#7767 Store at –80°C

HTScan™ PDGF Receptor α Kinase Assay Kit

☑ 100 Assays
(96 Well Format)

Cell Signaling TECHNOLOGY®

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

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

Web ■ **www.cellsignal.com**

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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
DTT (1000x, 1.25 M)		80 µl
ATP (10 mM)	9804	1 ml
FLT3 (Tyr589) Biotinylated Peptide Substrate	1305	1.25 ml
PDGFR-α Kinase	7766	1000 Units

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

Peptide Core Sequence: NEY*FY*V

Molecular Weights: Peptide substrate, Biotin-FLT3 (Tyr589): 1,945 Daltons, GST-PDGFR-α Kinase domain: 87,851 Daltons

Unit Definition: 10 Units is defined as the amount of PDGFR-α kinase required to maximally phosphorylate 75 pmol of FLT3 (Tyr589) biotinylated substrate peptide (#1305) in 30 minutes at 25°C in a total reaction volume of 50 µl quantified by DELFIA® to achieve signal/background=25 or greater.

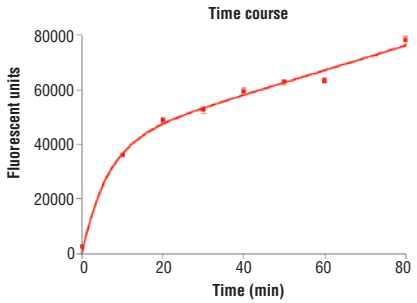

Figure 1. Time course of PDGFR-α kinase activity: DELFIA® data generated using Phospho-Tyrosine mAb P-Tyr-100 #9411 to detect phosphorylation of PDGFR-α substrate peptide (#1305) by PDGFR-α kinase. In a 50 µl reaction, 10 Units PDGFR-α and 1.5 µM substrate peptide were used per reaction well. Background reading is 2421. (DELFIA® is a registered trademark of PerkinElmer, Inc.)

Source/Purification: The GST-Kinase fusion protein was produced using a baculovirus expression system from a construct containing a fragment (Gln551-Leu1089) of the human PDGFR-α c-DNA (GenBank Accession No. NM_006206) fused to an amino-terminal GST-HIS_6-Thrombin cleavage site. The protein was then purified by one-step affinity purification using glutathione-agarose.

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

Quality Control: Biotinylated-FLT3 (Tyr589) peptide was selected as PDGFR-α kinase substrate from screening a panel of 150 pairs of tyrosine containing peptides as potential substrate candidates. Phospho-Tyrosine (P-Tyr-100) mAb #9411 was used for detection. The quality of the biotinylated peptides was evaluated by reverse-phase HPLC and by mass spectrometry.

Purified PDGFR-α kinase was quality controlled for purity by SDS-PAGE followed by silver stain and Western blot.

Assay conditions (time course [Fig.1], kinase dose-dependence [Fig.2], substrate dose-dependence [Fig.3] and staurosporine sensitivity [Fig.5]) for PDGFR-α kinase activity were verified using the PDGFR-α substrate peptide provided in this kit.

PDGFR-α kinase V_{max} and K_m values were measured by radiometric filtration assay using polyAEKY as a substrate [Fig.4].

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 carbonate buffer solution (3 mM Na_2CO_3, 7 mM $NaHCO_3$, pH 9.6). 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:

PDGF Receptor α Kinase #7766

Tyrosine Kinase Substrate Screening Kit #7450

FLT3 (Tyr589) Biotinylated Peptide #1305

HTScan™ Tyrosine Kinase Buffer (4X) #9805

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

Staurosporine #9953

ATP (10 mM) #9804

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

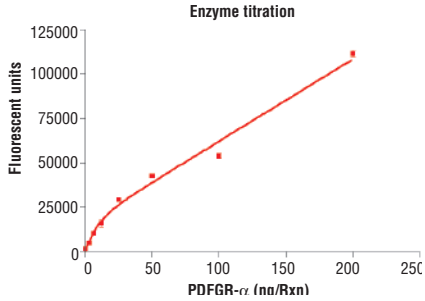

Figure 2. Dose dependence curve of PDGFR-α kinase activity: DELFIA® data generated using Phospho-Tyrosine mAb (P-Tyr-100) #9411 to detect phosphorylation of substrate peptide (#1305) by PDGFR-α kinase. In a 50 µl reaction, increasing amounts of PDGFR-α and 1.5 µM substrate peptide were used per reaction well at 25°C for 30 minutes. Background reading is 1624. (DELFIA® is a registered trademark of PerkinElmer, Inc.)

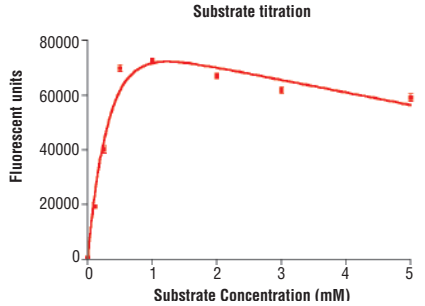

Figure 3. Peptide concentration dependence of PDGFR-α kinase activity: DELFIA® data generated using Phospho-Tyrosine mAb (P-Tyr-100) #9411 to detect phosphorylation of substrate peptide (#1305) by PDGFR-α kinase. In a 50 µl reaction, 10 Units of PDGFR-α and increasing concentrations of substrate peptide were used per reaction well at 25°C for 30 minutes. Background reading is 597. (DELFIA® is a registered trademark of PerkinElmer, Inc.)

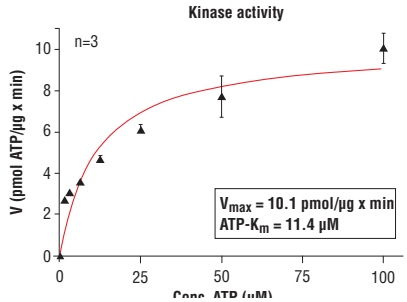

Figure 4. PDGFR-α kinase activity was measured in a radioisotopic filtration assay using the following reaction conditions: 60 mM HEPES-NaOH, pH 7.5, 3 mM $MgCl_2$, 3 mM $MnCl_2$, 3 µM Na-orthovanadate, 1.2 mM DTT, ATP (variable), 2.5 µg/50 µl PEG20.000, Substrate: Poly(AEKY) 2.5 µg/50 µl, Recombinant PDGFR-α: 20 Units/50 µl.

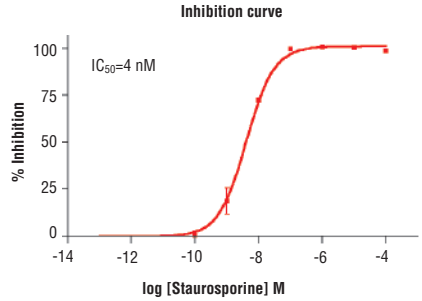

Figure 5. Staurosporine inhibition of PDGFR-α kinase activity: DELFIA® data generated using Phospho-Tyrosine mAb (P-Tyr-100) #9411 to detect phosphorylation of PDGFR-α substrate peptide (#1305) by PDGFR-α kinase. In a 50 µl reaction, 10 Units PDGFR-α kinase, 1.5 µM substrate peptide, 20 µM ATP and increasing amounts of staurosporine were used per reaction well at 25°C room temperature for 30 minutes. (DELFIA® is aregistered trademark of PerkinElmer, Inc.)

Background: The proteins of the PDGF family consist of several disulphide-bonded dimeric isoforms: PDGF-AA, PDGF-AB, PDGF-BB, PDGF-CC and PDGF-DD, which bind in a distinct pattern to two highly related RTKs: PDGFR-α and PDGFR-β. PDGFR-α and PDGFR-β show 85% and 75% identity between the two intracellular kinase domains, but the kinase insert and carboxy-terminal tail regions display only 27% and 28% identity. PDGFR-α binds all PDGF isoforms except PDGF-D, whereas PDGFR-β can only affiliate with PDGF-B and D (1). PDGFR-α and PDGFR-β not only form homo- and heterodimers, but also dimerize with EGFR, which can be stimulated by PDGF (2). The total number and the ratio of receptor subunits expressed varies between cell types, possibly accounting for the variable responsiveness of different cell types to PDGF (3). Ligand binding induces receptor dimerization and autophosphorylation, allowing binding and activation of cytoplasmic SH2 domain-containing signal transduction molecules including Grb2, Src, GAP, PI3 kinase, PLCγ and Nck. A number of different signaling pathways are thereby initiated leading to cell growth, actin reorganization, migration and differentiation (4). Tyr751 in the kinase-insert region of PDGFR-β is the docking site for PI3 kinase (5). Phosphorylated pentapeptides derived from Tyr751 of PDGFR-β (pTyr751-Val-Pro-Met-Leu) inhibit the association of the carboxy-terminal SH2 domain of the p85 subunit of PI3 kinase with PDGFR-β (6). Tyr740 is also required for PDGFR-β-mediated PI-3 kinase activation (7).

Background References:

(1) Deuel, T.F. et al. (1988) *Biofactors* 1, 213–217.

(2) Betsholtz, C. et al. (2001) *Bioessays* 23, 494–507.

(3) Coughlin, S.R. et al. (1988) *Prog. Clin. Biol. Res.* 266, 39–45.

(4) Ostman, A. and Heldin, C.H. (2001) *Adv. Cancer Res.* 80, 1–38.

(5) Panayotou, G. et al. (1992) *EMBO J.* 11, 4261–4272.

(6) Ramalingam, K. et al. (1995) *Bioorg. Med. Chem.* 3, 1263–1272.

(7) Kashishian, A. et al. (1992) *EMBO J.* 11, 1373–1382.

Protocol for HTScan™ PDGF Receptor α 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.

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. 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 pH7.5, 20 mM $MgCl_2$, 20 mM $MnCl_2$, 12 µM Na_3VO_4) to make DTT/Kinase buffer.
5. Transfer 1.25 ml of DTT/Kinase buffer to enzyme tube to make 4X reaction cocktail ([enzyme]=0.8 Units/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

10 Units Kinase

8. Incubate reaction plate at 25°C 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 Monoclonal Antibody (P-Tyr-100) #9411, 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. 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.

***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