Store at -80°C

#7549

HTScan® IKKβ Kinase Assay Kit

☑100 assays
(96 Well Format)

Cell Signaling TECHNOLOGY®

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Products Included	Products #	Kit Quantity
Phospho-IκBα (Ser32) (14D4) Rabbit mAb	2859	30 µl
Kinase Buffer (10X)	9802	15 ml
ATP (10 mM)	9804	1 ml
IκB-α (Ser32) Biotinylated Peptide	1146	1.25 ml
IKKβ Kinase (recombinant, human)	7548	5 µg

Description: The kit provides a means of performing kinase activity assays with recombinant human IKKβ kinase. It includes active IKKβ 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: DS*GLDS*M

Molecular Weights: Peptide substrate, Biotin-peptide: 2,085 Daltons. GST-IKKβ Kinase: 87 kDa.

Background: The NFκB/Rel transcription factors are present in the cytosol in an inactive state, complexed with the inhibitory IκB proteins (1-3). Most agents that activate NFκB do so through a common pathway based on phosphorylation-induced, proteasome-mediated degradation of IκB (3-7). The key regulatory step in this pathway involves activation of a high molecular weight IκB kinase (IKK) complex, whose catalysis is generally carried out by three tightly associated IKK subunits. IKKα and IKKβ serve as the catalytic subunits of the kinase. IKKγ serves as the regulatory subunit (8-9). Activation of IKK depends on phosphorylation; serines 177 and 181 in the activation loop of IKKβ (176 and 180 in IKKα) are the specific sites whose phosphorylation causes conformational changes resulting in kinase activation (10-13).

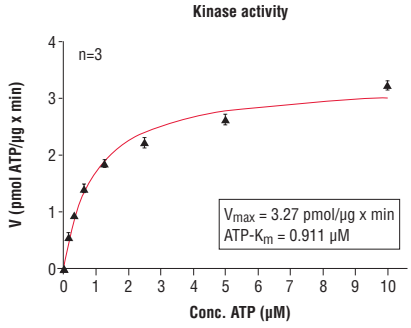

Figure 1. IKKβ kinase activity was measured in a radiometric assay using the following reaction conditions: 60 mM HEPES-NaOH, pH 7.5, 3 mM $MgCl_2$, 3 mM $MnCl_2$, 3 µM Na-orthovanadate, 1.2 mM DTT, ATP (variable), 2.5 µg/50 µl PEG20.000, Substrate: Rb CTF 1.5 µg/50 µl, recombinant IKKβ: 50 ng/50 µl.

Source/Purification: The GST-Kinase fusion protein was produced using a baculovirus expression system with a construct expressing full length human IKKβ (Met1-Ser756) (GenBank Accession No. AF029684) 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-IκBα (Ser32) (14D4) Rabbit mAb #2859 was used for detection. The quality of the biotinylated peptide was evaluated by reverse-phase HPLC and by mass spectrometry.

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

Background References:

(1) Baeuerle, P.A. et al. (1988) *Science* 242, 540–546.
(2) Beg, A.A. et al. (1993) *Genes Dev.* 7, 2064–2070.
(3) Finco, T.S. et al. (1994) *Proc. Natl. Acad. Sci. USA* 91, 11884–11888.
(4) Brown, K. et al. (1995) *Science* 267, 1485–1488.
(5) Brockman, J.A. et al. (1995) *Mol. Cell. Biol.* 15, 2809–2818.
(6) Traenckner, E.B. et al. (1995) *EMBO J.* 14, 2876–2883.
(7) Chen, Z.J. et al. (1996) *Cell* 84, 853–862.
(8) Zandi, E. et al. (1997) *Cell* 91, 243–252.
(9) Karin, M. et al. (1999) *Oncogene* 18, 6867–6874.
(10) DiDonato, J.A. et al. (1997) *Nature* 388, 548–554.
(11) Mercurio, F. et al. (1997) *Science* 278, 860–866.
(12) Johnson, L.N. et al. (1996) *Cell* 85, 149–158.
(13) Delhase, M. et al. (1999) *Science* 284, 309–313.

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

IKKβ Kinase #7548

Phospho-IκB-α (Ser32/36) (5A5) Mouse mAb #9246

IκB-α (Ser32) Biotinylated Peptide #1146

Staurosporine #9953

Kinase Buffer (10X) #9802

ATP (10 mM) #9804

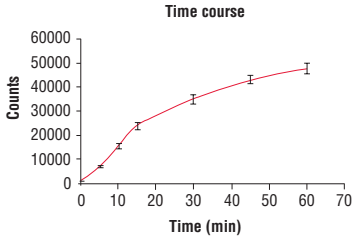

Figure 2. Time course of IKKβ kinase activity: DELFIA® data generated using Phospho-IκB-α (Ser32/36) (5A5) Mouse mAb #9246 to detect phosphorylation of IKKβ substrate peptide (#1146) by IKKβ kinase. In a 50 µl reaction, 50 ng IKKβ and 1.5 µM substrate peptide were used per reaction. (DELFIA® is a registered trademark of PerkinElmer, Inc.)

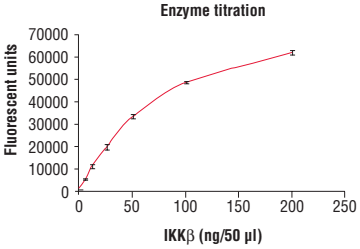

Figure 3. Dose dependence curve of IKKβ kinase activity: DELFIA® data generated using Phospho-IκB-α (Ser32/36) (5A5) Mouse mAb #9246 to detect phosphorylation of substrate peptide (#1146) by IKKβ kinase. In a 50 µl reaction, increasing amounts of IKKβ and 1.5 µM substrate peptide were used per reaction at room temperature for 30 minutes. (DELFIA® is a registered trademark of PerkinElmer, Inc.)

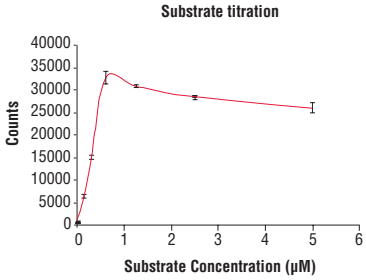

Figure 4. Peptide concentration dependence of IKKβ kinase activity: DELFIA® data generated using Phospho-IκB-α (Ser32/36) (5A5) Mouse mAb #9246 to detect phosphorylation of substrate peptide (#1146) by IKKβ kinase. In a 50 µl reaction, 50 ng of IKKβ and increasing concentrations of substrate peptide were used per reaction at room temperature for 30 minutes. (DELFIA® is a registered trademark of PerkinElmer, Inc.)

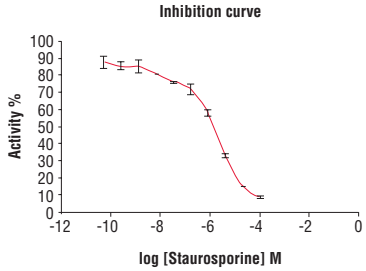

Figure 5. Staurosporine inhibition of IKKβ kinase activity: DELFIA® data generated using Phospho-IκB-α (Ser32/36) (5A5) Mouse mAb #9246 to detect phosphorylation of IKKβ substrate peptide (#1146) by IKKβ kinase. In a 50 µl reaction, 50 ng IKKβ, 1.5 µM substrate peptide, 20 µM ATP and increasing amounts of staurosporine were used per reaction at room temperature for 30 minutes. (DELFIA® is a registered trademark of PerkinElmer, Inc.)

Protocol for HTScan® IKKβ 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. 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 $MgCl_2$
5 mM β-glycerophosphate
0.1 mM Na_3VO_4
2 mM DTT
200 µM ATP
1.5 µM peptide
50 ng IKKβ 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-IκBα (Ser32) (14D4) 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