Store at -80°C

#7501

HTScan® Akt1 Kinase Assay Kit

☑ 100 assays
(96 Well Format)

Cell Signaling TECHNOLOGY®

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Products Included	Products #	Kit Quantity
Phospho-eNOS (Ser1177) (C9C3) Rabbit mAb	9570	30 µl
Kinase Buffer (10X)	9802	15 ml
ATP (10 mM)	9804	1 ml
eNOS (Ser1177) Biotinylated Peptide	1133	1.25 ml
Akt1 Kinase (recombinant, human)	7535	5 µg

Description: The kit provides a means of performing kinase activity assays with recombinant human Akt1 kinase. It includes active Akt1 kinase (supplied as a GST fusion protein), a biotinylated peptide substrate and a Phospho-serine antibody for detection of the phosphorylated form of the substrate peptide.

Peptide Core Sequence: TQS*FS

Molecular Weights: Peptide substrate, Biotin-peptide: 2,431 Daltons. GST-Akt1 Kinase: 85 kDa.

Background: Akt, also referred to as PKB or Rac, plays a critical role in controlling the balance between survival and apoptosis (1-3). This protein kinase is activated by insulin and various growth and survival factors and functions 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. Akt promotes cell survival by inhibiting apoptosis through its ability to phosphorylate and inactivate several targets, including Bad (5), Forkhead transcription factors (6), c-Raf (7) and caspase-9. PTEN phosphatase is a major negative regulator of the PI3 kinase/Akt signaling pathway (8). LY294002 is a specific PI3 kinase inhibitor (9).

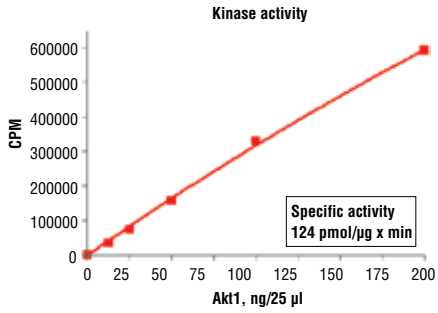

Figure 1. Akt1 kinase activity was measured in a radiometric assay using the following reaction conditions: 5 mM MOPS, pH 7.2, 2.5 mM β-glycerophosphate, 1 mM EGTA, 0.4 mM EDTA, 5 mM $MgCl_2$, 0.05 mM DTT, 50 µM ATP, Substrate: Akt-substrate peptide 200 ng/µL, and variable amount of recombinant Akt1.

One of the essential functions of Akt is the regulation of glycogen synthesis through phosphorylation and inactivation of GSK-3α and β (10,11). Akt may also play a role in insulin stimulation of glucose transport (10).

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 (12) and by negatively regulating the cyclin dependent kinase inhibitors p27 Kip (13) and p21 Waf1 (14). Akt also plays a critical role in cell growth by directly phosphorylating the mammalian target of rapamycin, mTOR (15), but more importantly through phosphorylation and inactivation of tuberin (TSC2), an mTOR inhibitor (16). 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 (17,18).

Source/Purification: The GST-Kinase fusion protein was produced using a baculovirus expression system with a construct expressing a fragment of human Akt1 (Met1-Ala480) (GenBank Accession No. NM_005163) with an amino-terminal GST tag. The protein was purified by one-step affinity chromatography using glutathione-agarose.

Quality Control: The substrate peptide was selected using our Serine/Threonine Kinase Substrate Screening Kit #7400. Phospho-eNOS (Ser1177) (C9C3) Rabbit mAb #9570 was used for detection. The quality of the biotinylated peptide was evaluated by reverse-phase HPLC and by mass spectrometry.

Purified Akt1 kinase was quality controlled for purity by SDS-PAGE followed by Coomassie stain. The specific activity of the Akt1 kinase was determined using a radiometric assay [Fig.1]. Time course [Fig.2], kinase dose dependency [Fig.3] and substrate dose-dependency [Fig.4] assays were performed to verify Akt1 activity using the Akt1 substrate peptide provided in this kit. Akt1 sensitivity to the inhibitor staurosporine was measured using the Akt1 substrate peptide provided in this kit [Fig.5].

Storage: Storage: Antibodies are supplied in 10 mM sodium HEPES (pH 7.5), 150 mM NaCl, 100 µg/ml BSA and 50% glycerol. Do not aliquot the antibodies. Peptides are supplied at 6 µM in 0.001% DMSO.

Enzyme is supplied in 50 mM Tris-HCl, pH7.5; 150 mM NaCl, 0.25 mM DTT, 0.1mM EGTA, 0.1 mM EDTA, 0.1 mM PMSF, 25% glycerol, 7 mM glutathione.

Companion Products:
Serine/Threonine Kinase Substrate Screening Kit #7400

Akt1 Kinase #7535

Phospho-eNOS (Ser1177) (C9C3) Rabbit mAb #9570

eNOS (Ser1177) Biotinylated Peptide #1133

Staurosporine #9953

Kinase Buffer (10X) #9802

ATP (10 mM) #9804

Background References:

(1) Franke, T.F. (1997) *Cell* 88, 435–437.

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

(3) Franke, T.F. et al. (1995) *Cell* 81, 727–736.

(4) Alessi, D.R. et al. (1996) *EMBO J.* 15, 6541–6551.

(5) Cardone, M.H. et al. (1998) *Science* 282, 1318–1321.

(6) Brunet, A. et al. (1999) *Cell* 96, 857–868.

(7) Zimmerman, S. et al. (1999) *Science* 286, 1741–1744.

(8) Cantley, L.C. et al. (1999) *Proc. Natl. Acad. Sci. USA* 96, 4240–4245.

(9) Vlahos, C. et al. (1994) *J. Biol. Chem.* 269, 5241–5248.

(10) Hajduch, E. et al. (2000) *FEBS Lett.* 492, 199–203.

(11) Cross, D.A. et al. (1995) *Nature* 373, 785–789.

(12) Diehl, J.A. et al. (1998) *Genes Dev.* 12, 3499–3511.

(13) Gesbert, F. et al. (2000) *J. Biol. Chem.* 275, 39223–39230.

(14) Zhou, B.P. et al. (2001) *Nat. Cell Biol.* 3, 245–252.

(15) Nave, B.T. et al. (1999) *Biochem. J.* 344, 427–431.

(16) Manning, B.D. et al. (2000) *Mol. Cell* 4, 648–657.

(17) Manning, B.D. et al. (2002) *Mol. Cell* 10, 151–162.

(18) Inoki, K. et al. (2002) *Nat. Cell Biol.* 4, 648–657.

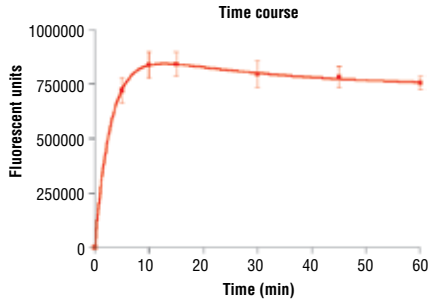

Figure 2. Time course of Akt1 kinase activity: DELFIA® data generated using Phospho-eNOS (Ser1177) (C9C3) Rabbit mAb #9570 to detect phosphorylation of Akt1 substrate peptide (#1133) by Akt1 kinase. In a 50 µl reaction, 10 ng Akt1 and 1.5 µM substrate peptide were used per reaction. (DELFIA® is a registered trademark of PerkinElmer, Inc.)

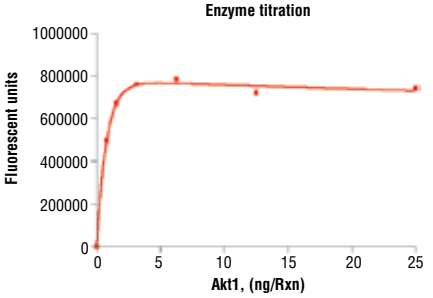

Figure 3. Dose dependence curve of Akt1 kinase activity: DELFIA® data generated using Phospho-eNOS (Ser1177) (C9C3) Rabbit mAb #9570 to detect phosphorylation of substrate peptide (#1133) by Akt1 kinase. In a 50 µl reaction, increasing amounts of Akt1 and 1.5 µM substrate peptide were used per reaction at room temperature for 10 minutes. (DELFIA® is a registered trademark of PerkinElmer, Inc.)

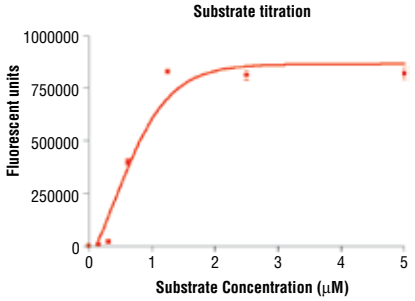

Figure 4. Peptide concentration dependence of Akt1 kinase activity: DELFIA® data generated using Phospho-eNOS (Ser1177) (C9C3) Rabbit mAb #9570 to detect phosphorylation of substrate peptide (#1133) by Akt1 kinase. In a 50 µl reaction, 10 ng of Akt1 and increasing concentrations of substrate peptide were used per reaction at room temperature for 10 minutes. (DELFIA® is a registered trademark of PerkinElmer, Inc.)

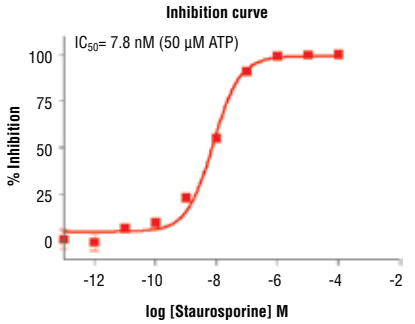

Figure 5. Staurosporine inhibition of Akt1 kinase activity: DELFIA® data generated using Phospho-eNOS (Ser1177) (C9C3) Rabbit mAb #9570 to detect phosphorylation of Akt1 substrate peptide (#1133) by Akt1 kinase. In a 50 µl reaction, 10 ng Akt1, 1.5 µM substrate peptide, 50 µM ATP and increasing amounts of staurosporine were used per reaction at room temperature for 10 minutes. (DELFIA® is a registered trademark of PerkinElmer, Inc.)

Protocol for HTScan® Akt1 Kinase Assay Kit

Kinase

Note: Lot-specific information for this kinase is provided on the enzyme vial. Optimal assay incubation times and enzyme concentrations must be determined empirically for each lot of kinase under specified conditions.

A Additional Solutions and Reagents (Not included)

1. **Wash Buffer:** 1X PBS, 0.05% Tween-20 (PBS/T)
2. Bovine Serum Albumin (BSA)
3. **Stop Buffer:** 50 mM EDTA pH 8

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B Suggested Protocol for 100 Assays

1. Add 100 µl 10 mM ATP to 1.25 ml 6 µM substrate peptide. Dilute the mixture with dH_20 to 2.5 ml to make 2X ATP/substrate cocktail ([ATP]=400 µM, [substrate] = 3 µm).
2. Transfer enzyme from –80°C to ice. Allow enzyme to thaw on ice.
3. **Microcentrifuge briefly at 4°C to bring liquid to the bottom of the vial. Return immediately to ice.**
4. Add 1 ml 10X kinase buffer [1 ml 10X Kinase Buffer 250 mM Tris-HCl pH 7.5, 100 mM $MgCl_2$, 1 mM Na_3VO_4, 50 mM β-glycerophosphate, 20 mM dithiothreitol (DTT)] to 1.5 ml dH_20 to make 2.5 ml 4X reaction buffer.
5. Mix 1.5 ml of 4X Reaction buffer with 12 µl enzyme (100 ng/µl) to make 4X reaction cocktail ([enzyme]) = 0.8 ng/µl in 4X reaction cocktail).
6. Add 12.5 µl of the 4X reaction cocktail to 12.5 µl/well of prediluted compound of interest (usually around 10 µM) and incubate for 5 minutes at room temperature.
7. Add 25 µl of 2X ATP/substrate cocktail to 25 µl/well preincubated reaction cocktail/compound.

Final Assay Conditions for a 50 µl Reaction

25 mM Tris-HCl (pH 7.5)
10 mM $MgCl_2$
5 mM β-glycerophosphate
0.1 mM Na_3VO_4
2 mM DTT
200 µM ATP
1.5 µM peptide
10 ng Akt1 Kinase

8. Incubate reaction plate at room temperature for 30 minutes.
9. Add 50 µl/well Stop Buffer (50 mM EDTA, pH 8) to stop the reaction.
10. Transfer 25 µl of each reaction to a 96-well streptavidin-coated plate containing 75 µl dH_2O/well and incubate at room temperature for 60 minutes.
11. *Wash three times with 200 µl/well PBS/T.
12. Dilute primary antibody, Phospho-eNOS (Ser1177) (C9C3) Rabbit mAb, 1:1000 in PBS/T with 1% BSA. Add 100 µl/well primary antibody.
13. Incubate at room temperature for 120 minutes.
14. *Wash three times with 200 µl/well PBS/T.
15. For DELFIA® or Colorimetric ELISA detection methods please use the following protocols.

DELFIA® Assay

1. Prepare appropriate dilution of Europium labeled secondary antibody in PBS/T with 1% BSA (1:500 dilution for anti-mouse IgG or 1:1000 for anti-rabbit IgG).
2. Add 100 µl/well secondary antibody solution.
3. Incubate at room temperature for 30 minutes.
4. *Wash five times with 200 µl/well PBS/T.
5. Add 100 µl/well DELFIA® Enhancement Solution.
6. Incubate at room temperature for 5 minutes.
7. Read plate using a Time Resolved Fluorescent plate reader using the following settings;
   - **a.** Excitation Filter: 340 nm
   - **b.** Emission Filter: 615 nm
   - **c.** Delay++: 400 µs

++ Delay time is the delay from the excitation pulse to the beginning of the measurement.

Companion Products for DELFIA®

DELFIA® Europium-labeled Anti-mouse IgG (PerkinElmer Life Sciences #AD0124)
DELFIA® Europium-labeled Anti-rabbit IgG (PerkinElmer Life Sciences #AD0105)
DELFIA® Enhancement Solution (PerkinElmer Life Sciences #1244-105)
DELFIA® Streptavidin coated, 96-well, yellow plate (PerkinElmer Life Sciences AAAND-0005)

Colorimetric ELISA Assay

1. Prepare appropriate dilution of HRP labeled secondary antibody in PBS/T with 1% BSA (1:500 dilution for anti-mouse IgG or 1:1000 for anti-rabbit IgG).
2. Add 100 µl/well secondary antibody solution.
3. Incubate at room temperature for 30 minutes.
4. *Wash five times with 200 µl/well PBS/T.
5. Add 100 µl/well TMB substrate.
6. Incubate at room temperature for 15 minutes.
7. Add 100 µl/well of stop solution.
8. Mix well.
9. Read the absorbance at 450 nm with a microtiter plate reader.

Companion Products For Colorimetric ELISA Assay

Anti-mouse IgG, HRP Linked Antibody #7076
Anti-rabbit IgG, HRP Linked Antibody #7074
TMB Solution #7004
Stop Solution #7002

***NOTE:** Use of an automated microplate washer as well as centrifugation of plates when appropriate, greatly improves reproducibility.

Please contact Cell Signaling Technology for HTS-ready antibodies (PBS formulated and carrier-free), and detailed peptide substrate sequence information.
Email: drugdiscovery@cellsignal.com