

HTScan® NEK2 Kinase Assay Kit

✓ 100 assays
(96 Well Format)

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rev. 09/05/07

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-PKA Substrate (RRXS/T) (100G7) Rabbit mAb	9624	30 µl
Kinase Buffer (10X)	9802	15 ml
ATP (10 mM)	9804	1 ml
CREB (Ser133) Biotinylated Peptide	1331	1.25 ml
NEK2 Kinase (recombinant, human)	7554	5 µg

Description: The kit provides a means of performing kinase activity assays with recombinant human NEK2 kinase. It includes full-length human active NEK2 kinase (supplied as a GST fusion protein), a biotinylated peptide substrate and a phospho-serine/threonine antibody for detection of the phosphorylated form of the substrate peptide.

Peptide Core Sequence: RRPS*Y

Molecular Weights: Peptide substrate, Biotin-CREB (Ser133) peptide: 2,326 Daltons. GST-NEK2 Kinase: 83 kDa.

Background: The NEK family of protein kinases is composed of 11 members in humans that share an amino-terminal catalytic domain related to NIMA, a serine/threonine kinase identified in *Aspergillus nidulans*. While NIMA is critical for cell cycle progression in fungus, the function of NEKs in mammalian cells is largely unknown. NEK1 was first identified by screening mouse cDNA expression libraries and was demonstrated to have dual kinase activity on both tyrosine and serine/threonine sites (1). NEK2 most closely resembles fungal NIMA in its primary structure and is believed to promote the splitting of duplicated centrosomes at the onset of mitosis (2,3). NEK3 is predominantly a cytoplasmic enzyme and its activity shows marginal variation throughout the cell cycle (4). NEK4 is ubiquitously expressed and its expression

and subcellular location are not associated with cell cycle (5). NEK6/7 have been suggested to phosphorylate and activate p70 S6 kinase *in vitro* (6). Expression of an inactive NEK6 mutant arrests cells in M phase and interferes with chromosome segregation (7). NEK8 activity is not cell cycle regulated and may play a role in cell cycle independent microtubule dynamics (8). NEK9 is activated during mitosis and may participate in the activation of NEK6/7 during mitosis (9,10).

Source/Purification: The GST-Kinase fusion protein was produced using a baculovirus expression system with a construct expressing full-length human NEK2 (Met1-Arg445) (GenBank Accession No. NM_002497) 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-PKA Substrate (RRXS/T) (100G7) Rabbit mAb #9624 was used for detection. The quality of the biotinylated peptide was evaluated by reverse-phase HPLC and by mass spectrometry.

Purified NEK2 kinase was quality controlled for purity by SDS-PAGE followed by Coomassie stain and Western blot. The specific activity of the NEK2 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 NEK2 activity using the NEK2 substrate peptide provided in this kit. Nek2 sensitivity to the inhibitor staurosporine was measured using the NEK2 substrate peptide provided in this kit [Fig.5].

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

NEK2 Kinase #7554

Phospho-PKA Substrate (RRXS/T) (100G7) Rabbit mAb #9624

CREB (Ser133) Biotinylated Peptide #1331

Staurosporine #9953

Background References:

- (1) Letwin, K. et al. (1992) *EMBO J.* 11, 3521–3531.
- (2) Fry, A.M. et al. (1995) *J. Biol. Chem.* 270, 12899–12905.
- (3) Fry, A.M. (2002) *Oncogene* 21, 6184–6194.
- (4) Tanaka, K. and Nigg, E.A. (1999) *J. Biol. Chem.* 274, 13491–13497.
- (5) Hayashi, K. et al. (1999) *Biochem. Biophys. Res. Commun.* 264, 449–456.
- (6) Belham, C. et al. (2001) *Curr. Biol.* 11, 1155–1167.
- (7) Yin, M.J. et al. (2003) *J. Biol. Chem.* 278, 52454–52460.
- (8) Holland, P.M. et al. (2002) *J. Biol. Chem.* 277, 16229–16240.
- (9) Belham, C. et al. (2003) *J. Biol. Chem.* 278, 34897–34909.
- (10) Roig, J. et al. (2002) *Genes Dev.* 16, 1640–1658.

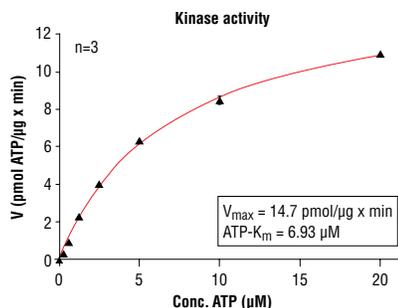


Figure 1. NEK2 kinase activity was measured in a radiometric assay using the following reaction conditions: 60 mM HEPES-NaOH, pH 7.5, 3 mM MgCl₂, 3 mM MnCl₂, 3 µM Na-orthovanadate, 1.2 mM DTT, 1 µM ATP, 2.5 µg/50 µl PEG20,000, Substrate: Myelin basic protein, 5 µg/50 µl, and recombinant NEK2: 100 ng/50 µl.

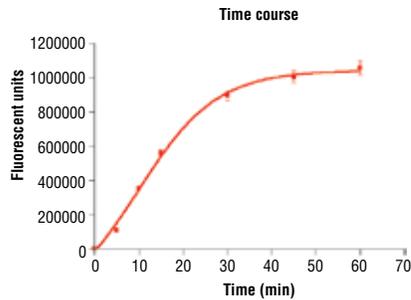


Figure 2. Time course of NEK2 kinase activity: DELFIA® data generated using Phospho-PKA Substrate (RRXS/T) (100G7) Rabbit mAb #9624 to detect phosphorylation of NEK2 substrate peptide (#1331) by NEK2 kinase. In a 50 µl reaction, 50 ng NEK2 and 1.5 µM substrate peptide were used per reaction. (DELFIA® is a registered trademark of PerkinElmer, Inc.)

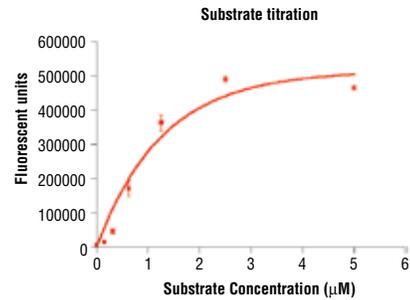


Figure 4. Peptide concentration dependence of NEK2 kinase activity: DELFIA® data generated using Phospho-PKA Substrate (RRXS/T) (100G7) Rabbit mAb #9624 to detect phosphorylation of substrate peptide (#1331) by NEK2 kinase. In a 50 µl reaction, 50 ng of NEK2 and increasing concentrations of substrate peptide were used per reaction at room temperature for 30 minutes. (DELFIA® is a registered trademark of PerkinElmer, Inc.)

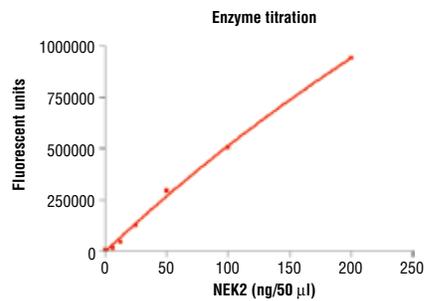


Figure 3. Dose dependence curve of NEK2 kinase activity: DELFIA® data generated using Phospho-PKA Substrate (RRXS/T) (100G7) Rabbit mAb #9624 to detect phosphorylation of substrate peptide (#1331) by NEK2 kinase. In a 50 µl reaction, increasing amounts of NEK2 and 1.5 µM substrate peptide were used per reaction at room temperature for 30 minutes. (DELFIA® is a registered trademark of PerkinElmer, Inc.)

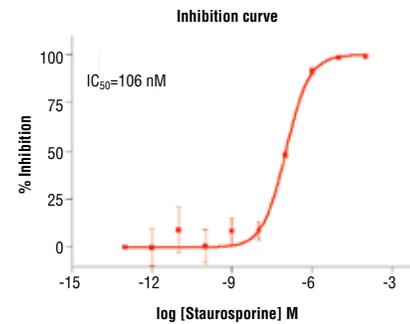


Figure 5. Staurosporine inhibition of NEK2 kinase activity: DELFIA® data generated using Phospho-PKA Substrate (RRXS/T) (100G7) Rabbit mAb #9624 to detect phosphorylation of NEK2 substrate peptide (#1331) by NEK2 kinase. In a 50 µl reaction, 50 ng NEK2, 1.5 µM substrate peptide, 20 µM ATP and increasing amounts of staurosporine were used per reaction at room temperature for 30 minutes. (DELFIA® is a registered trademark of PerkinElmer, Inc.)

Protocol for HTScan® NEK2 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 dH₂O 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 MgCl₂, 1 mM Na₃VO₄, 50 mM β-glycerophosphate, 20 mM dithiothreitol (DTT)] to 1.5 ml dH₂O 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 MgCl₂
 5 mM β-glycerophosphate
 0.1 mM Na₃VO₄
 2 mM DTT
 200 µM ATP
 1.5 µM peptide
 50 ng NEK2 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 dH₂O/well and incubate at room temperature for 60 minutes.
11. *Wash three times with 200 µl/well PBS/T.
12. Dilute primary antibody, Phospho-PKA Substrate (RRXS/T) (100G7) 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 DELFLIA® or Colorimetric ELISA detection methods please use the following protocols.

DELFLIA® 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 DELFLIA® 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 DELFLIA®

DELFLIA® Europium-labeled Anti-mouse IgG (PerkinElmer Life Sciences #AD0124)
 DELFLIA® Europium-labeled Anti-rabbit IgG (PerkinElmer Life Sciences #AD0105)
 DELFLIA® Enhancement Solution (PerkinElmer Life Sciences #1244-105)
 DELFLIA® 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