#7773 Store at –80°C

HTScan® Ret 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
Ret Kinase (recombinant, human)	7772	5 µg

Description: The kit provides a means of performing kinase activity assays with recombinant human Ret kinase. It includes active Ret 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-peptide: 2,853Daltons. GST-Ret Kinase: 84 kDa.

Background: The Ret proto-oncogene (c-Ret), a receptor tyrosine kinase, functions as a multicompetent receptor complex in conjunction with other membrane-bound ligand-binding GDNF family receptors (aGFRs) (1). The ligands for c-Ret have been identified as the glial cell line-derived neurotropic factor (GDNF) and its congeners neurturin, persephin and artemin (2-4). Gene alterations in c-Ret are associated with diseases including papillary thyroid carcinoma, multiple endocrine neoplasma type 2A (MEN2A), MEN2B, familial medullary thyroid carcinoma and a congenital developmental defect called Hirschsprung's disease (1,3). Tyr905 is located in the kinase domain of Ret and plays crucial roles in its catalytic and biological activity. Substitution of Phe for Tyr905 dramatically inhibits Ret autophosphorylation activity (5).

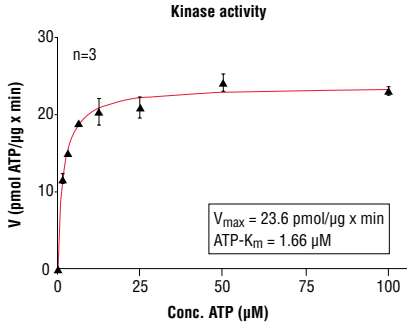

Figure 1. Ret kinase activity was measured in a radiometric assay using the following reaction conditions: 60 mM HEPES-NaOH, pH 7.5, 3 mM $MgCl_2$, 3 mM $MnCl_2$, 3 µM Na-orthovanadate, 1.2 mM DTT, ATP (variable), 2.5 µg/50 µl PEG20,000, Substrate: PolyEY, 2 µg/50 µl, recombinant Ret: 200 ng/50 µl.

Source/Purification: The GST-Kinase fusion protein was produced using a baculovirus expression system with a construct expressing a fragment of human Ret (His658-Asp1110) (GenBank Accession No. NM_000323) 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 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 Ret kinase was quality controlled for purity by SDS-PAGE followed by Coomassie stain and Western blot. The specific activity of the Ret kinase 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 Ret activity using the Ret substrate peptide provided in this kit. Ret sensitivity to the inhibitor staurosporine was measured using the Ret substrate peptide provided in this kit [Fig.5].

Background References:

(1) Airaksinen, M.S. et al. (1999) *Mol. Cell. Neurosci.* 13, 313–325.

(2) Takahashi, M. et al. (1989) *Oncogene* 4, 805–806.

(3) Manie, S. et al. (2001) *Trends Genet.* 17, 580–589.

(4) Tallini, G. and Asa, S. (2001) *Adv. Anat. Pathol.* 8, 345–354.

(5) Iwashita, T. et al. (1999) *Oncogene* 18, 3919–3922.

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:
Ret Kinase #7772

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

Gastrin Precursor (Tyr87) Biotinylated Peptide #1310

Tyrosine Kinase Substrate Screening Kit #7450

Staurosporine #9953

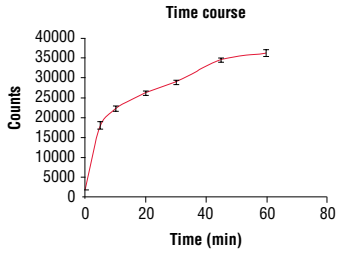

Figure 2. Time course of Ret kinase activity: DELFIA® data generated using Phospho-Tyrosine Mouse mAb (P-Tyr-100) #9411 to detect phosphorylation of Ret substrate peptide (#1310) by Ret kinase. In a 50 µl reaction, 50 ng Ret and 1.5 µM substrate peptide were used per reaction. (DELFIA® is a registered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

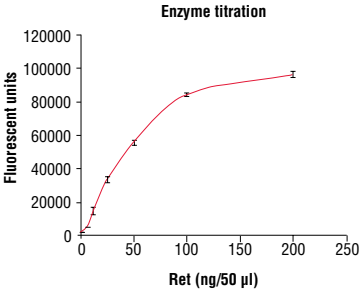

Figure 3. Dose dependence curve of Ret kinase activity: DELFIA® data generated using Phospho-Tyrosine Mouse mAb (P-Tyr-100) #9411 to detect phosphorylation of substrate peptide (#1310) by Ret kinase. In a 50 µl reaction, increasing amounts of Ret and 1.5 µM substrate peptide were used per reaction at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

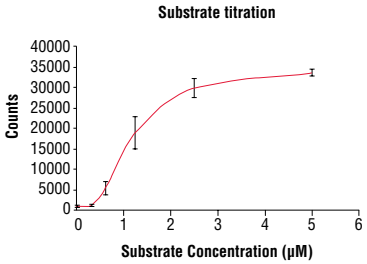

Figure 4. Peptide concentration dependence of Ret kinase activity: DELFIA® data generated using Phospho-Tyrosine Mouse mAb (P-Tyr-100) #9411 to detect phosphorylation of substrate peptide (#1310) by Ret kinase. In a 50 µl reaction, 50 ng of Ret and increasing concentrations of substrate peptide were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

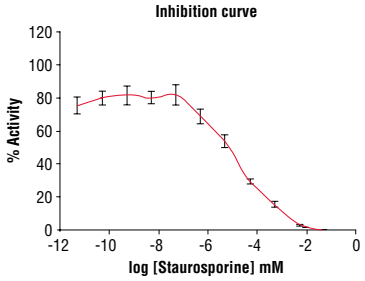

Figure 5. Staurosporine inhibition of Ret kinase activity: DELFIA® data generated using Phospho-Tyrosine Mouse mAb (P-Tyr-100) #9411 to detect phosphorylation of substrate peptide (#1310) by Ret kinase. In a 50 µl reaction, 50 ng Ret, 1.5 µM substrate peptide, 20 µM ATP and increasing amounts of staurosporine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

Protocol for HTScan® Ret 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
50 ng Ret 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