Store at -80°C

#7922

c-Kit T670I Kinase

☑5 µg

Cell Signaling TECHNOLOGY®

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new 09/06

Description: Purified recombinant human c-Kit T670I (Thr544-Val976) kinase, supplied as a GST fusion protein.

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).

Gleevec (Imatinib) has been successfully used to treat c-Kit kinase associated chronic myeloid leukemia (CML). One of the identified mutation associated with resistance to Gleevec in CML patients is T670I in the c-Kit kinase domain (8,9).

Source/Purification: The GST-Kinase fusion protein was produced using a baculovirus expression system with a construct expressing human c-Kit T670I (Thr544-Val976) (GenBank Accession No. NM_000222) with an amino-terminal GST tag. The protein was purified by one-step affinity chromatography using glutathione-agarose.

Quality Control: The theoretical molecular weight of the GST-c-Kit T670I fusion protein is 75 kDa. The purified kinase was quality controlled for purity using SDS-PAGE followed by Coomassie stain [Fig.1]. c-Kit T670I kinase activity was determined using a radiometric assay [Fig.2]. A kinase dose dependency assay was performed to measure c-Kit T670I activity using HTScan® Kit Kinase Assay Kit #7755 [Fig.3]. Sensitivity of c-Kit T670I to Gleevec was determined using the c-Kit substrate peptide (#1364) and detection antibody (#9411) provided in HTScan® Kit Kinase Assay Kit #7755 [Fig.4].

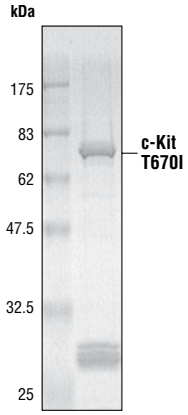

Figure 1. The purity of the GST-c-Kit T670I fusion protein was analyzed using SDS/PAGE followed by Coomassie stain.

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.

(8) Tamborini, E. et al. (2004) *Gastroenterology* 127, 294–299.

(9) Carter, T.A. et al. (2005) *Proc. Natl. Acad. Sci. USA* 102, 11011–11016.

Storage: Enzyme is supplied in 50 mM Tris-HCl, pH 8.0, 150 mM NaCl, 2 mM DTT, 15 mM reduced glutathione, 20% glycerol. Store at –80°C.

Keep on ice during use.

Avoid repeated freeze-thaw cycles.

Companion Products:

HTScan® KIT Kinase Assay Kit #7755

KDR (Tyr996) Biotinylated Peptide #1364

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

HTScan® Tyrosine Kinase Buffer (4X) #9805

ATP (10 mM) #9804

Staurosporine #9953

Tyrosine Kinase Substrate Screening Kit #7450

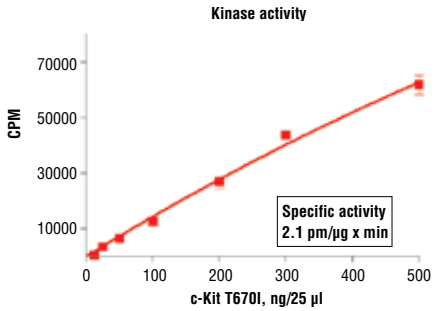

Figure 2. c-Kit T670I kinase activity was measured in a radiometric assay using the following reaction conditions: 60 mM HEPES-NaOH, pH 7.5, 5 mM $MgCl_2$, 5 mM $MnCl_2$, 3 µM Na-orthovanadate, 1.2 mM DTT, 100 µM ATP, 100 µM KDR (Tyr996) Biotinylated Peptide #1364 and variable amount of recombinant c-Kit T670I. The reaction mixture was incubated at room temperature for 15 minutes.

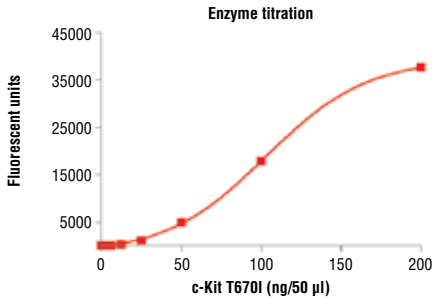

Figure 3. Dose dependence curve of c-Kit T670I kinase activity: DELFIA® data generated using Phospho-Tyrosine Mouse mAb (P-Tyr-100) #9411 to detect phosphorylation of substrate peptide (#1364) by c-KIT T670I kinase. In a 50 µl reaction, increasing amounts of c-KIT T670I and 1.5 µM substrate peptide were used per reaction at room temperature for 30 minutes. (DELFIA® is a registered trademark of PerkinElmer, Inc.)

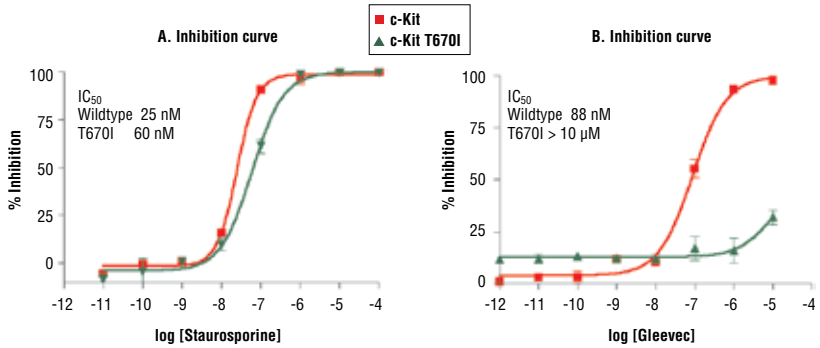

Figure. 4. Sensitivities of wild type and mutant c-Kit T670I kinase to Staurosporine (A) and Gleevec (B) were compared: DELFIA® data generated using Phospho-Tyrosine mAb (P-Tyr-100) #9411 to detect phosphorylation of c-Kit substrate peptide (#1364) by the c-Kit kinases. In a 50 µl reaction, 100 ng of c-Kit T670I kinase, 1.5 µM substrate peptide, 5 µM ATP and increasing concentration of indicated inhibitors were used per reaction well at room temperature for 30 minutes. (DELFIA® is a registered trademark of PerkinElmer, Inc.)

Protocol for c-Kit T670I Kinase Assay

***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
4. Phospho-Tyrosine Mouse mAb (P-Tyr-100) #9411
5. HTScan™ Kinase Buffer (10X) #9805
6. ATP (10 mM) #9804
7. KDR (Tyr996) Biotinylated Peptide #1364
8. DTT (1000X, 1.25 M)
9. DELFIA® Europium-labeled Anti-mouse IgG (PerkinElmer Life Sciences #AD0124)
10. DELFIA® Enhancement Solution (PerkinElmer Life Sciences #1244-105)
11. DELFIA® Streptavidin coated, 96-well, yellow plate (PerkinElmer Life Sciences AAAND-0005)

DELFIA® 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_20 to 2.5 ml to make 2X ATP/substrate cocktail ([ATP]=400 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_3V0_4) to make DTT/Kinase buffer.
5. Transfer 1.25 ml of DTT/Kinase buffer to enzyme tube to make 4X reaction cocktail ([enzyme]=8.0 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
200 µM ATP
1.5 µM peptide
100 ng c-Kit T670I 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 dH_20/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 in PBS/T with 1% BSA. Add 100 µl/well primary antibody.
    Please note: This protocol was validated using a KDR (Tyr996) Biotinylated Peptide and Phospho-Tyrosine Mouse mAb (P-Tyr-100) diluted 1:1000 (see additional reagents). Primary antibody chosen should be specific to the substrate used.
13. Incubate at room temperature for 120 minutes.
14. *Wash three times with 200 µl/well PBS/T.
15. Dilute Europium labeled anti-mouse IgG 1:500 in PBS/T with 1% BSA. Add 100 µl/well diluted antibody.
16. Incubate at room temperature for 30 minutes.
17. *Wash five times with 200 µl/well PBS/T.
18. Add 100 µl/well DELFIA® Enhancement Solution.
19. Incubate at room temperature for 5 minutes.
20. Detect 615 nm fluorescence emission with appropriate Time-Resolved Plate Reader.

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