#7910 Store at -80°C

EGF Receptor T790M/L858R Kinase

☑ 5 µg

Cell Signaling TECHNOLOGY®

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Description: Purified recombinant EGFR T790M/L858R mutant kinase (His672-Ala1210), supplied as a GST fusion protein.

Background: Epidermal growth factor (EGF) receptor is a 170 kDa tyrosine kinase. Ligand binding results in receptor dimerization, autophosphorylation, activation of downstream signaling and lysosomal degradation (1,2). Phosphorylation of Tyr845 in the kinase domain may stabilize the activation loop, maintain the enzyme in an active state and provide a binding surface for substrate proteins (3,4). c-Src is involved in phosphorylation of Tyr845 (5). Phospho-tyrosine 992 is a direct binding site for the PLC-γ SH2 domain, resulting in activation of PLC-γ-mediated downstream signaling (6). Phosphorylation of Tyr1045 creates a major docking site for c-Cbl (7). Binding of c-Cbl to the activated EGFR leads to receptor ubiquitination and degradation (8). Phospho-Tyr1068 of activated EGFR is a direct binding site for Grb2 (9). Phospho-tyrosine 1148 and 1173 provide a docking site for SHC (2). Both sites are involved in the activation of MAP kinase signaling. Phosphorylation of EGFR on serine and threonine residues attenuates EGFR kinase activity. Ser1046/1047 in the carboxy-terminal region of EGFR are sites phosphorylated by CaM kinase II. Mutations of Ser1046/1047 upregulate tyrosine autokinase activity of EGFR (10).

Mutations in EGFR have been identified in patients with non-small cell lung cancer. The L858R gain-of-function mutation is associated with a therapeutic response to the EGFR inhibitor, Iressa. A secondary T790M mutation results in resistance to Iressa inhibition (11,12).

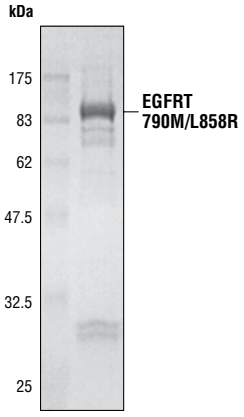

Figure 1. The purity of the GST-EGFR T790M/L858R fusion protein was analyzed using SDS/PAGE followed by Coomassie stain.

Source/Purification: The GST-kinase fusion protein was produced using a baculovirus expression system with a construct expressing human EGFR T790M/L858R (His672-Ala1210) (GenBank Accession No. NM-005228) 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-EGFR T790M/L858R kinase fusion protein is 87 kDa. The purified kinase was quality controlled for purity using SDS-PAGE followed by Coomassie stain [Fig.1]. The specific activity of the EGFR T790M/L858R kinase was determined using a radiometric assay [Fig.2]. A kinase dose dependency assay was performed to measure EGFR T790M/L858R activity using EGFR Kinase substrate (#1325) [Fig.3]. Sensitivity of EGFR T790M/L858R to Iressa was measured using the EGFR substrate peptide #1325 and detection antibody #9411 provided in HTScan™ EGFR Kinase Assay Kit #7909 [Fig.4].

Background References:

(1) Hackel, P.O. et al. (1999) *Curr. Opin. Cell Biol.* 11, 184–189.

(2) Zwick, E. et al. (1999) *Trends Pharmacol. Sci.* 20, 408–412.

(3) Cooper, J.A. and Howell, B. (1993) *Cell* 73, 1051–1054.

(4) Hubbard, S.R. et al. (1994) *Nature* 372, 746–754.

(5) Biscardi, J.S. et al. (1999) *J. Biol. Chem.* 274, 8335–8343.

(6) Emlet, D.R. et al. (1997) *J. Biol. Chem.* 272, 4079–4086.

(7) Levkowitz, G. et al. (1999) *Mol. Cell* 4, 1029–1040.

(8) Ettenberg, S.A. et al. (1999) *Oncogene* 18, 1855–1866.

(9) Rojas, M. et al. (1996) *J. Biol. Chem.* 271, 27456–27461.

(10) Feinmesser, R.L. et al. (1999) *J. Biol. Chem.* 274, 16168–16173.

(11) Kwak, E.L. et al. (2005) *Proc. Natl. Acad. Sci. USA* 102, 7665–70.

(12) Fabian, M.A. et al. (2005) *Nat. Biotechnol.* 23, 329–36.

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

Keep enzymes on ice during use.

Avoid repeated freeze-thaw cycles.

Companion Products:

HTScan™ EGFR Kinase Assay Kit #7909

PTP1B (Tyr66) Biotinylated peptide #1325

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

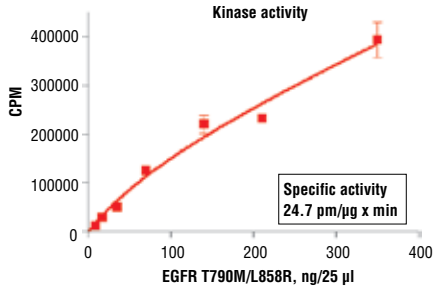

Figure 2. EGFR T790M/L858R kinase activity was measured in a radiometric assay using the following reaction conditions: 60 mM HEPES-NaOH, pH 7.5, 5 mM $MgCl_2$, 5 mM $MnCl_2$, 3 µM Na-orthovanadate, 1.2 mM DTT, 100 µM ATP, 250 µM PTP1B(Tyr66) biotinylated peptide (#1325) and variable amount of recombinant EGFR T790M/L858R. Reaction mixture incubated at room temperature for 10 minutes.

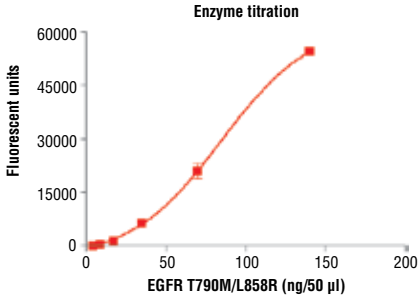

Figure 3. Dose dependence curve of EGFR T790M/L858R kinase activity: DELFIA® data generated using Phospho-Tyrosine mAb (P-Tyr-100) #9411 to detect phosphorylation of substrate peptide (#1325) by EGFR T790M/L858R kinase. In a 50 µl reaction, increasing amounts of EGFR T790M/L858R and 1.5 µM substrate peptide were used per reaction at room temperature for 30 minutes. (DELFIA® is a registered trademark of PerkinElmer, Inc.)

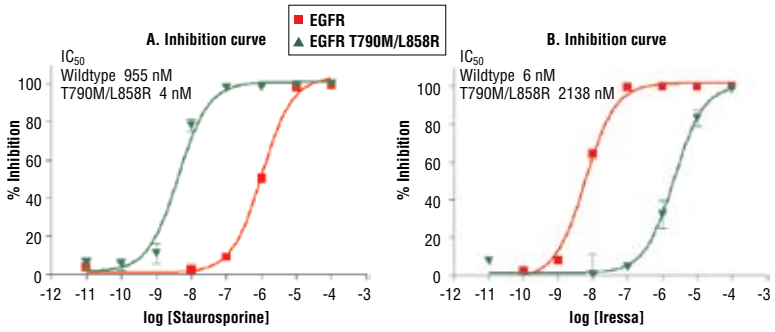

Figure. 4. EGFR T790M/L858R kinase sensitivity to staurosporine (A) and Iressa (B) inhibition was compared to wild type EFGR. DELFIA® data generated using Phospho-Tyrosine mAb (P-Tyr-100) #9411 to detect phosphorylation of EGFR substrate peptide (#1325) by the EGFR T790M/L858R kinase. In a 50 µl reaction, 100 ng EGFR T790M/L858R, 1.5 µM substrate peptide, 20 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 EGF Receptor T790M/L858R 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. PTP1B (Tyr66) Biotinylated peptide #1325
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)

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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. 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 EGF Receptor T790M/L858R 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_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 PTP1B (Tyr66) 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