#9840 Store at -20°C

Akt Kinase Assay Kit (Nonradioactive)

☑ 1 Kit 40 assays

Cell Signaling TECHNOLOGY®

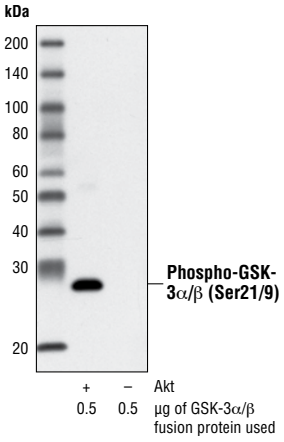
Orders ■ 877-616-CELL (2355)
orders@cellsignal.com
Support ■ 877-678-TECH (8324)
info@cellsignal.com
Web ■ www.cellsignal.com

rev. 05/02/16

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

Description: Nonradioactive Akt Kinase Assay Kit provides all the reagents necessary to measure Akt kinase activity in the cell. Immobilized Phospho-Akt (Ser473) (D9E) Rabbit mAb is used to immunoprecipitate Akt from cell extracts. Then, an *in vitro* kinase assay is performed using GSK-3 Fusion Protein as a substrate. Phosphorylation of GSK-3 is measured by Western blotting, using Phospho-GSK-3α/β (Ser21/9) Antibody.

Species Cross Reactivity: H, M, R



AKT Kinase activity of PDGF-treated NIH/3T3 cell extracts was analyzed by IP/Kinase assay. Cell extracts (200 µl) were incubated overnight with Immobilized Phospho-Akt (Ser473) (D9E) Rabbit mAb #4070. After extensive washing the kinase reaction was performed in the presence of 200 µM of cold ATP and 1 µg of GSK-substrate. Phosphorylation of GSK-3 was measured by Western blot using Phospho-GSK-3 α/β (Ser21/9) Antibody #9327.

Molecular Weight: 27 kDa

Kit Components:

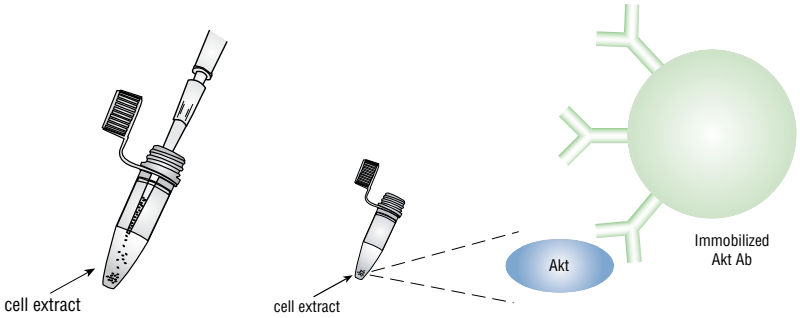
Immobilized Phospho-Akt (Ser473) (D9E) Rabbit mAb (Bead Conjugate): Phospho-Akt (Ser473) (D9E) Rabbit mAb (Bead Conjugate) immunoprecipitates endogenous levels of Akt only when phosphorylated at Ser473. Store at -20ºC. *Do not aliquot the antibody.*

Phospho-GSK-3α/β (Ser21/9) (37F11) Rabbit mAb (GSK-3α Preferred): Phospho-GSK-3α/β (Ser21/9) (37F11) Rabbit mAb (GSK-3α Preferred) preferentially detects endogenous levels of GSK-3α when phosphorylated at Ser21 and also detects GSK-3β when phosphorylated at Ser9. Store at –20°C. *Do not aliquot the antibody.*

Products Included	Product #	Kit Quantity
Immobilized Phospho-Akt (Ser473) (D9E) Rabbit mAb (Bead Conjugate)	4070	1 x 800 µl (40 immunoprecipitations)
Phospho-GSK-3α/β (Ser21/9) (37F11) Rabbit mAb (GSK-3α Preferred)	9327	1 x 100 µl (10 Western mini-blots)
GSK 3 Fusion Protein	9237	1 x 40 µg
Kinase Buffer (10X)	9802	1 x 15 ml
Cell Lysis Buffer (10X)	9803	1 x 15 ml
ATP (10 mM)	9804	1 x 50 µl
Anti-rabbit IgG, HRP-linked Antibody	7074	1 x 100 µl
Anti-biotin, HRP-linked Antibody	7075	1 x 100 µl
20X LumiGLO® Reagent and 20X Peroxide	7003	5 ml each
Biotinylated Protein Ladder Detection Pack	7727	1 x 100 µl

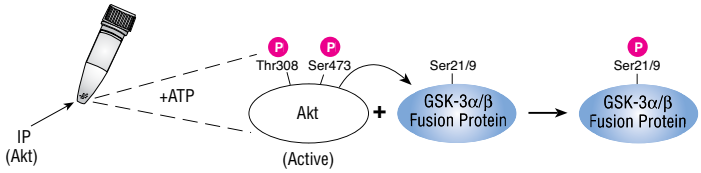
Akt Kinase Assay Kit Overview

Step 1: Selective IP of Akt using Immobilized Akt Antibody.



a) Add immobilized Akt Antibody *b) IP Akt from cell extracts using Immobilized Akt Antibody.*

Step 2: Incubate IP pellets in Kinase Buffer containing GSK-3 fusion protein and cold ATP.



Step 3: Detect GSK-3 phosphorylation using phospho-GSK-3α/β (Ser21/9) antibodies by Western blotting and chemiluminescent detection.

Species Cross-Reactivity Key: **H**—human **M**—mouse **R**—rat **Hm**—hamster **Mk**—monkey **Mi**—mink **C**—chicken **X**—Xenopus **Z**—zebra fish **B**—bovine **All**—all species expected
Species enclosed in parentheses are predicted to react based on 100% sequence homology.

GSK-3 Fusion Protein: GSK-3α/β crosstide corresponding to residues surrounding GSK-3α/β (Ser21/9) (CGPKGPGRRGRRRTSSFAEG) is expressed as a GST fusion protein.

10X Kinase Buffer: 1X concentration: 25 mM Tris (pH 7.5), 5 mM β-Glycerophosphate, 2 mM DTT, 0.1 mM Na_3VO_4, 10 mM $MgCl_2$.

10X Cell Lysis Buffer: 1X concentration: 20 mM Tris (pH 7.5), 150 mM NaCl, 1 mM EDTA, 1 mM EGTA, 1% Triton, 2.5 mM sodium pyrophosphate, 1 mM β-glycerophosphate, 1 mM Na_3VO_4, 1 µg/ml Leupeptin.

10 mM ATP (50 µl): Adenosine-5' triphosphate (ATP) supplied as a 10 mM solution in sterile, doubly distilled water as a disodium salt.

Phototope-HRP Western Detection Kit: The Phototope Western Detection System contains sufficient reagents for the chemiluminescent detection of rabbit antibodies on 10 (10 cm x 10 cm) Western blots. It includes a secondary anti-rabbit antibody conjugated to horseradish peroxidase, anti-biotin antibody conjugated to horseradish peroxidase for the detection of the biotinylated protein ladder (included), LumiGLO® chemiluminescent reagent, and peroxide.

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 a critical role in cell growth by directly phosphorylating mTOR in a rapamycin-sensitive complex containing raptor (17). More importantly, Akt phosphorylates and inactivates tuberin (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).

Selected Application References:

Hisamoto, K. et al. (2000) Estrogen induces the Akt-dependent activation of endothelial nitric oxide synthase in vascular endothelial cells. *J. Biol. Chem.* 276, 3459–3467. Applications: Kinase Assay, W.

Deregibus, M.C. et al. (2003) CD40-dependent activation of phosphatidylinositol 3-kinase/Akt pathway mediates endothelial cell survival and *in vitro* angiogensis. *J. Biol. Chem.* 278, 18008–18014. Applications: kinase assay, W.

Jost, M. et al. (2001) Epidermal growth factor receptor-dependent control of keratinocyte survival and Bcl-xL expression through a MEK-dependent pathway. *J. Biol. Chem.* 276, 6320–6326. Applications: Kinase Assay, W.

Thimmaiah, K.N. et al. (2003) Insulin-like growth factor I-mediated protection from rapamycin-induced apoptosis is independent of Ras-Erk1-Erk2 and phosphatidylinositol 3'-kinase-Akt signaling pathways. *Cancer Res.* 63, 364–374. Applications: Kinase Assay, W.

Kim, S. et al. (2004) Akt activation in platelets depends on Gi signaling pathways. *J. Biol. Chem.* 279, 4186–4195. Applications: Kinase Assay, W.

Cavin, L.G. et al. (2005) Transforming growth factor-α inhibits the intrinsic pathway of c-Myc-induced apoptosis through activation of nuclear factor-κB in murine hepatocellular carcinomas. *Mol. Cancer Res.* 3, 403–412. Applications: Kinase Assay, W.

Hu, H. et al. (2005) PKB/AKT and ERK regulation of caspase-mediated apoptosis by methylseleninic acid in LNCaP prostate cancer cells. *Carcinogenesis* 26, 1374–1381. Applications: Kinase Assay, W.

Companion Products:

Wortmannin #9951

LY294002 (PI3 Kinase Inhibitor) #9901

PhosphoPlus® Akt (Ser473) Antibody Kit #9270

Phospho-Akt (Ser473) Antibody #9271

Phospho-Akt (Ser473) (587F11) Mouse mAb #4051

Phospho-Akt (Thr308) Antibody #9275

Phospho-Bad (Ser112) Antibody #9291

Phospho-Bad (Ser136) Antibody #9295

Phospho-GSK-3β (Ser9) Antibody #9336

Phospho-Tuberin/TSC2 (Thr1462) Antibody #3611

Phospho-Tuberin/TSC2 (Tyr1571) Antibody #3614

GSK-3β (27C10) Rabbit mAb #9315

Akt Antibody #9272

Prestained Protein Marker, Broad Range (Premixed Format) #7720

Biotinylated Protein Ladder Detection Pack #7727

Background References:

(1) Franke, T.F. et al. (1997) *Cell* 88, 435–7.

(2) Burgering, B.M. and Coffer, P.J. (1995) *Nature* 376, 599–602.

(3) Franke, T.F. et al. (1995) *Cell* 81, 727–36.

(4) Alessi, D.R. et al. (1996) *EMBO J* 15, 6541–51.

(5) Sarbassov, D.D. et al. (2005) *Science* 307, 1098–101.

(6) Jacinto, E. et al. (2006) *Cell* 127, 125–37.

(7) Cardone, M.H. et al. (1998) *Science* 282, 1318–21.

(8) Brunet, A. et al. (1999) *Cell* 96, 857–68.

(9) Zimmermann, S. and Moelling, K. (1999) *Science* 286, 1741–4.

(10) Cantley, L.C. and Neel, B.G. (1999) *Proc Natl Acad Sci USA* 96, 4240–5.

(11) Vlahos, C.J. et al. (1994) *J Biol Chem* 269, 5241–8.

(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.

Nonradioactive IP-Kinase Assay Protocol

A Solutions and Reagents

1. **Note:** Prepare solutions with Milli-Q or equivalently purified water.
2. **1X Cell Lysis Buffer:** May be stored at 4°C for short-term use (1–2 weeks). Note: Supplied 10X Cell Lysis Buffer should be vortexed before being used to make 1X solution.
3. **1X Kinase Buffer:** Store at –20°C. May be stored at 4°C for short-term use (1–2 weeks).
4. **GSK-3 Fusion Protein:** Concentration = 0.5 mg/ml. Use 0.5 µg/assay.
5. **10 mM ATP Adenosine-5' triphosphate (ATP)** supplied as a 10 mM solution in sterile, doubly distilled water as a disodium salt.
6.* **Transfer Buffer:** 25 mM Tris base, 0.2 M glycine, 20% methanol (pH=8.5).
7.* **3X SDS Sample Buffer:** 187.5 mM Tris-HCl (pH 6.8 at 25°C), 6% w/v sodium dodecyl sulfate (SDS), 30% glycerol, 150 mM dithiothreitol (DTT), 0.03% w/v bromophenol blue. For 100 mL, use 2.27 g Tris-HCl, 6g SDS, 30 mL glycerol and 30mg w/v bromophenol blue or bromophenol blue dye. Store at –20°C. Add DTT fresh just before use.
8.* **10X Tris-Buffered Saline with Tween®20 (TBS/T):** 0.2 M Tris base, 1.36 M NaCl, 1.0% Tween®20. To prepare 1 liter, dissolve 24.2 g Tris, 80 g NaCl in dH_2O and adjust pH to 7.6 with HCl. Store at room temperature.
9.* **Blocking Buffer:** 1X TBS/T with 5% w/v nonfat dry milk. For 150 mL, dissolve 7.5g nonfat dry milk in 15 mL 10X TBS/T and 135 mL dH_2O. Mix well. Prepare freshly for each experiment.
10.* **Wash Buffer:** 1X TBS, 0.1% Tween®20 (TBS/T). Store at room temperature.
11.* **Primary Antibody Dilution Buffer:** 1X TBS/T with 5% BSA.
12. **Phototope-HRP Western Blot Detection System #7071:** Includes biotinylated protein marker, secondary anti-rabbit (#7074) antibody conjugated to horseradish peroxidase (HRP), Anti-biotin, HRP-linked (D5A7) Rabbit mAb (#5571), 20X LumiGLO® chemiluminescent reagent and 20X peroxide (#7003).
13. **LumiGLO® Substrate #7003:** 0.5 mL 20X LumiGLO, 0.5 mL 20X peroxide and 9.0 mL Milli-Q water.

B Preparing Cell Lysates

1. Aspirate media. Treat cells by adding fresh media containing regulator for desired time.
2. To harvest cells under nondenaturing conditions, remove media and rinse cells once with ice-cold PBS.
3. Remove PBS and add 0.5 mL ice-cold 1X Cell Lysis Buffer plus 1 mM phenylmethylsulfonyl fluoride (PMSF) to each plate (10 cm^2) and incubate the plate on ice for 5 minutes.
4. Scrape cells off the plate and transfer to an appropriate tube. Keep on ice.
5. Sonicate lysates on ice.
6. Microcentrifuge for 10 minutes at 4°C and transfer the supernatant to a new tube. The supernatant is the cell lysate. Store at –80°C.

C IP with Immobilized Antibodies

1. Instructions for use: Prior to use, put tube on ice for 5 minutes to lower viscosity of buffer. Then Beads should be resuspended to a 50% slurry be inversion or *gentle* vortexing.
2. For immunoprecipitations with immobilized Akt primary antibody: Add 20 µl of immobilized antibody bead slurry to 200 µl cell lysate. Incubate with gentle rocking overnight at 4°C.

D Kinase Assay

1. Microcentrifuge cell lysate/immobilized antibody at 14,000 x G for 30 seconds at 4°C. Wash pellet two times with 500 µL of 1X Cell Lysis Buffer. Keep on ice during washes.
2. Wash pellet twice with 500 µL of 1X Kinase Buffer. Keep on ice.
3. Suspend pellet in 50 µL of 1X Kinase Buffer supplemented with 1 µl of 10 mM ATP and appropriate quantity of kinase substrate (1µl).
4. Incubate for 30 minutes at 30°C.
5. Terminate reaction with 25 µL 3X SDS Sample Buffer. Vortex, then microcentrifuge for 30 seconds at 14,000 x G.

E Western Immunoblotting

1. Heat the sample to 95–100°C for 2–5 minutes.
2. Load 5-15 µl of sample per well sample on SDS-PAGE gel.
3. Note: CST recommends loading prestained molecular weight markers (#7720, 10 µL/lane) to verify electrotransfer and biotinylated protein marker (#7727, 10 µL/lane) to estimate molecular weights.
4. Run SDS-page and electrotransfer to nitrocellulose or PVDF membrane.
5. Note: Volumes for all the following steps are for 10 cm x 10 cm membrane; for different sized membranes, adjust volumes accordingly.
6. (Optional) After transfer, wash nitrocellulose membrane with 25 mL TBST for 5 minutes at room temperature.
7. Incubate membrane in 10 mL Blocking Buffer for 1-2 hours at room temperature.
8. Wash three times for 5 minutes each with 15 mL Wash Buffer.
9. Incubate membrane and Phospho-GSK-3a/b (Ser21/9) Antibody (1:1000 dilution) in 10 mL Primary Antibody Dilution Buffer with gentle agitation overnight at 4°C.
10. Wash three times for 5 minutes each with 15 mL Wash Buffer.
11. 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.
12. Wash three times for 5 minutes each with 15 mL Wash Buffer.
13. Incubate membrane with 10 mL LumiGLO Substrate with gentle agitation for 1 minute at room temperature.
14. Drain membrane of excess LumiGLO® Substrate (but 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.
15. **Note:** Due to the kinetics of the detection reaction, signal is most intense immediately following LumiGLO® incubation and declines over the following 2 hours. LumiGLO® Substrate can be further diluted if signal response is too fast.