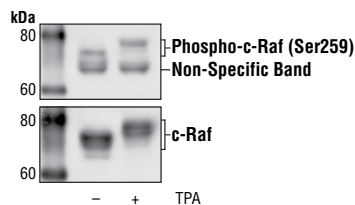
See www.cellsignal.com for individual component applications, species cross-reactivity, dilutions and additional application protocols.

Description: The Phospho-Akt Pathway Sampler Kit provides an economical means to evaluate the activation status of the Akt signaling pathway, including PTEN and phosphorylated Akt, GSK-3β, c-Raf and PDK1. The kit includes enough primary and secondary antibodies to perform four mini-blot experiments, as well as a specific inhibitor of PI3 kinase (LY294002).

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-3α and β (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-3β mediated phosphorylation and degradation of cyclin D1 (14) and by negatively regulating the cyclin dependent kinase inhibitors p27 Kip (15) and p21 Waf1 (16). Akt also plays



Western blot analysis of extracts from HeLa cells, untreated or TPA-treated, using **Phospho-Raf (Ser259) Antibody #9421** (upper), or a total c-Raf antibody (lower).

a critical role in cell growth by directly phosphorylating mTOR in a rapamycin-sensitive complex containing raptor (17). More importantly, Akt phosphorylates and inactivates tuberlin (TSC2), an inhibitor of mTOR within the mTOR-raptor complex (18). Inhibition of mTOR stops the protein synthesis machinery due to inactivation of its effector, p70 S6 kinase and activation of the eukaryotic initiation factor 4E binding protein 1 (4E-EP1), an inhibitor of translation (18,19).

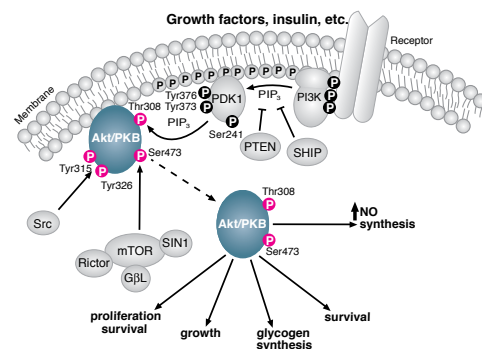
Specificity/Sensitivity: Each phospho-specific antibody recognizes the phosphorylated form of its target. Akt Antibody recognizes total Akt protein, independent of its phosphorylation state.

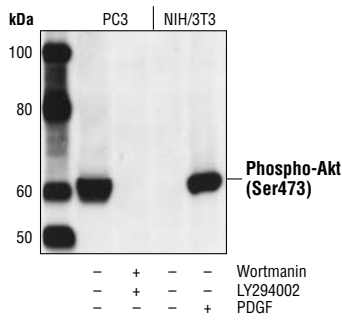
Source/Purification: Antibodies are produced by immunizing rabbits with synthetic phospho-peptides corresponding to residues surrounding Ser473 or Thr308 of mouse Akt, Ser9 of human GSK-3β, Ser259 of human c-Raf, Ser380 of human PTEN or Ser241 of human PDK1. Polyclonal antibodies are purified by protein A and peptide affinity chromatography.

Storage: Supplied in 10 mM sodium HEPES (pH 7.5), 150 mM NaCl, 100 µg/ml BSA and 50% glycerol. Store at -20°C. *Do not aliquot the antibodies.*

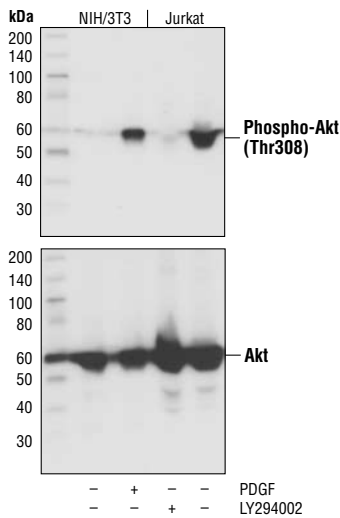
Recommended Antibody Dilutions:
Western blotting 1:1000

Please visit www.cellsignal.com for a complete listing of recommended companion products.

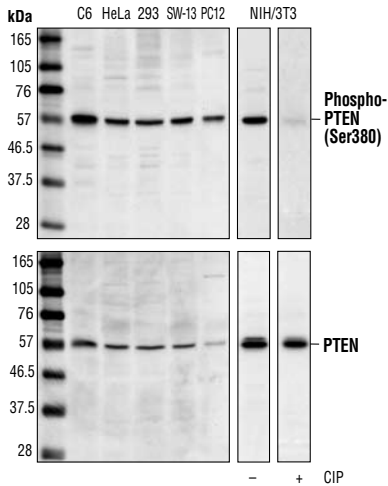




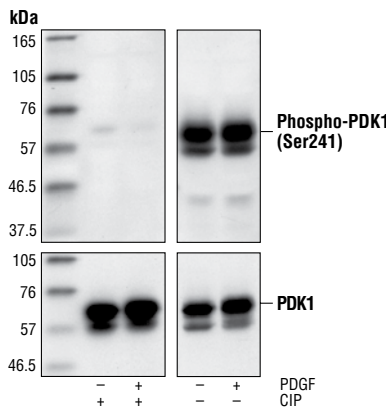
Western blot analysis of extracts from PC3 cells, untreated or LY294002/wortmannin-treated, and NIH/3T3 cells, serum-starved or PDGF-treated, using **Phospho-Akt (Ser473) (D9E) Rabbit mAb #4060**.



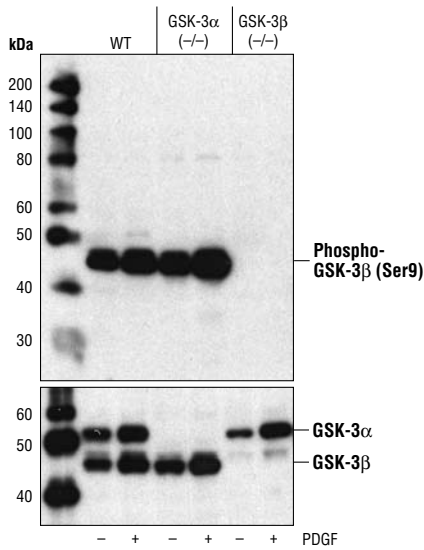
Western blot analysis of extracts from NIH/3T3 and Jurkat cells, untreated, PDGF-treated or LY294002-treated as indicated, using **Phospho-Akt (Thr308) (C31E5) Rabbit mAb #2965** (upper) or **Akt (pan) (C67E7) Rabbit mAb #4691** (lower).



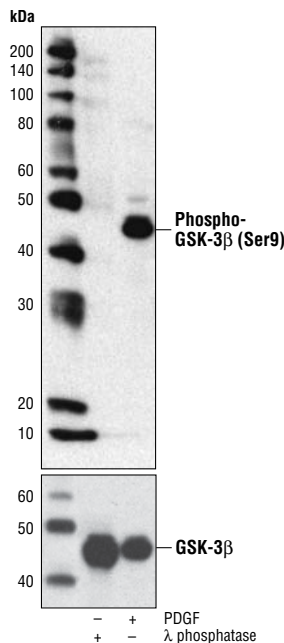
Western blot analysis of various cell lines with **Phospho-PTEN (Ser380) Antibody #9551** (upper) or **PTEN Antibody #9552** (lower). The phospho-specificity of the antibody was characterized by treating the membrane without (-) or with (+) calf intestinal alkaline phosphatase (CIP) after Western transfer.



Western blot analysis of extracts from NIH/3T3 cells (starved for 16 hours) treated with PDGF (50 ng/ml), using **Phospho-PDK1 (Ser241) Antibody #3061** (upper) or control PDK1 antibody #3062 (lower). The phospho-specificity of the antibody was confirmed by treating the membrane with calf intestinal alkaline phosphatase (CIP) after Western transfer.



Western blot analysis of extracts from wild type (lanes 1,2), GSK-3α(-/-) (lanes 3,4) and GSK-3β(-/-) (lanes 5,6) mouse embryonic fibroblast cells (MEF), untreated or PDGF treated, using **Phospho-GSK-3β (Ser9) (5B3) Rabbit mAb #9323** (upper) and **GSK-3α/β Antibody** (lower). (MEF wild type, GSK-3α(-/-) and GSK-3β(-/-) cells were kindly provided by Dr. Jim Woodgett, University of Toronto, Canada).



Western blot analysis of extracts from NIH/3T3 cells, λ-phosphatase or PDGF treated, using **Phospho-GSK-3β (Ser9) (5B3) Rabbit mAb #9323** (upper) or **GSK-3β (27C10) Rabbit mAb #9315** (lower).

Selected Application References:

Crowder, R.J. and Freeman, R.S. (2000) Glycogen synthase kinase-3 β activity is critical for neuronal death caused by inhibiting phosphatidylinositol 3-kinase or Akt but not for death caused by nerve growth factor withdrawal. *J Biol Chem* 275, 34266–71. Application: W.

Guo, M. et al. (2000) Suppression of 2,3,7,8-tetrachlorodibenzo-p-dioxin (TCDD)-mediated aryl hydrocarbon receptor transformation and CYP1A1 induction by the phosphatidylinositol 3-kinase inhibitor 2-(4-morpholinyl)-8-phenyl-4H-1-benzopyran-4-one (LY294002). *Biochem Pharmacol* 60, 635–42. Application: PI3K inhibition.

Background References:

- (1) Franke, T.F. et al. (1997) *Cell* 88, 435–7.
- (2) Burgering, B.M. and Coffey, P.J. (1995) *Nature* 376, 599–602.
- (3) Franke, T.F. et al. (1995) *Cell* 81, 727–36.
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- (12) Hajduch, E. et al. (2001) *FEBS Lett* 492, 199–203.
- (13) Cross, D.A. et al. (1995) *Nature* 378, 785–9.
- (14) Diehl, J.A. et al. (1998) *Genes Dev* 12, 3499–511.
- (15) Gesbert, F. et al. (2000) *J Biol Chem* 275, 39223–30.
- (16) Zhou, B.P. et al. (2001) *Nat Cell Biol* 3, 245–52.
- (17) Navé, B.T. et al. (1999) *Biochem J* 344 Pt 2, 427–31.
- (18) Inoki, K. et al. (2002) *Nat Cell Biol* 4, 648–57.
- (19) Manning, B.D. et al. (2002) *Mol Cell* 10, 151–62.

Western Immunoblotting Protocol (Primary Antibody Incubation in BSA)

For Western blots, incubate membrane with diluted antibody in 5% w/v BSA, 1X TBS, 0.1% Tween-20 at 4°C with gentle shaking, overnight.

A Solutions and Reagents

NOTE: Prepare solutions with Milli-Q or equivalently purified water.

- 1X Phosphate Buffered Saline (PBS)
- 1X SDS Sample Buffer:** 62.5 mM Tris-HCl (pH 6.8 at 25°C), 2% w/v SDS, 10% glycerol, 50 mM DTT, 0.01% w/v bromophenol blue or phenol red
- Transfer Buffer:** 25 mM Tris base, 0.2 M glycine, 20% methanol (pH 8.5)
- 10X Tris Buffered Saline (TBS):** To prepare 1 liter of 10X TBS: 24.2 g Tris base, 80 g NaCl; adjust pH to 7.6 with HCl (use at 1X).
- Nonfat Dry Milk (weight to volume [w/v])
- Blocking Buffer:** 1X TBS, 0.1% Tween-20 with 5% w/v nonfat dry milk; for 150 ml, add 15 ml 10X TBS to 135 ml water, mix. Add 7.5 g nonfat dry milk and mix well. While stirring, add 0.15 ml Tween-20 (100%).
- Wash Buffer:** 1X TBS, 0.1% Tween-20 (TBS/T)
- Bovine Serum Albumin (BSA)
- Primary Antibody Dilution Buffer:** 1X TBS, 0.1% Tween-20 with 5% BSA; for 20 ml, add 2 ml 10X TBS to 18 ml water, mix. Add 1.0 g BSA and mix well. While stirring, add 20 µl Tween-20 (100%).
- Phototope[®]-HRP Western Blot Detection System #7071:** Includes biotinylated protein ladder, secondary anti-rabbit (#7074) antibody conjugated to horseradish peroxidase (HRP), anti-biotin antibody conjugated to HRP, LumiGLO[®] chemiluminescent reagent and peroxide.
- Prestained Protein Marker, Broad Range (Premixed Format) #7720
- Biotinylated Protein Ladder Detection Pack #7727
- Blotting Membrane:** This protocol has been optimized for nitrocellulose membranes, which CST recommends. PVDF membranes may also be used.

B Protein Blotting

A general protocol for sample preparation is described below.

- Treat cells by adding fresh media containing regulator for desired time.
- Aspirate media from cultures; wash cells with 1X PBS; aspirate.
- Lyse cells by adding 1X SDS sample buffer (100 µl per well of 6-well plate or 500 µl per plate of 10 cm diameter plate). Immediately scrape the cells off the plate and transfer the extract to a microcentrifuge tube. Keep on ice.
- Sonicate for 10–15 seconds to shear DNA and reduce sample viscosity.
- Heat a 20 µl sample to 95–100°C for 5 minutes; cool on ice.
- Microcentrifuge for 5 minutes.
- Load 20 µl onto SDS-PAGE gel (10 cm x 10 cm).

NOTE: CST recommends loading prestained molecular weight markers (#7720, 10 µl/lane) to verify electrotransfer and biotinylated protein ladder (#7727, 10 µl/lane) to determine molecular weights.

- Electrotransfer to nitrocellulose or PVDF membrane.

C Membrane Blocking and Antibody Incubations

NOTE: Volumes are for 10 cm x 10 cm (100 cm²) of membrane; for different sized membranes, adjust volumes accordingly.

- (Optional) After transfer, wash nitrocellulose membrane with 25 ml TBS for 5 minutes at room temperature.
- Incubate membrane in 25 ml of blocking buffer for 1 hour at room temperature.
- Wash three times for 5 minutes each with 15 ml of TBS/T.
- Incubate membrane and primary antibody (at the appropriate dilution) in 10 ml primary antibody dilution buffer with gentle agitation overnight at 4°C.
- Wash three times for 5 minutes each with 15 ml of TBS/T.
- Incubate membrane with HRP-conjugated secondary antibody (1:2000) and HRP-conjugated anti-biotin antibody (1:1000) to detect biotinylated protein markers in 10 ml of blocking buffer with gentle agitation for 1 hour at room temperature.
- Wash three times for 5 minutes each with 15 ml of TBS/T.

D Detection of Proteins

- Incubate membrane with 10 ml LumiGLO[®] (0.5 ml 20X LumiGLO[®], 0.5 ml 20X Peroxide and 9.0 ml Milli-Q water) with gentle agitation for 1 minute at room temperature.

NOTE: LumiGLO[®] substrate can be further diluted if signal response is too fast.

- Drain membrane of excess developing solution (do not let dry), wrap in plastic wrap and expose to x-ray film. An initial 10-second exposure should indicate the proper exposure time.

NOTE: Due to the kinetics of the detection reaction, signal is most intense immediately following LumiGLO[®] incubation and declines over the following 2 hours.