

# HTScan® PDGF Receptor $\alpha$ Kinase Assay Kit

- ✓ 100 Assays (96 Well Format)



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This product is for *in vitro* research use only and is not intended for use in humans or animals.

Products Included	Product #	Kit Quantity
Phospho-Tyrosine Mouse mAb (P-Tyr-100)	9411	30 $\mu$ l
HTScan® Tyrosine Kinase Buffer (4X)	9805	15 ml
ATP (10 mM)	9804	1 ml
DTT (1000x, 1.25M)		80 $\mu$ l
FLT3 (Tyr589) Biotinylated Peptide	1305	1.25 ml
PDGF Receptor $\alpha$ Kinase	7912	2 X 5 $\mu$ g

**Description:** The kit provides a means of performing kinase activity assays with recombinant human PDGFR $\alpha$  kinase. It includes active PDGFR $\alpha$  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:** DNEY\*FYV

**Molecular Weights:** Peptide substrate, Biotin-FLT3 (Tyr589): 1945 Daltons. GST-PDGFR $\alpha$  Kinase: 76 kDa.

**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 $\alpha$  and PDGFR $\beta$ . PDGFR $\alpha$  and PDGFR $\beta$  contain between 75% and 85% sequence homology between their two intracellular kinase domains. The kinase insert and carboxy-terminal tail regions display approximately 27% to 28% homology. PDGFR $\alpha$  binds all PDGF isoforms except PDGF D, whereas PDGFR $\beta$  binds only PDGF B and D (1). PDGFR $\alpha$  and PDGFR $\beta$  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 difference in responsiveness of various 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, PLC $\gamma$  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 $\beta$  is the docking site for PI3 kinase (5). Phosphorylated pentapeptides derived from Tyr751 of PDGFR $\beta$  (pTyr751-Val-Pro-Met-Leu) inhibit the association of the carboxy-terminal SH2 domain of the p85 subunit of PI3 kinase with PDGFR $\beta$  (6). Tyr740 is also required for PDGFR $\beta$  mediated PI-3 kinase activation (7).

**Source/Purification:** The GST-kinase fusion protein was produced using a baculovirus expression system with a construct expressing human PDGFR $\alpha$  (Lys550-Leu1090) (GenBank Accession No. NM\_006206) 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 by screening a panel of 150 pairs of tyrosine containing peptides as potential substrate candidates. 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 PDGFR $\alpha$  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 PDGFR $\alpha$  activity using the PDGFR $\alpha$  substrate peptide provided in this kit. PDGFR $\alpha$  sensitivity to the inhibitor staurosporine was measured using the PDGFR substrate peptide provided in this kit [Fig.5].

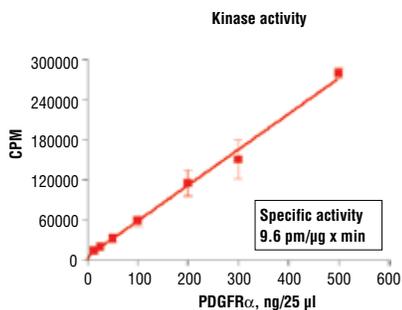


Figure 1. PDGFR $\alpha$  kinase activity was measured in a radiometric assay using the following reaction conditions: 60 mM HEPES-NaOH, pH 7.5, 5 mM MgCl<sub>2</sub>, 5 mM MnCl<sub>2</sub>, 3  $\mu$ M Na-orthovanadate, 1.2 mM DTT, 100  $\mu$ M ATP, 100  $\mu$ M FLT3 (Tyr589) Biotinylated Peptide (#1305) and variable amount of recombinant PDGFR $\alpha$ . 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  $\mu$ g/ml BSA and 50% glycerol. Do not aliquot the antibodies. Peptides are supplied at 6  $\mu$ M 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  $-80^{\circ}$ C.

Keep enzymes on ice during use.

Avoid repeated freeze-thaw cycles.

**Companion Products:**

- Tyrosine Kinase Substrate Screening Kit #7450
- PDGF Receptor  $\alpha$  Kinase #7912
- Phospho-Tyrosine Mouse mAb (P-Tyr-100) #9411
- FLT3 (Tyr589) Biotinylated Peptide #1305
- Staurosporine #9953

**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.

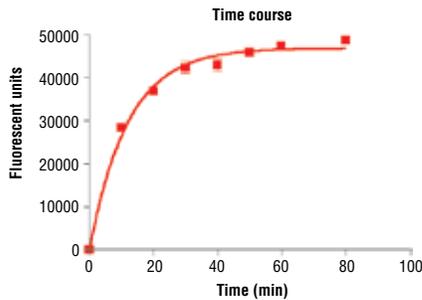


Figure 2. Time course of PDGFR $\alpha$  kinase activity: DELFIA® data generated using Phospho-Tyrosine Mouse mAb (P-Tyr-100) #9411 to detect phosphorylation of PDGFR $\alpha$  substrate peptide (#1305) by PDGFR $\alpha$  kinase. In a 50  $\mu$ l reaction, 100 ng PDGFR $\alpha$  and 1.5  $\mu$ M substrate peptide were used per reaction. (DELFIA® is a registered trademark of PerkinElmer, Inc.)

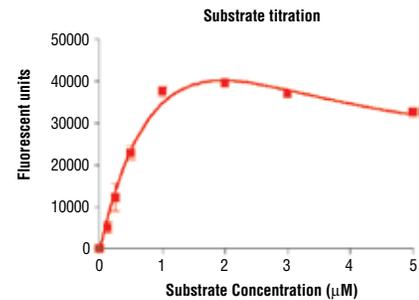


Figure 4. Peptide concentration dependence of PDGFR $\alpha$  kinase activity: DELFIA® data generated using Phospho-Tyrosine Mouse mAb (P-Tyr-100) #9411 to detect phosphorylation of substrate peptide (#1305) by PDGFR $\alpha$  kinase. In a 50  $\mu$ l reaction, 100 ng of PDGFR $\alpha$  and increasing concentrations of substrate peptide were used per reaction at room temperature for 30 minutes. (DELFIA® is a registered trademark of PerkinElmer, Inc.)

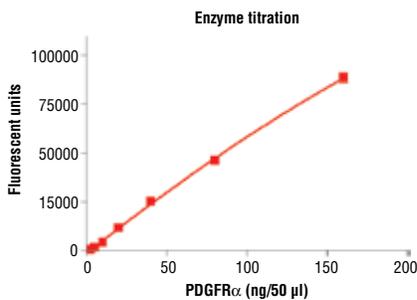


Figure 3. Dose dependence curve of PDGFR $\alpha$  kinase activity: DELFIA® data generated using Phospho-Tyrosine Mouse mAb (P-Tyr-100) #9411 to detect phosphorylation of substrate peptide (#1305) by PDGFR $\alpha$  kinase. In a 50  $\mu$ l reaction, increasing amounts of PDGFR $\alpha$  and 1.5  $\mu$ M substrate peptide were used per reaction at room temperature for 30 minutes. (DELFIA® is a registered trademark of PerkinElmer, Inc.)

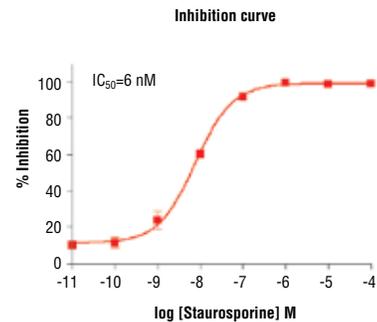


Figure 5. Staurosporine inhibition of PDGFR $\alpha$  kinase activity: DELFIA® data generated using Phospho-Tyrosine Mouse mAb (P-Tyr-100) #9411 to detect phosphorylation of PDGFR $\alpha$  substrate peptide (#1305) by PDGFR $\alpha$  kinase. In a 50  $\mu$ l reaction, 100 ng PDGFR $\alpha$ , 1.5  $\mu$ M substrate peptide, 5  $\mu$ M 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® PDGF Receptor $\alpha$ 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  $\mu$ l 10 mM ATP to 1.25 ml 6  $\mu$ M substrate peptide. Dilute the mixture with dH<sub>2</sub>O to 2.5 ml to make 2X ATP/substrate cocktail ([ATP]=40  $\mu$ M, [substrate]=3  $\mu$ M).
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  $\mu$ l of DTT (1.25 M) to 2.5 ml of 4X HTScan® Tyrosine Kinase Buffer (240 mM HEPES pH 7.5, 20 mM MgCl<sub>2</sub>, 20 mM MnCl<sub>2</sub>, 12  $\mu$ M Na<sub>3</sub>VO<sub>4</sub>) 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/ $\mu$ L in 4X reaction cocktail).
6. Incubate 12.5  $\mu$ l of the 4X reaction cocktail with 12.5  $\mu$ l/well of prediluted compound of interest (usually around 10  $\mu$ M) for 5 minutes at room temperature.
7. Add 25  $\mu$ l of 2X ATP/substrate cocktail to 25  $\mu$ l/well preincubated reaction cocktail/compound.

#### Final Assay Conditions for a 50 $\mu$ l Reaction

60 mM HEPES pH 7.5  
5 mM MgCl<sub>2</sub>  
5 mM MnCl<sub>2</sub>  
3  $\mu$ M Na<sub>3</sub>VO<sub>4</sub>  
1.25 mM DTT  
20  $\mu$ M ATP  
1.5  $\mu$ M peptide  
100 ng PDGF Receptor  $\alpha$  Kinase

8. Incubate reaction plate at room temperature for 30 minutes.
9. Add 50  $\mu$ l/well Stop Buffer (50 mM EDTA, pH 8) to stop the reaction.
10. Transfer 25  $\mu$ l of each reaction and 75  $\mu$ l dH<sub>2</sub>O/well to a 96-well streptavidin-coated plate and incubate at room temperature for 60 minutes.
11. \*Wash three times with 200  $\mu$ l/well PBS/T
12. Dilute primary antibody, Phospho-Tyrosine mAb (P-Tyr-100), 1:1000 in PBS/T with 1% BSA. Add 100  $\mu$ l/well primary antibody.
13. Incubate at room temperature for 60 minutes.
14. \*Wash three times with 200  $\mu$ l/well PBS/T
15. For DELFLIA® or Colorimetric ELISA detection methods please use the following protocols.

### DELFLIA® 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  $\mu$ l/well secondary antibody solution.
3. Incubate at room temperature for 30 minutes.
4. \*Wash five times with 200  $\mu$ l/well PBS/T.
5. Add 100  $\mu$ l/well DELFLIA® 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  $\mu$ s
 \*\* Delay time is the delay from the excitation pulse to the beginning of the measurement.

### Companion Products for DELFLIA®

DELFLIA® Europium-labeled Anti-mouse IgG (PerkinElmer Life Sciences #AD0124)  
DELFLIA® Europium-labeled Anti-rabbit IgG (PerkinElmer Life Sciences #AD0105)  
DELFLIA® Enhancement Solution (PerkinElmer Life Sciences #1244-105)  
DELFLIA® 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  $\mu$ l/well secondary antibody solution.
3. Incubate at room temperature for 30 minutes.
4. \*Wash five times with 200  $\mu$ l/well PBS/T.
5. Add 100  $\mu$ l/well TMB substrate.
6. Incubate at room temperature for 15 minutes.
7. Add 100  $\mu$ l/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