#7755 Store at -80°C

HTScan® c-Kit Kinase Assay Kit

☑ 100 assays
(96 Well Format)

Cell Signaling TECHNOLOGY®

Orders ■ 877-616-CELL (2355)
orders@cellsignal.com
Support ■ 877-678-TECH (8324)
info@cellsignal.com
Web ■ www.cellsignal.com

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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
KDR (Tyr996) Biotinylated Peptide	1364	1.25 ml
c-Kit Kinase (recombinant, human)	7754	5 µg

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

Peptide Core Sequence: DLY*KD

Molecular Weights: Peptide substrate, Biotin-KDR (Tyr996): 2,164 Daltons, GST-c-Kit Kinase: 75 KDa.

Background: c-Kit is a member of the subfamily of receptor tyrosine kinases that includes PDGF, CSF-1 and FLT3/flk-2 receptors (1,2). It plays critical controlling roles in a number of cell types such as hematopoietic stem cells, mast cells, melanocytes and germ cells (3). Upon binding with its ligand, stem cell factor (SCF), c-Kit undergoes dimerization/oligomerization and autophosphorylation. Activation of c-Kit results in the recruitment and tyrosine phosphorylation of downstream SH2-containing signaling components including PLCγ, the p85 subunit of PI3 kinase, SHP2 and CrkL (4). Molecular lesions that impair the kinase activity of c-Kit are associated with a variety of developmental disorders (5), while mutations that constitutively activate c-Kit can lead to pathogenesis of mastocytosis and gastrointestinal stromal tumors (6). Tyr719 is located in the kinase insert region of the catalytic domain. c-Kit phosphorylated at Tyr719 binds to the p85 subunit of PI3 kinase *in vitro* and *in vivo* (7).

Source/Purification: The GST-c-Kit fusion protein was produced using a baculovirus expression system with a construct expressing a fragment of human c-Kit (Thr544-Val976) 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 c-Kit #7450. Phospho-Tyrosine mAb (P-Tyr-100) #7450 was used for detection. The quality of the biotinylated peptide was evaluated by reverse-phase HPLC and by mass spectrometry.

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

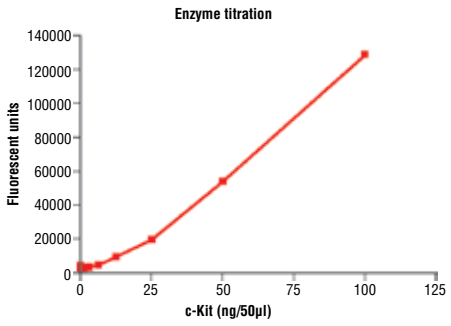

Figure 1. Dose dependence curve of c-Kit kinase activity: DELFIA data gencerated using Phospho-Tyrosine mAb (P-Tyr-100) #9411 to detect phosphorylation of substrate peptide (#1364) by c-Kit kinase. In a 50 µl reaction, increasing amounts of c-Kit and 1.5 µM substrate peptide were used per reaction at room temperature for 30 minutes. (DELFIA is a registered trademark of PerkinElmer, Inc.)

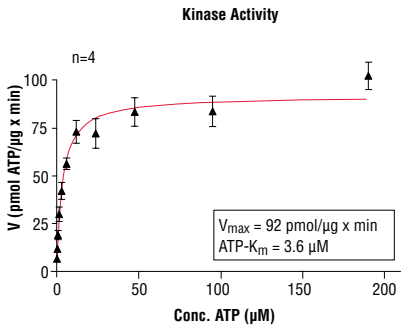

Figure 2. c-Kit kinase activity was measured in a radiometric assay using the following reaction conditions: 60 mM HEPES-NaOH, pH 7.5, 3 mM $MgCl_2$, 3 mM $MnCl_2$, 3 µM Na-orthovanadate, 1.2 mM DTT, ATP (variable), 2.5 µg/50 µl PEG20.000, Substrate: Tetra (LRRWSLG), 5 µg/50 µl, recombinant c-Kit: 200 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:
Tyrosine Kinase Substrate Screening Kit #7450

c-Kit Kinase #7754

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

KDR (Tyr996) Biotinylated Peptide #1364

Staurosporine #9953

HTScan® Tyrosine Kinase Buffer (4X) #9805

ATP (10 mM) #9804

Background References:

(1) Martin, F.H. et al. (1990) *Cell* 63, 203–211.

(2) Yarden, Y. et al. (1987) *EMBO J.* 6, 3341–3351.

(3) Gommerman, J.L. et al. (1997) *J. Biol. Chem.* 272, 30519–30525.

(4) Sattler, M. et al. (1997) *J. Biol. Chem.* 272, 10248–10253.

(5) Nocka, K. et al. (1990) *EMBO J.* 9, 1805–1813.

(6) Hirota, S. et al. (1998) *Science* 279, 577–580.

(7) Blume-Jensen, P. et al. (2000) *Nat. Genet.* 24, 157–162.

Protocol for HTScan® c-Kit Kinase Assay Kit

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

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 0.6 ml of DTT/Kinase buffer to each enzyme tube to make 4X reaction cocktail ([enzyme] = 8 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
100 ng c-Kit 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 detection methods, DELFIA® or ELISA please use the following steps.

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. Incubate at room temperature for 30 minutes.
3. *Wash five times with 200 ml/well PBS/T.
4. Add 100 ml/well DELFIA® Enhancement Solution.
5. Incubate at room temperature for 5 minutes.
6. Read plate using a Time Resolves Fluorescent plate reader using the following settings;
   - **a.** Excitation Filter: 340 nm
   - **b.** Emission Filter: 615 nm
   - **c.** Delay[++]: 400 ms

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

[++] 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)

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. Incubate at room temperature for 30 minutes.
3. *Wash five times with 200 ml/well PBS/T.
4. Add 100 ml TMB substrate.
5. Incubate at room temperature for 15 minutes.
6. Add 100 ml of stop solution.
7. Mix well.
8. Read the absorbance at 405 nm with a microtiter plate reader.

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

Companion Products For ELISA

Anti-Mouse IgG, HRP Linked Antibody #7076
Anti-Rabbit IgG HRP Linked Antibody #7074
TMB Solution #7004
Stop Solution #7002

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