

c-Kit D816V Kinase

✓ 5 µg

Orders ■ 877-616-CELL (2355)
orders@cellsignal.com

Support ■ 877-678-TECH (8324)
info@cellsignal.com

Web ■ www.cellsignal.com

new 09/06

This product is for *in vitro* research use only and is not intended for use in humans or animals.

Description: Purified recombinant human c-Kit D816V (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 PLC γ , 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).

c-Kit D816V, a constitutively active Kit mutant, has been found in adult-type human mastocytosis and is resistant to Gleevec (Imatinib), which has been successfully used to treat c-Kit kinase associated chronic myeloid leukemia (CML) (8,9).

Source/Purification: The GST-Kinase fusion protein was produced using a baculovirus expression system with a construct expressing human c-Kit D816V (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 D816V fusion protein is 75 kDa. The purified kinase was quality controlled for purity using SDS-PAGE followed by Coomassie stain [Fig.1]. c-Kit D816V kinase activity was determined using a radiometric assay [Fig.2]. A kinase dose dependency assay was performed to measure c-Kit D816V activity using HTScan® c-Kit Kinase Assay Kit #7755 [Fig.3]. Sensitivity of c-Kit D816V to Gleevec was determined using the c-Kit substrate peptide #1364 and detection antibody #9411 provided in HTScan® c-Kit Kinase Assay Kit #7755 [Fig.4].

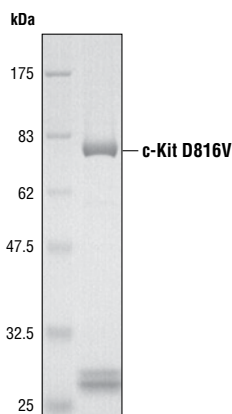


Figure 1. The purity of the GST-c-Kit D816V fusion protein was analyzed using SDS-PAGE followed by Coomassie stain.

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) Foster, R. et al. (2004) *J. Mol. Graph Model* 23, 139–152.
- (9) Ma, Y. et al. (2002) *Blood* 99, 1741–1744.

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

c-Kit Kinase #7754

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

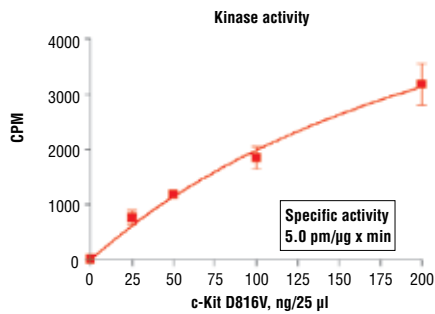


Figure 2. c-Kit D816V kinase activity was measured in a radiometric assay using the following reaction conditions: 60 mM HEPES-NaOH, pH 7.5, 5 mM MgCl₂, 5 mM MnCl₂, 3 µM Na-orthovanadate, 1.2 mM DTT, 100 µM ATP, 100 µM KDR (Tyr996) Biotinylated Peptide #1364 and variable amount of recombinant c-Kit D816V. The reaction mixture was incubated at room temperature for 15 minutes.

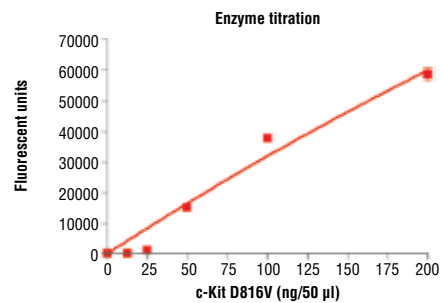


Figure 3. Dose dependence curve of c-Kit D816V kinase activity: DELFIA® data generated using Phospho-Tyrosine Mouse mAb (P-Tyr-100) #9411 to detect phosphorylation of substrate peptide (#1364) by c-Kit D816V kinase. In a 50 µl reaction, increasing amounts of c-Kit D816V and 1.5 µM substrate peptide were used per reaction at room temperature for 30 minutes. (DELFIA® is a registered trademark of PerkinElmer, Inc.)

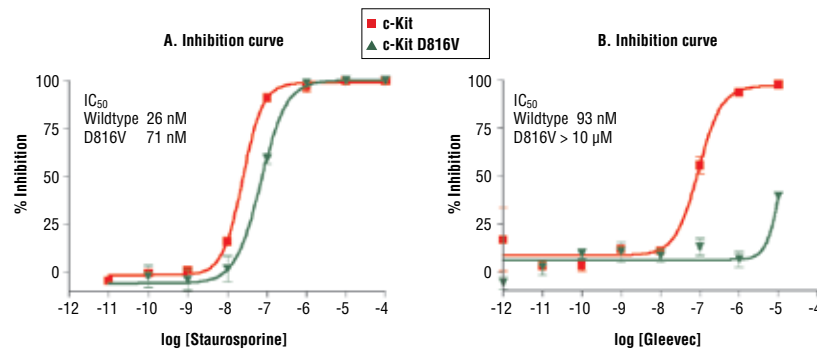


Figure 4. Sensitivities of wild type and mutant c-Kit D816V kinase to Staurosporine (A) and Gleevec (B) were compared: DELFIA® data generated using Phospho-Tyrosine mAb (P-Tyr-100) #9411 to detect phosphorylation of c-Kit substrate peptide (#1364) by the c-Kit kinases. In a 50 µl reaction, 100 ng of c-Kit D816V 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 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)
- Bovine Serum Albumin (BSA)
- 3. Stop Buffer:** 50 mM EDTA pH 8
- Phospho-Tyrosine Mouse mAb (P-Tyr-100) #9411
- HTScan™ Kinase Buffer (10X) #9805
- ATP (10 mM) #9804
- KDR (Tyr996) Biotinylated Peptide #1364
- DTT (1000X, 1.25 M)
- DELFI[®] Europium-labeled Anti-mouse IgG (PerkinElmer Life Sciences #AD0124)
- DELFI[®] Enhancement Solution (PerkinElmer Life Sciences #1244-105)
- DELFI[®] Streptavidin coated, 96-well, yellow plate (PerkinElmer Life Sciences AAAND-0005)

DELFI[®] is a registered trademark of PerkinElmer Life Sciences

B Suggested Protocol for 100 Assays

- Add 100 μ l 10 mM ATP to 1.25 ml 6 μ M substrate peptide. Dilute the mixture with dH₂O to 2.5 ml to make 2X ATP/substrate cocktail ([ATP]=400 μ M, [substrate] = 3 μ M).
- Immediately transfer enzyme from -80°C to ice. Allow enzyme to thaw on ice.
- Microcentrifuge briefly at 4°C to bring liquid to the bottom of the vial. Return immediately to ice.**
- Add 10 μ l of DTT (1.25 M) to 2.5 ml of 4X HTScan™ Tyrosine Kinase Buffer (240 mM HEPES pH 7.5, 20 mM MgCl₂, 20 mM MnCl₂, 12 μ M Na₃VO₄) to make DTT/Kinase buffer.
- Transfer 1.25 ml of DTT/Kinase buffer to enzyme tube to make 4X reaction cocktail ([enzyme]=8.0 ng/ μ l in 4X reaction cocktail).
- Incubate 12.5 μ l of the 4X reaction cocktail with 12.5 μ l/well of prediluted compound of interest (usually around 10 μ M) for 5 minutes at room temperature.
- Add 25 μ l of 2X ATP/substrate cocktail to 25 μ l/well preincubated reaction cocktail/compound.

Final Assay Conditions for a 50 μ l Reaction

- 60 mM HEPES pH 7.5
 - 5 mM MgCl₂
 - 5 mM MnCl₂
 - 3 μ M Na₃VO₄
 - 1.25 mM DTT
 - 200 μ M ATP
 - 1.5 μ M peptide
 - 100 ng c-Kit D816V Kinase
- Incubate reaction plate at room temperature for 30 minutes.
 - Add 50 μ l/well Stop Buffer (50 mM EDTA, pH 8) to stop the reaction.
 - Transfer 25 μ l of each reaction and dH₂O/well to a 96-well streptavidin-coated plate and incubate at room temperature for 60 minutes.
 - *Wash three times with 200 μ l/well PBS/T.
 - Dilute primary antibody in PBS/T with 1% BSA. Add 100 μ l/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.
 - Incubate at room temperature for 120 minutes.
 - *Wash three times with 200 μ l/well PBS/T.
 - Dilute Europium labeled anti-mouse IgG 1:500 in PBS/T with 1% BSA. Add 100 μ l/well diluted antibody.
 - Incubate at room temperature for 30 minutes.
 - *Wash five times with 200 μ l/well PBS/T.
 - Add 100 μ l/well DELFI[®] Enhancement Solution.
 - Incubate at room temperature for 5 minutes.
 - 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