Store at –80°C

#7909

HTScan® EGF Receptor Kinase Assay Kit

☑ 100 Assays
(96 Well Format)

Cell Signaling TECHNOLOGY®

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Products Included	Product #	Kit Quantity
Phospho-Tyrosine Mouse mAb (P-Tyr-100)	9411	30 µl
HTScan® Tyrosine Kinase Buffer (4X)	9805	15 ml
ATP (10mM)	9804	1 ml
PTP1B (Tyr66) Biotinylated Peptide	1325	1.25 ml
EGF Receptor Kinase	7908	5 µg

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

Peptide Core Sequence: DNDY*INA

Molecular Weights: Peptide substrate, Biotin-PTP1B (Tyr66): 2141 Daltons. GST-EGFR Kinase: 87 kDa.

Source/Purification: The GST-kinase fusion protein was produced using a baculovirus expression system with a construct expressing human EGFR (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 substrate peptide was selected using our Tyrosine Kinase Substrate Screening Kit #7450. Phospho-Tyrosine mAb (P-Tyr-100) #9411 was used for detection. The quality of the biotinylated peptides was evaluated by reverse-phase HPLC and by mass spectrometry.

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

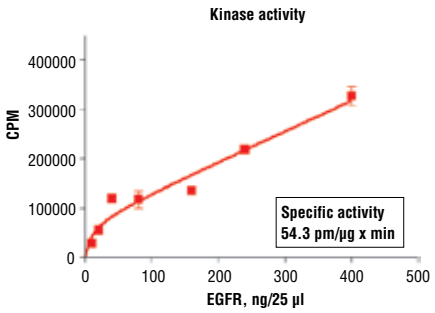

Figure 1. EGFR kinase activity was measured in a radiometric assay using the following reaction conditions: 60 mM HEPES-NaOH, pH 7.5, 5 mM $MgCl_2$, 5 mM $MnCl_2$, 3 µM Na-orthovanadate, 1.2 mM DTT, 100 µM ATP, 250 µM PTP1B (Tyr66) Biotinylated Peptide (#1325) and variable amount of recombinant EGFR. Reaction mixture incubated at room temperature for 10 minutes.

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), 150 mM NaCl, 2 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

EGF Receptor Kinase #7908

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

PTP1B (Tyr66) Biotinylated Peptide #1325

Staurosporine #9953

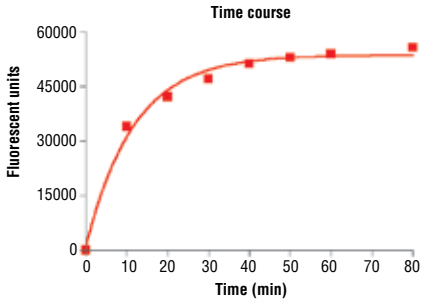

Figure 2. Time course of EGFR kinase activity: DELFIA® data generated using Phospho-Tyrosine Mouse mAb (P-Tyr-100) #9411 to detect phosphorylation of EGFR substrate peptide (#1325) by EGFR kinase. In a 50 µl reaction, 50 ng EGFR and 1.5 µM substrate peptide were used per reaction. (DELFIA® is a registered trademark of PerkinElmer, Inc.)

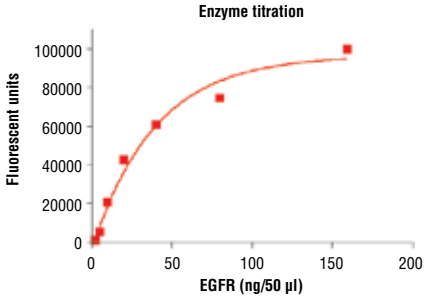

Figure 3. Dose dependence curve of EGFR kinase activity: DELFIA® data generated using Phospho-Tyrosine Mouse mAb (P-Tyr-100) #9411 to detect phosphorylation of substrate peptide (#1325) by EGFR kinase. In a 50 µl reaction, increasing amounts of EGFR and 1.5 µM substrate peptide were used per reaction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

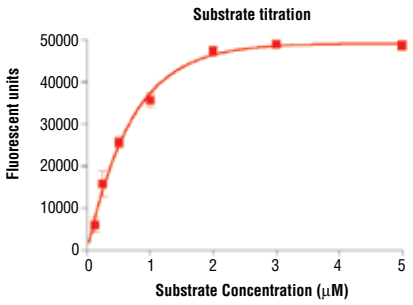

Figure 4. Peptide concentration dependence of EGFR kinase activity: DELFIA® data generated using Phospho-Tyrosine Mouse mAb (P-Tyr-100) #9411 to detect phosphorylation of substrate peptide (#1325) by EGFR kinase. In a 50 µl reaction, 50 ng of EGFR and increasing concentrations of substrate peptide were used per reaction at room temperature for 30 minutes. (DELFIA® is a registered trademark of PerkinElmer, Inc.)

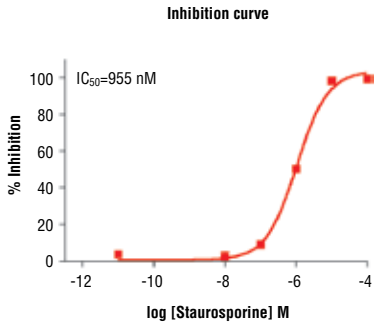

Figure 5. Staurosporine inhibition of EGFR kinase activity: DELFIA® data generated using Phospho-Tyrosine Mouse mAb (P-Tyr-100) #9411 to detect phosphorylation of EGFR substrate peptide (#1366) by EGFR kinase. In a 50 µl reaction, 50 ng EGFR, 1.5 µM substrate peptide, 20 µM ATP and increasing amounts of staurosporine were used per reaction at room temperature for 30 minutes. (DELFIA® is a registered trademark of PerkinElmer, Inc.)

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-gamma SH2 domain, resulting in activation of PLC-gamma-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).

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.

Protocol for HTScan® EGF Receptor 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 EGF Recpetor 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