Store at –80°C

#7716

HTScan® EPHB3 Kinase Assay Kit

☑ 100 assays
(96 Well Format)

Cell Signaling TECHNOLOGY®

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Products Included	Products #	Kit Quantity
Phospho-Tyrosine Mouse mAb (P-Tyr-100)	9411	30 µl
HTScan® Tyrosine Kinase Buffer (4X)	9805	15 ml
DTT (1000X, 1.25 M)		80 µl
ATP (10 mM)	9804	1 ml
BTK (Tyr223) Biotinylated Peptide	1330	1.25 ml
EphB3 Kinase (recombinant, human)	7715	5 µg

Description: The kit provides a means of performing kinase activity assays with recombinant human EphB3 kinase. It includes active EphB3 kinase (supplied as a GST fusion protein), a biotinylated peptide substrate and a phospho-tyrosine antibody for detection of the phosphorylated form of the substrate peptide.

Peptide Core Sequence: LY*DY*M

Molecular Weights: Peptide substrate, Biotin-peptide 2,052 Daltons. GST-EphB3 Kinase: 80 kDa.

Background: The Eph receptors are the largest known family of receptor tyrosine kinases (RTKs). They can be divided into two groups based on sequence similarity and on their preference for a subset of ligands: EphA receptors bind to a glycosylphosphatidylinositol-anchored ephrin A ligand, and EphB receptors bind to ephrin B proteins that have a transmembrane and cytoplasmic domain (1,2). Eph receptors and ligands may be involved in many diseases including cancer (3). Both ephrin A and ephrin B ligands have dual functions. As RTK ligands, the ephrins stimulate the kinase activity of the Eph receptors and activate signaling pathways in receptor-expressing cells. The ephrin extracellular domain is sufficient for this function as long as it is clustered (4). The second function of ephrins has been described as "reverse signaling," whereby the cytoplasmic domain becomes tyrosine phosphorylated, allowing interactions with other proteins that may activate signaling pathways in the ligand-expressing cells (5). Various stimuli can induce tyrosine phosphorylation of ephrin B, including binding to EphB receptors, activation of Src kinase and stimulation by PDGF and FGF (6). Tyrosines 324/327 have been identified as major phosphorylation sites of ephrin B1 *in vivo* (7).

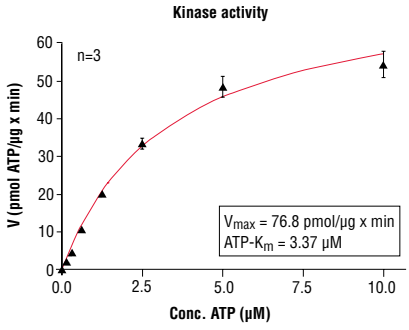

Figure 1. EphB3 kinase activity was measured in a radiometric assay using the following reaction conditions: 60 mM HEPES-NaOH, pH 7.5, 3 mM $MgCl_2$, 3 mM $MnCl_2$, 3 µM Na-orthovanadate, 1.2 mM DTT, ATP (variable), 2.5 µg/50 µl PEG20.000, Substrate: PolyEY, 0.5 µg/50 µl, recombinant EphB3: 25 ng/50 µl.

Source/Purification: The GST-Kinase fusion protein was produced using a baculovirus expression system with a construct expressing a fragment of human EphB3 (Gln585-Val998) (GenBank accession No. NM_004443) 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 Tyrosine Kinase Substrate Screening Kit #7450. Phospho-Tyrosine mAb (P-Y-100) #9411 was used for detection. The quality of the biotinylated peptide was evaluated by reverse-phase HPLC and by mass spectrometry.

Purified EphB3 kinase was quality controlled for purity by SDS-PAGE followed by Coomassie stain and Western blot. The specific activity of the EphB3 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 EphB3 activity using the EphB3 substrate peptide provided in this kit. EphB3 sensitivity to the inhibitor staurosporine was measured using the EphB3 substrate peptide provided in this kit [Fig.5].

Background References:

(1) Wilkinson, D.G. (2000) *Int. Rev. Cytol.* 196, 177–244.

(2) Klein, R. (2001) *Curr. Opin. Cell Biol.* 13, 196–203.

(3) Dodelet, V.C. and Pasquale, E.B. (2000) *Oncogene* 19, 5614–5619.

(4) Holder, N. and Klein, R. (1999) *Development* 126, 2033–2044.

(5) Bruckner, K. et al. (1997) *Science* 275, 1640–1643.

(6) Palmer, A. et al. (2002) *Mol. Cell* 9, 1–20.

(7) Kalo, M.S. et al. (2001) *J. Biol. Chem.* 276, 38940–38948.

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:

Tyrosine Kinase Substrate Screening Kit #7450

EphB3 Kinase #7715

Phospho-Tyrosine Mouse mAb (P-Tyr-100) #9411

BTK (Tyr223) Biotinylated Peptide #1330

HTScan® Profiling Kit (Tyrosine Kinase Set I) #7405

Staurosporine #9953

HTScan® Tyrosine Kinase Buffer (4X) #9805

ATP (10 mM) #9804

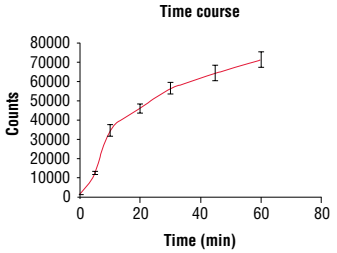

Figure 2. Time course of EphB3 kinase activity: DELFIA® data generated using Phospho-Tyrosine mAb (P-Tyr-100) #9411 to detect phosphorylation of EphB3 substrate peptide (#1330) by EphB3 kinase. In a 50 µl reaction, 50 ng EphB3 and 1.5 µM substrate peptide were used per reaction. (DELFIA® is a registered trademark of PerkinElmer, Inc.)

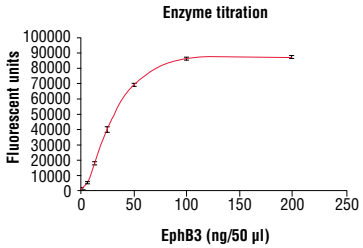

Figure 3. Dose dependence curve of EphB3 kinase activity: DELFIA® data generated using Phospho-Tyrosine mAb (P-Tyr-100) #9411 to detect phosphorylation of substrate peptide (#1330) by EphB3 kinase. In a 50 µl reaction, increasing amounts of EphB3 and 1.5 µM substrate peptide were used per reaction at room temperature for 30 minutes. (DELFIA® is a registered trademark of PerkinElmer, Inc.)

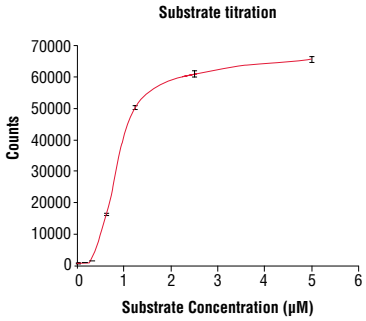

Figure 4. Peptide concentration dependence of EphB3 kinase activity: DELFIA® data generated using Phospho-Tyrosine mAb (P-Tyr-100) #9411 to detect phosphorylation of substrate peptide (#1330) by EphB3 kinase. In a 50 µl reaction, 50 ng of EphB3 and increasing concentrations of substrate peptide were used per reaction at room temperature for 30 minutes. (DELFIA® is a registered trademark of PerkinElmer, Inc.)

Protocol for HTScan® EphB3 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 10 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]=40 µM, [substrate]=3 µm).
2. Immediately 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 10 µl of DTT (1.25 M) to 2.5 ml of 4X HTScan® Tyrosine Kinase Buffer (240 mM HEPES pH 7.5, 20 mM $MgCl_2$, 20 mM $MnCl_2$, 12 µM Na_3VO_4) to make DTT/Kinase buffer.
5. Transfer 1.2 ml of DTT/Kinase buffer to each enzyme tube to make 4X reaction cocktail ([enzyme] = 4 ng/µL in 4X reaction cocktail).
6. Incubate 12.5 µl of the 4X reaction cocktail with 12.5 µl/well of prediluted compound of interest (usually around 10 µM) 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

60 mM HEPES pH 7.5
5 mM $MgCl_2$
5 mM $MnCl_2$
3 µM Na_3VO_4
1.25 mM DTT
20 µM ATP
1.5 µM peptide
50 ng EphB3 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 and 75 µl dH_2O/well to a 96-well streptavidin-coated plate and incubate at room temperature for 60 minutes.
11. *Wash three times with 200 µl/well PBS/T
12. Dilute primary antibody, Phospho-Tyrosine mAb (P-Tyr-100), 1:1000 in PBS/T with 1% BSA. Add 100 µl/well primary antibody.
13. Incubate at room temperature for 60 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