Store at -80°C

#7412

HTScan® PKCμ Kinase Assay Kit

☑ 100 assays
(96 Well Format)

Cell Signaling TECHNOLOGY®

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Products Included	Products #	Kit Quantity
Phospho-PKA Substrate (RRXS/T) (100G7) Rabbit mAb	9624	30 µl
Kinase Buffer (10X)	9802	15 ml
ATP (10 mM)	9804	1 ml
CREB (Ser133) Biotinylated Peptide	1331	1.25 ml
PKCμ Kinase (recombinant, human)	7602	5 µg

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

Peptide Core Sequence: RRPS*YRK

Molecular Weights: Peptide substrate, Biotin-peptide: 2,326 Daltons. GST-PKD/PKCμ Kinase domain: 133 kDa.

Background: Activation of PKC is one of the earliest events in a cascade leading to a variety of cellular responses such as secretion, gene expression, proliferation and muscle contraction (1,2). Protein kinase D (PKD), also called PKCμ, is a serine/ threonine kinase whose activation is dependent on the phosphorylation of two activation loop sites, Ser744 and Ser748, via a PKC-dependent signaling pathway (3,4,5). In addition to the two activation loop sites, the carboxy-terminal Ser916 has been identified as an autophosphorylation site for PKD/PKCμ. Phosphorylation at Ser916 correlates with PKD/PKCμ catalytic activity (6).

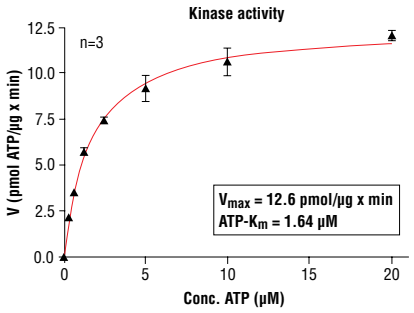

Figure 1. PKD/PKCμ kinase activity was measured in a radiometric assay using the following reaction conditions: 60 mM HEPES-NaOH, pH 7.5, 5 mM $MgCl_2$, 1.32 mM $CaCl_2$, 1 mM EDTA, 1.25 mM EGTA, 0.25 µg/50 µl phosphatidylserine, 50 ng/50 µl 1.2 Dioleyl-glycerol, 1.2 mM DTT, ATP (variable), 2.5 µg/50 µl PEG20.000, Substrate: tetra (LRRWSLG), 0.5 µg/50 µl and recombinant PKD/PKCμ: 200 ng/50 µl.

Source/Purification: The GST-Kinase fusion protein was produced using a baculovirus expression system with a construct expressing full length human PKD/PKCμ (Met1-Leu912) (GenBank Accession No. NM_002742) 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 Serine/Threonine Kinase Substrate Screening Kit #7400. Phospho-PKA Substrate (RRXS/T) (100G7) Rabbit mAb #9624 was used for detection. The quality of the biotinylated peptide was evaluated by reverse-phase HPLC and by mass spectrometry.

Purified PKD/PKCμ kinase was quality controlled for purity by SDS-PAGE followed by Coomassie stain and Western blot. PKD/PKCμ kinase specific 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 PKD/PKCμ activity using the PKD/PKCμ substrate peptide provided in this kit. PKD/PKCμ sensitivity to the inhibitor staurosporine was measured using the PKD/PKCμ substrate peptide provided in this kit [Fig.5].

Background References:

(1) Nishizuka, Y. (1984) *Nature* 308, 693–698.

(2) Keranen, L.M. et al. (1995) *Curr. Biol.* 5, 1394–1403.

(3) Valverde, A.M. et al. (1994) *Proc. Natl. Acad. Sci.* 91, 8572–8576.

(4) Johannes, F.J. et al. (1994) *J. Biol. Chem.* 269, 6140–6148.

(5) Iglesias, T. et al. (1998) *J. Biol. Chem.* 273, 27662–27667.

(6) Matthews, S.A. et al. (1999) *J. Biol. Chem.* 274, 26543–26549.

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:

Serine/Threonine Kinase Substrate Screening Kit #7400

PKCμ Kinase #7602

Phospho-PKA Substrate (RRXS/T) (100G7) Rabbit mAb #9624

CREB (Ser133) Biotinylated Peptide #1331

Staurosporine #9953

Kinase Buffer (10X) #9802

ATP (10 mM) #9804

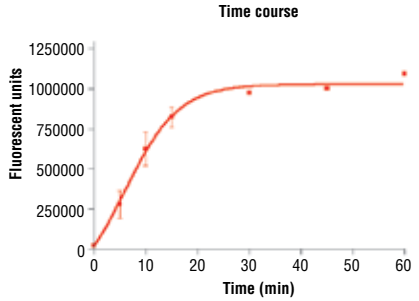

Figure 2. Time course of PKD/PKCμ kinase activity: DELFIA® data generated using Phospho-PKA Substrate (RRXS/T) (100G7) Rabbit mAb #9624 to detect phosphorylation of PKD/PKCμ substrate peptide (#1331) by PKD/PKCμ kinase. In a 50 µl reaction, 10 ng PKD/PKCμ and 1.5 µM substrate peptide were used per reaction. (DELFIA® is a registered trademark of PerkinElmer, Inc.)

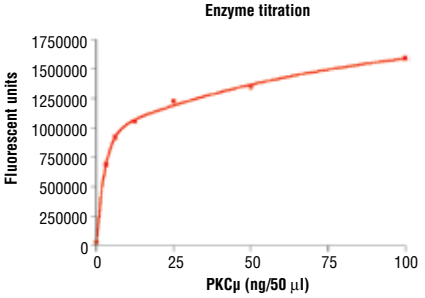

Figure 3. Dose dependence curve of PKD/PKCμ kinase activity: DELFIA® data generated using Phospho-PKA Substrate (RRXS/T) (100G7) Rabbit mAb #9624 to detect phosphorylation of substrate peptide (#1331) by PKD/PKCμ kinase. In a 50 µl reaction, increasing amounts of PKD/PKCμ and 1.5 µM substrate peptide were used per reaction at room temperature for 15 minutes. (DELFIA® is a registered trademark of PerkinElmer, Inc.)

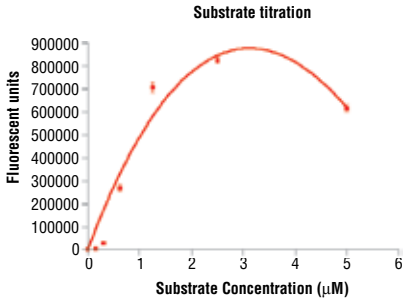

Figure 4. Peptide concentration dependence of PKD/PKCμ kinase activity: DELFIA® data generated using Phospho-PKA Substrate (RRXS/T) (100G7) Rabbit mAb #9624 to detect phosphorylation of substrate peptide (#1331) by PKD/PKCμ kinase. In a 50 µl reaction, 10 ng of PKD/PKCμ and increasing concentrations of substrate peptide were used per reaction at room temperature for 15 minutes. (DELFIA® is a registered trademark of PerkinElmer, Inc.)

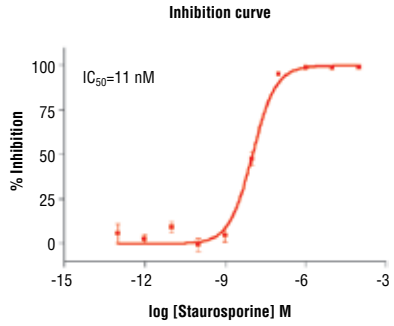

Figure 5. Staurosporine inhibition of PKD/PKCμ kinase activity: DELFIA® data generated using Phospho-PKA Substrate (RRXS/T) (100G7) Rabbit mAb #9624 to detect phosphorylation of PKD/PKCμ substrate peptide (#1331) by PKD/PKCμ kinase. In a 50 µl reaction, 10 ng PKD/PKCμ, 1.5 µM substrate peptide, 20 µM ATP and increasing amounts of staurosporine were used per reaction at room temperature for 15 minutes. (DELFIA® is a registered trademark of PerkinElmer, Inc.)

Protocol for HTScan® PKCμ 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 100 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]=400 µM, [substrate] = 3 µm).
2. 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 1 ml 10X kinase buffer [1 ml 10X Kinase Buffer 250 mM Tris-HCl pH 7.5, 100 mM $MgCl_2$, 1 mM Na_3VO_4, 50 mM β-glycerophosphate, 20 mM dithiothreitol (DTT)] to 1.5 ml dH_20 to make 2.5 ml 4X reaction buffer.
5. Transfer 1.2 ml of 4X Reaction buffer to each enzyme tube to make 4X reaction cocktail ([enzyme]) = 4 ng/µl in 4X reaction cocktail).
6. Add 12.5 µl of the 4X reaction cocktail to 12.5 µl/well of prediluted compound of interest (usually around 10 µM) and incubate 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

25 mM Tris-HCl (pH 7.5)
10 mM $MgCl_2$
5 mM β-glycerophosphate
0.1 mM Na_3VO_4
2 mM DTT
200 µM ATP
1.5 µM peptide
10 ng PKCμ 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 to a 96-well streptavidin-coated plate containing 75 µl dH_2O/well and incubate at room temperature for 60 minutes.
11. *Wash three times with 200 µl/well PBS/T.
12. Dilute primary antibody, Phospho-PKA Substrate (RRXS/T) (100G7) Rabbit mAb, 1:1000 in PBS/T with 1% BSA. Add 100 µl/well primary antibody.
13. Incubate at room temperature for 120 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