#7410 Store at –80°C

HTScan™ EGF-R Kinase Assay Kit

☑ 100 Assays
(96 Well Format)

Cell Signaling TECHNOLOGY®

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Products Included	Product #	Kit Quantity
Phospho-Tyrosine Monoclonal Antibody (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
Peptide Substrate Biotin-PTP1B (Tyr66) (6 µM)		1.25 ml
EGF-R Kinase (recombinant, human)	7706	1000 Units

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

Peptide Core Sequence: NDY*IN

Molecular Weights: Peptide Substrate, Biotin-PTP1B (Tyr66): 2141 Daltons, GST-EGF-R Kinase domain: 91,163 Daltons

Unit Definition: 10 Units is defined as the amount of EGF-R kinase required to maximally phosphorylate 75 pmol of PTP1B (Tyr66) #C03-1727 biotinylated substrate peptide in 30 minutes at 25°C in a total reaction volume of 50 µl quantified by DELFIA®.

Background: Epidermal growth factor receptor (EGF-R) is a 170 kDa membrane receptor tyrosine kinase. The receptor molecule consists of an extracellular ligand binding domain, a single transmembrane domain, an intracellular tyrosine kinase domain and a cytoplasmic tail. EGF ligand binding to this receptor results in receptor dimerization, autophosphorylation (in trans), activation of various downstream signaling molecules and lysosomal degradation (1,2). EGF-R has substrate specificity similar to other receptor tyrosine kinase members, prefering acidic residues at the -1 to -4 positions and large hydrophobic amino acids at positions +1 and +3 (3).

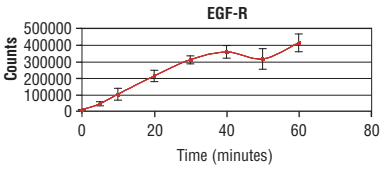

Figure 1. Time course of EGF-R kinase activity: DELFIA® data generated using Phospho-Tyrosine Monoclonal Antibody P-Tyr-100 #9411 to detect phosphorylation of EGF-R substrate peptide by GST-EGF-R kinase. In a 50 µl reaction, 10 Units GST-EGF-R and 5 µM substrate peptide were used per reaction well. Background reading is 5144. (DELFIA® is a registered trademark of PerkinElmer, Inc.).

Source/Purification: The GST-Kinase fusion protein was produced by using a baculovirus expression system from a construct containing a human EGF-R cDNA kinase domain fragment amino-terminally fused to a GST-HIS_6-Thrombin cleavage site. The protein was then purified by one-step affinity purification using glutathione-agarose.

The detection antibody, Phospho-Tyrosine Monoclonal Antibody (P-Tyr-100) #9411, was derived from mice immunized with phospho-tyrosine-containing peptides (KLH-coupled).

Quality Control: PTP1B peptide was selected by using Tyrosine Kinase Substrate Screening Kit #7450 to screen for EGF-R kinase substrates. Phospho-Tyrosine Monoclonal Antibody #9411 was used for detection (fig.2). The quality of the biotinylated peptides was evaluated by reverse-phase HPLC and by mass spectrometry.

Purified EGF-R kinase was quality controlled for purity by silver stain SDS-PAGE and Western blot. EGF-R kinase V_{max} and K_m values were measured to determine specific enzymatic activity (fig.5).

Assay conditions (time course [fig.1], kinase dose-dependence [fig.3], substrate dose-dependence [fig.4] and inhibitor sensitivity [fig.6]) for EGF-R kinase activity were verified using the EGF-R substrate peptide provided in this kit.

Background References:

(1) Muthuswamy, S.K. et al. (1999) *Mol. Cell. Biol.* 19, 6845–6857.

(2) Qian, X. et al. (1994) *Proc. Natl. Acad. Sci. U S A.* 91, 1500–1504.

(3) Songyang, Z. and Cantley, L.C. (1995) *TIBS* 20, 470–475.

Storage: Antibodies are supplied in 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 glutathion and 20% glycerol. Store at –80°C.

Keep enzymes on ice during use.

Avoid repeated freeze-thaw cycles.

Companion Products:
EGF-R Kinase #7706

Kinase Substrate Screening Kit #7400

Tyrosine Kinase Substrate Screening Kit #7450

Staurosporine #9953

Cell Signaling Technology offers a full line of protein kinases, substrates, and antibody detection reagents for high throughput screening. Please direct all inquiries to: drugdiscovery@cellsignal.com

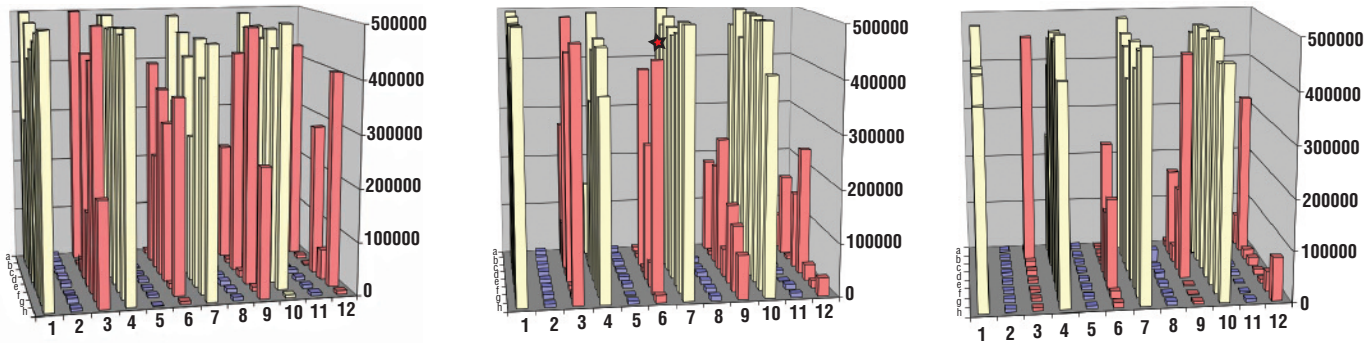

Figure 2. Treatment of Tyrosine Kinase Substrate Screening Kit #7450 with EGF-R Kinase. Positive control is in yellow and negative control is in blue. Several positive hits for EGF-R were identified using this approach (in red). The star indicates the peptide chosen for this HTScan™ EGF-R Kinase Assay Kit.

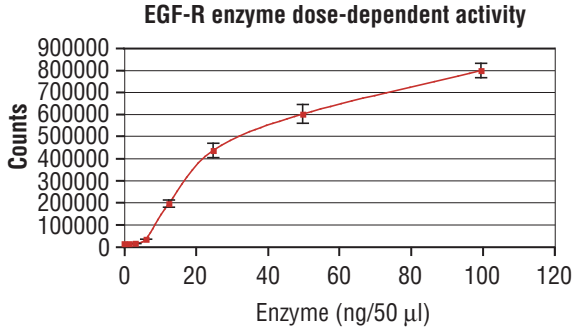

Figure 3. Dose dependence curve of EGF-R kinase activity: DELFIA® data generated using Phospho-Tyrosine Monoclonal Antibody P-Tyr-100 #9411 to detect phosphorylation of peptide C03-1727 by GST-EGF-R kinase. In a 50 µl reaction, increasing amounts of GST-EGF-R and 5 µM substrate peptide were used per reaction well at 25°C for 30 minutes. Background reading is 11152. (DELFIA® is a registered trademark of PerkinElmer, Inc.)

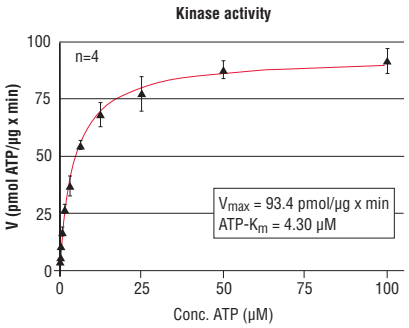

Figure 5. EGF-R kinase activity was measured in a radioisotopic filtration assay using the following reaction conditions: 60 mM HEPES-NaOH, pH 7.5, 3 mM MgCl2, 3 mM MnCl2, 3 µM Na-orthovanadate, 1.2 mM DTT, ATP (variable), 2.5 µg / 50 ul PEG20.000, Substrate:PolyEY, 10 µg/50 µl, Recombinant EGF-R: 5 Units/50 µl.

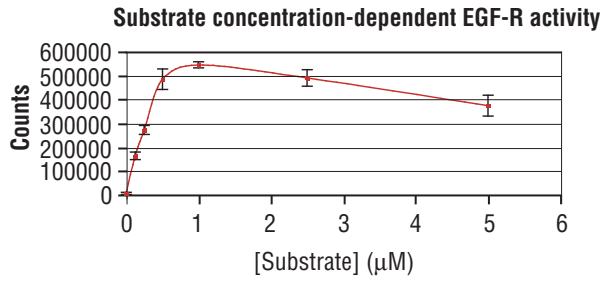

Figure 4. Peptide concentration dependence of EGF-R kinase activity: DELFIA® data generated using Phospho-Tyrosine Monoclonal Antibody P-Tyr-100 #9411 to detect phosphorylation of peptide C03-1727 by GST-EGF-R kinase. In a 50 µl reaction, 10 Units of GST-EGF-R and increasing concentrations of substrate peptide were used per reaction well at 25°C for 30 minutes. Background reading is 8985. (DELFIA® is a registered trademark of PerkinElmer, Inc.)

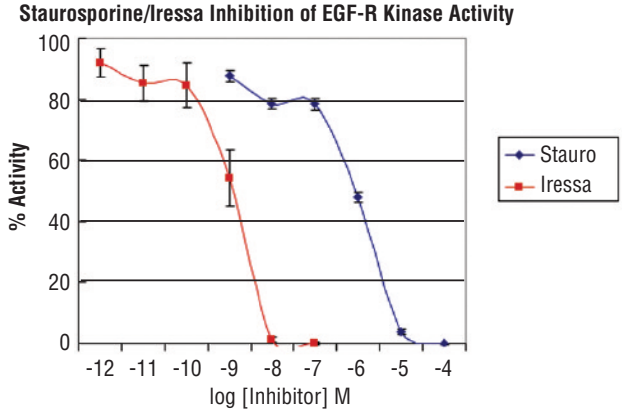

Figure 6. Iressa and staurosporine inhibition of EGF-R kinase activity: DELFIA® data generated using Phospho-Tyrosine Monoclonal Antibody P-Tyr-100 #9411 to detect phosphorylation of EGF-R substrate peptide (C03-1727) by GST-EGF-R kinase. In a 50 µl reaction, 10 Units GST-EGF-R, 1.5 µM substrate peptide, 20 µM ATP and increasing amount of inhibitor (iressa or staurosporine) were used per reaction well at 25°C for 30 minutes. (DELFIA® is a registered trademark of PerkinElmer, Inc.)

Protocol for HTScan™ EGF-R Kinase Assay Kit

Suggested Kinase Assay Conditions:

■ Combine 12.5 µl of 6 µM peptide substrate and 12.5 µl H_2O with kinase inhibitor or activator of interest. Add 2x kinase reaction cocktail (see below). Incubate at 25°C for 30 minutes.

Peptide substrate is supplied in H_2O plus 0.001 % DMSO.

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 kinase.

Additional Solutions and Reagents (Not included)

■ Wash Buffer: 1X PBS, 0.05% Tween-20 (PBS/T)

■ Bovine Serum Albumin (BSA)

■ Stop Buffer: 50 mM EDTA pH 8

■ DELFIA® Europium-labeled Anti-mouse IgG (PerkinElmer Life Sciences #AD0124)

■ DELFIA® Enhancement Solution (PerkinElmer Life Sciences #1244-105)

■ DELFIA® Streptavidin coated, 96-well, yellow plate (PerkinElmer Life Sciences AAAND-0005)

DELFIA® is a registered trademark of PerkinElmer Life Sciences

Suggested Protocol For 100 Assays

1. Transfer enzyme from -80°C immediately to ice. Allow enzyme to thaw on ice.
2. **Microcentrifuge briefly at 4°C to bring all liquid to the bottom of the vial. Return immediately to ice.**
3. Add 1.25 ml 6 µM peptide substrate to 1.25 ml H_2O (include kinase inhibitor or activator of interest if desired). Aliquot 25 µl/well into a 96 well plate.
4. Set up 2x Reaction cocktails (Mock/No Kinase and Plus Kinase) in tubes on ice.

   2.5 ml 4X HTScan™ Tyrosine Kinase Buffer (240 mM HEPES pH 7.5, 20 mM $MgCl_2$, 20 mM $MnCl_2$, 12 µM Na_3VO_4)

   20–200 µl ATP (10 mM)

   10 µl DTT (1.25 M)

   Bring to 5 ml with H_20. Mix gently by inversion

   Transfer enzyme to 2.5 ml 2X reaction cocktail. Final enzyme concentration should be 10 Units/25 µl in 2X reaction cocktail.
5. Add 25 µl 2x kinase reaction cocktail (containing kinase) to 25 µl diluted peptide in each well. Mix by gentle agitation.

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

20–200 µM ATP

1.5 µM peptide

10 Units Kinase

4. Incubate reaction plate at 25°C for 30 minutes.
5. Add 50 µl Stop Buffer (50 mM EDTA, pH 8) to each well and mix by pipetting.
6. Transfer 25 µl of each reaction and 75 µl H_2O/well to a 96-well streptavidin-coated plate and incubate at room temperature for 60 minutes.
7. *Wash three times with 200 µl/well PBS/T.
8. Dilute primary antibody, Phospho-Tyrosine Monoclonal Antibody (P-Tyr-100) #9411, 1:1000 in PBS/T with 1% BSA. Add 100 µl primary antibody per well.
9. Mix and incubate at room temperature with rocking for 60 minutes.
10. *Wash three times with 200 µl/well PBS/T.
11. Dilute Europium labeled anti-mouse IgG 1:500 in PBS/T with 1% BSA. Add 100 µl diluted antibody/well.
12. Mix and incubate at room temperature for 30 minutes.
13. *Wash five times with 200 µl/well PBS/T.
14. Add 100 µl/well DELFIA® Enhancement Solution.
15. Incubate at room temperature for 5 minutes.
16. Detect 615 nm fluorescence emission with appropriate Time-Resolved Plate Reader.

***IMPORTANT: 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