

# HTScan® Akt3 Kinase Assay Kit

✓ 100 assays  
(96 Well Format)

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This product is for *in vitro* research use only and is not intended for use in humans or animals.

Products Included	Products #	Kit Quantity
Phospho-eNOS (Ser1177) Antibody	9571	30 µl
Kinase Buffer (10X)	9802	15 ml
ATP (10 mM)	9804	1 ml
eNOS (Ser1177) Biotinylated Peptide	1133	1.25 ml
Akt3 Kinase (recombinant, human)	7506	5 µg

**Description:** The kit provides a means of performing kinase activity assays with recombinant human Akt3 kinase. It includes active Akt3 kinase (supplied as a GST fusion protein), a biotinylated peptide substrate and a phosphoserine antibody for detection of the phosphorylated form of the substrate peptide.

**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 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. 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 through its ability to phosphorylate and inactivate 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).

One of the essential functions of Akt 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 (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 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 (19,20).

**Peptide Core Sequence:** TQS\*FS

**Molecular Weights:** Peptide substrate, Biotin-peptide: 2,431 Daltons. GST-Akt3 Kinase: 84 kDa.

**Source/Purification:** The GST-Kinase fusion protein was produced using a baculovirus expression system with a construct expressing full length human Akt3 (Met1-Glu479) (GenBank Accession No. NM\_005465) 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) Antibody #9571 was used for detection. The quality of the biotinylated peptide was evaluated by reverse-phase HPLC and by mass spectrometry.

Purified Akt3 kinase was quality controlled for purity by SDS-PAGE followed by Coomassie stain and Western blot. The specific activity of the Akt3 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 Akt3 activity using the Akt3 substrate peptide provided in this kit. Akt3 sensitivity to the inhibitor staurosporine was measured using the Akt3 substrate peptide provided in this kit [Fig.5].

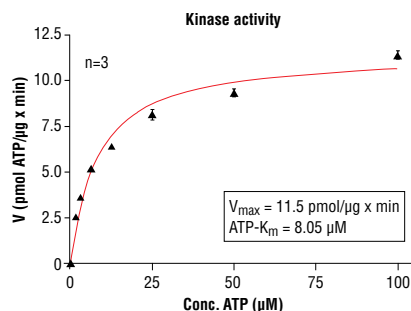


Figure 1. Akt3 kinase activity was measured in a radiometric assay using the following reaction conditions: 60 mM HEPES-NaOH, pH 7.5, 3 mM MgCl<sub>2</sub>, 3 mM MnCl<sub>2</sub>, 3 µM Na-orthovanadate, 1.2 mM DTT, ATP (variable), 2.5 µg/50 µl PEG20,000, Substrate: GSK3 (14-27), 5 µg/50 µl, recombinant Akt3: 200 ng/50 µl.

**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. Enzymes are supplied in 50 mM Tris-HCL (pH 8.0), 100 mM NaCl, 5 mM DTT, 15 mM reduced glutathione and 20% glycerol. Store at -80°C.

Keep enzymes on ice during use.

Avoid repeated freeze-thaw cycles.

#### Companion Products:

Serine/Threonine Kinase Substrate Screening Kit #7400

Akt3 Kinase #7506

Phospho-eNOS (Ser1177) Antibody #9571

eNOS (Ser1177) Biotinylated Peptide #1133

Staurosporine #9953

#### Background References:

- (1) Franke, T.F. (1997) *Cell* 88, 435-437.
- (2) Burgering, B.T. and Coffey, 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) Sarbassov, D.D. et al. (2005) *Science* 307, 1098-1101.
- (6) Jacinto, E. et al. (2006) *Cell* 127, 125-137.
- (7) Cardone, M.H. et al. (1998) *Science* 282, 1318-1321.
- (8) Brunet, A. et al. (1999) *Cell* 96, 857-868.
- (9) Zimmerman, S. et al. (1999) *Science* 286, 1741-1744.
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- (11) Vlahos, C. et al. (1994) *J. Biol. Chem.* 269, 5241-5248.
- (12) Hajdich, E. et al. (2000) *FEBS Lett.* 492, 199-203.
- (13) Cross, D.A. et al. (1995) *Nature* 373, 785-789.
- (14) Diehl, J.A. et al. (1998) *Genes Dev.* 12, 3499-3511.
- (15) Gesbert, F. et al. (2000) *J. Biol. Chem.* 275, 39223-39230.
- (16) Zhou, B.P. et al. (2001) *Nat. Cell Biol.* 3, 245-252.
- (17) Nave, B.T. et al. (1999) *Biochem. J.* 344, 427-431.
- (18) Manning, B.D. et al. (2000) *Mol. Cell* 4, 648-657.
- (19) Manning, B.D. et al. (2002) *Mol. Cell* 10, 151-162.
- (20) Inoki, K. et al. (2002) *Nat. Cell Biol.* 4, 648-657.

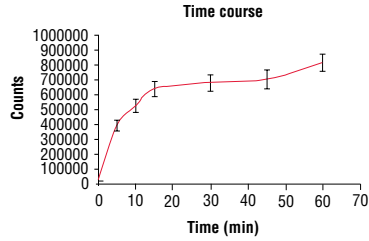


Figure 2. Time course of Akt3 kinase activity: DELFIA® data generated using Phospho-eNOS (Ser1177) Antibody #9571 to detect phosphorylation of Akt3 substrate peptide (#1133) by Akt3 kinase. In a 50 µl reaction, 50 ng Akt3 and 1.5 µM substrate peptide were used per reaction. (DELFIA® is a registered trademark of PerkinElmer, Inc.)

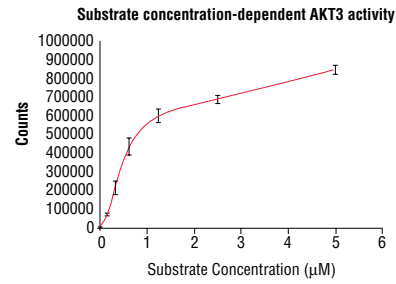


Figure 4. Peptide concentration dependence of Akt3 kinase activity: DELFIA® data generated using Phospho-eNOS (Ser1177) Antibody #9571 to detect phosphorylation of substrate peptide (#1133) by Akt3 kinase. In a 50 µl reaction, 50 ng of Akt3 and increasing concentrations of substrate peptide were used per reaction at room temperature for 30 minutes. (DELFIA® is a registered trademark of PerkinElmer, Inc.)

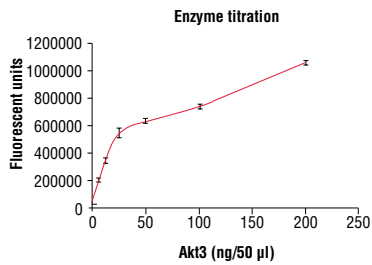


Figure 3. Dose dependence curve of Akt3 kinase activity: DELFIA® data generated using Phospho-eNOS (Ser1177) Antibody #9571 to detect phosphorylation of substrate peptide (#1133) by Akt3 kinase. In a 50 µl reaction, increasing amounts of Akt3 and 1.5 µM substrate peptide were used per reaction at room temperature for 30 minutes. (DELFIA® is a registered trademark of PerkinElmer, Inc.)

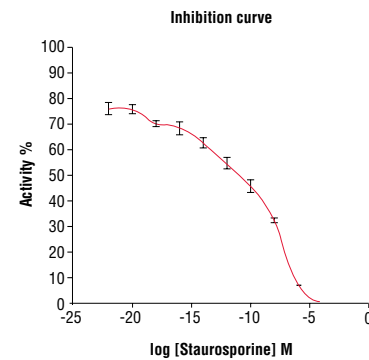


Figure 5. Staurosporine inhibition of Akt3 kinase activity: DELFIA® data generated using Phospho-eNOS (Ser1177) Antibody #9571 to detect phosphorylation of Akt3 substrate peptide (#1133) by Akt3 kinase. In a 50 µl reaction, 50 ng Akt3, 1.5 µM substrate peptide, 20 µM ATP and increasing amounts of staurosporine were used per reaction at room temperature for 30 minutes. (DELFIA® is a registered trademark of PerkinElmer, Inc.)

## Protocol for HTScan® Akt3 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

*DELFI<sup>®</sup> is a registered trademark of PerkinElmer Life Sciences*

### 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<sub>2</sub>O 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<sub>2</sub>, 1 mM Na<sub>3</sub>VO<sub>4</sub>, 50 mM β-glycerophosphate, 20 mM dithiothreitol (DTT)] to 1.5 ml dH<sub>2</sub>O to make 2.5 ml 4X reaction buffer.
5. Transfer 1.2 ml of 4X Reaction buffer to each enzyme tube to make 4X reaction cocktail ([enzyme] = 4 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<sub>2</sub>  
 5 mM β-glycerophosphate  
 0.1 mM Na<sub>3</sub>VO<sub>4</sub>  
 2 mM DTT  
 200 µM ATP  
 1.5 µM peptide  
 50 ng Akt3 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<sub>2</sub>O/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) Antibody, 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<sup>®</sup> or Colorimetric ELISA detection methods please use the following protocols.

### DELFI<sup>®</sup> 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<sup>®</sup> 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<sup>\*\*</sup>: 400 µs
- <sup>\*\*</sup> Delay time is the delay from the excitation pulse to the beginning of the measurement.

### Companion Products for DELFIA<sup>®</sup>

DELFI<sup>®</sup> Europium-labeled Anti-mouse IgG (PerkinElmer Life Sciences #AD0124)  
 DELFI<sup>®</sup> Europium-labeled Anti-rabbit IgG (PerkinElmer Life Sciences #AD0105)  
 DELFI<sup>®</sup> Enhancement Solution (PerkinElmer Life Sciences #1244-105)  
 DELFI<sup>®</sup> 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