Store at -80°C

#7788

HTScan® VEGF Receptor 2 Kinase Assay Kit

☑ 100 assays
(96 Well Format)

Cell Signaling TECHNOLOGY®

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Products Included	Products #	Kit Quantity
Phospho-Tyrosine Mouse mAb (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
Gastrin Precursor (Tyr87) Biotinylated Peptide	1310	1.25 ml
VEGF Receptor 2 Kinase (recombinant, human)	7407	2 X 5 µg

Description: The kit provides a means of performing kinase activity assays with recombinant human VEGFR-2 kinase. It includes active VEGFR-2 kinase (supplied as a GST fusion protein), a biotinylated peptide substrate and a phospho-tyrosine antibody for detection of the phosphorylated form of the substrate peptide.

Peptide Core Sequence: EAY*GW

Molecular Weights: Peptide substrate, Biotin-Gastrin Precursor (Tyr87) peptide: 2,853Daltons. GST-VEGFR-2 Kinase: 110 kDa.

Background: Vascular endothelial growth factor receptor 2 (VEGFR-2, KDR, Flk-1) is a major receptor transducing VEGF-induced signaling in endothelial cells. Upon ligand binding, VEGFR-2 undergoes autophosphorylation and becomes activated (1). Major autophosphorylation sites of VEGFR-2 are located in the kinase insert domain (Tyr951/996) and in the tyrosine kinase catalytic domain (Tyr1054/1059) (2). Activation of the receptor leads to rapid recruitment of adaptor proteins, including Shc, GRB2, PI-3 kinase, Nck and the protein tyrosine phosphatases SHP-1 and SHP-2 (3). The phosphorylation of Tyr1212 provides a docking site for Grb2 binding and phospho-Tyr1175 binds with the p85 subunit of PI-3 kinase and PLCγ, as well as Shb (5,6). Signaling from VEGFR-2 is necessary for the execution of VEGF-stimulated proliferation, chemotaxis and sprouting, as well as survival of cultured endothelial cells *in vitro* and angiogenesis *in vivo* (4).

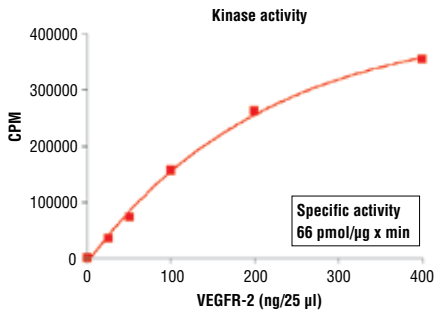

Figure 1. VEGFR2 kinase activity was measured in a radiometric assay using the following reaction conditions: 4 mM MOPS, pH 7.2, 2.5 mM β-glycerophosphate, 1 mM EGTA, 0.4 mM EDTA, 4 mM $MgCl_2$, 0.05 mM DTT, 50 µM ATP, Substrate: MBP 200 ng/µL, and variable amounts of recombinant VEGFR-2.

Source/Purification: The GST-Kinase fusion protein was produced using a baculovirus expression system with a construct expressing a fragment of human VEGFR-2 (Val789-Val1356) (GenBank accession No. NM_002253) 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 Substrates Screening Kit #7450. Phospho-Tyrosine Mouse mAb (P-Tyr-100) #9411 was used for detection. The quality of the biotinylated peptide was evaluated by reverse-phase HPLC and by mass spectrometry.

Purified VEGFR-2 kinase was quality controlled for purity by SDS-PAGE followed by Coomassie stain. The specific activity of the VEGFR-2 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 VEGFR-2 activity using the VEGFR-2 substrate peptide provided in this kit. VEGFR-2 sensitivity to the inhibitor staurosporine was measured using the VEGFR-2 substrate peptide provided in this kit [Fig.5].

Background References:

(1) Meyer, M. et al. (1999) *EMBO J.* 18, 363–374.

(2) Dougher-Vermazen, M. et al. (1994) *Biochem. Biophys. Res. Commun.* 205, 728–738.

(3) Kroll, J. and Waltenberger, J. (1997) *J. Biol. Chem.* 272, 32521–32527.

(4) Karkkainen, M.J. and Petrova, T. (2000) *Oncogene* 19, 5598–5605.

(5) Rahimi, N. et al. (2000) *J. Biol. Chem.* 275, 16986–16992.

(6) Claesson-Welsh, L. (2003) *Biochem. Soc. Transact.* 31, 20–24.

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), 100 mM NaCl, 5 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

VEGF Receptor 2 Kinase #7407

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

Gastrin Precursor (Tyr87) Biotinylated Peptide #1310

Staurosporine #9953

HTScan® Tyrosine Kinase Buffer (4X) #9805

ATP (10 mM) #9804

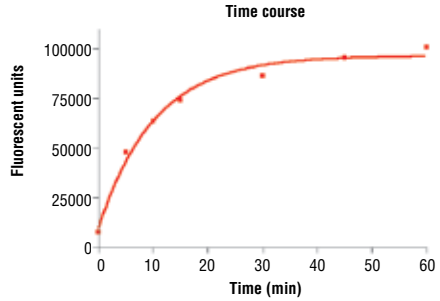

Figure 2. Time course of VEGFR-2 kinase activity: DELFIA® data generated using Phospho-Tyrosine Mouse mAb (P-Tyr-100) #9411 to detect phosphorylation of VEGFR-2 substrate peptide #1310 by VEGFR-2 kinase. In a 50 µl reaction, 100 ng VEGFR-2 and 1.5 µM substrate peptide were used per reaction. (DELFIA® is a registered trademark of PerkinElmer, Inc.)

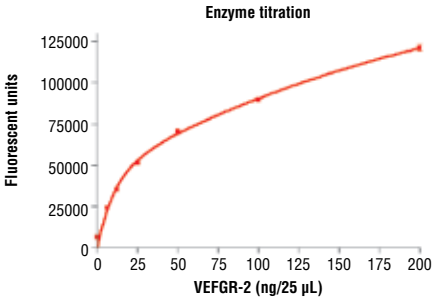

Figure 3. Dose dependence curve of VEGFR-2 kinase activity: DELFIA® data generated using Phospho-Tyrosine Mouse mAb (P-Tyr-100) #9411 to detect phosphorylation of substrate peptide #1310 by VEGFR-2 kinase. In a 50 µl reaction, increasing amounts of VEGFR-2 and 1.5 µM substrate peptide were used per reaction at room temperature for 30 minutes. (DELFIA® is a registered trademark of PerkinElmer, Inc.)

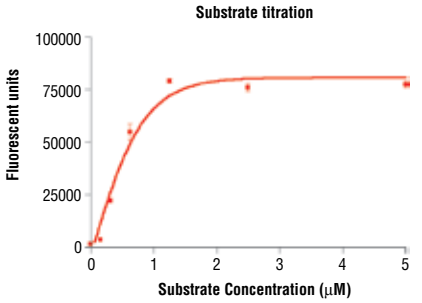

Figure 4. Peptide concentration dependence of VEGFR-2 kinase activity: DELFIA® data generated using Phospho-Tyrosine Mouse mAb (P-Tyr-100) #9411 to detect phosphorylation of substrate peptide #1310 by VEGFR-2 kinase. In a 50 µl reaction, 100 ng of VEGFR-2 and increasing concentrations of substrate peptide were used per reaction at room temperature for 30 minutes. (DELFIA® is a registered trademark of PerkinElmer, Inc.)

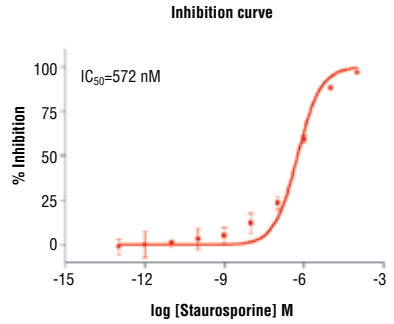

Figure 5. Staurosporine inhibition of VEGFR-2 kinase activity: DELFIA® data generated using Phospho-Tyrosine Mouse mAb (P-Tyr-100) #9411 to detect phosphorylation of VEGFR-2 substrate peptide #1310 by VEGFR-2 kinase. In a 50 µl reaction, 100 ng VEGFR-2, 1.5 µM substrate peptide, 20 µM ATP and increasing amounts of staurosporine were used per reaction at room temperature for 30 minutes. (DELFIA® is a registered trademark of PerkinElmer, Inc.)

Protocol for HTScan® VEGF Receptor 2 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 0.6 ml of DTT/Kinase buffer to each enzyme tube to make 4X reaction cocktail ([enzyme] = 8 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
100 ng VEGF Receptor 2 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