Unit#253 5489 Byrne Rd.
Burnaby BC Canada
V5J 3J1
Orders@immunechem.com
www.immunechem.com

PKC Kinase Assay Kit

Catalog#: ICP0211

ASSAY DESCRIPTION:

Protein kinase C, also known as PKC, is a family of protein kinase enzymes that are involved in controlling the function of other proteins through the phosphorylation of hydroxyl groups of serine and threonine amino acid residues on these proteins. PKC enzymes in turn are activated by signals such as increases in the concentration of diacylglycerol (DAG) or calcium ions (Ca2+). Hence PKC enzymes play important roles in several signal transduction cascades. The non-radioactive PKC Kinase Activity Assay provides a safe, rapid and reliable method for screening of inhibitors or activators of PKC and for quantitating the activity of PKC in partially purified, purified or crude enzyme preparations.

The PKC Kinase Activity Assay is based on a solid phase enzyme-linked immuno-absorbent assay (ELISA) that utilizes a specific synthetic peptide as a substrate for PKC and a polyclonal antibody that recognizes the phosphorylated form of the substrate. The assay is designed for the analysis of PKC activity in the solution phase.

In the assay, the substrate, which is readily phosphorylated by PKC, is pre-coated on the wells of the provided PKC Substrate Microtiter Plate. The samples to be assayed are added to the appropriate wells, followed by the addition of ATP to initiate the reaction. The kinase reaction is terminated and a Phosphospecific Substrate Antibody is added to the wells which binds specifically to the phosphorylated peptide substrate. The phosphospecific antibody is subsequently bound by a peroxidase conjugated secondary antibody. The assay is developed with tetramethylbenzidine substrate (TMB) and a color develops in proportion to PKC phosphotransferase activity. The color development is stopped with acid stop solution and the intensity of the color is measured in a microplate reader at 450 nm.

Unit#253 5489 Byrne Rd.
Burnaby BC Canada
V5J 3J1
Orders@immunechem.com
www.immunechem.com

ASSAY PROCEDURE SUMMARY:

1. Bring to room temperature: Substrate Microtiter Plate, Antibody Dilution Buffer, Kinase Assay Dilution Buffer, TMB Substrate and Stop Solution.
2. Add 60 µL samples to appropriate wells of the Substrate Microtiter Plate.
3. Initiate reaction by adding 10 µL of diluted ATP to each well.
4. Incubate for 30 minutes at 37°C.
5. Stop reaction by emptying contents of each well.
6. Wash wells ONCE with 300 µL 1X Wash Buffer.
7. Add 60 µL of Phosphospecific Substrate Antibody to each well, except the blank.
8. Incubate at room temperature for 60 minutes.
9. Wash wells 4 times with 300 µL 1X Wash Buffer.
10. Add 60 µL of diluted Anti-Rabbit IgG:HRP Conjugate to each well.
11. Incubate at room temperature for 30 minutes.
12. Wash wells 4 times with 300 µL 1X Wash Buffer.
13. Add 60 µL of TMB Substrate to each well.
14. Incubate at room temperature for 15-30 minutes. Incubation time should be determined by the investigator according to color development.
15. Add 60 µL of Stop Solution to each well.
16. Measure absorbance at 450 nm.

PRECAUTIONS:

FOR RESEARCH USE ONLY. NOT FOR USE IN DIAGNOSTIC PROCEDURES.

- The activity of the Anti-Rabbit IgG: HRP Conjugate (*Part# ICP0211-5*) is affected by nucleophiles such as azide, cyanide and hydroxylamine.

Please read the complete kit insert before performing this assay.

Unit#253 5489 Byrne Rd.
Burnaby BC Canada
V5J 3J1
Orders@immunechem.com
www.immunechem.com

KIT COMPONENTS:

The PKC Assay Kit contains the following components in sufficient quantities for a 96-well plate.

Item #	Part #	COMPONENT	SIZE	DESCRIPTION
1	ICP0211-1	Substrate Microtiter Plate	96-well plate	12x8 removable strips and frame; pre-coated plate with substrate peptide for PKC
2	ICP0211-2	Kinase Assay Dilution Buffer	15 mL	Buffer for the dilution of ATP, standard and samples
3	ICP0211-3	Phosphospecific Substrate Antibody	6.5 mL	Rabbit polyclonal antibody specific for phosphorylated substrate
4	ICP0211-4	Antibody Dilution Buffer	10 mL	Buffer for the dilution of Anti-Rabbit IgG: HRP Conjugate
5	ICP0211-5	Anti-Rabbit IgG: HRP Conjugate	15 µL	1 mg/mL solution of horseradish peroxidase conjugated goat anti-rabbit IgG containing 0.05% ProClin as a preservative
6	ICP0211-6	ATP	1 mg	Adenosine triphosphate
7	ICP0211-7	20X Wash Buffer	20 mL	Concentrated solution of buffer and surfactant
8	ICP0211-8	TMB Substrate	6.5 mL	Stabilized tetramethylbenzidine substrate
9	ICP0211-9	Stop Solution	6.5 mL	Acid solution to stop color reaction

STORAGE OF MATERIALS:

- The kit's expiry date is 6 months from the date of shipping.
- Anti-Rabbit IgG: HRP Conjugate (ICP0211-5) and ATP (ICP0211-6) should be stored at -20°C.
- All other reagents are stable as supplied at 4°C until the kit's expiry date.
- Unused wells of the Substrate Microtiter Plate should be resealed with the desiccant in the foil pouch provided and stored at 4°C until the kit's expiry date.

Any remaining diluted ATP can be stored at -20°C for up to 6 months or until the kit's expiry date, whichever is earlier.

MATERIALS REQUIRED BUT NOT PROVIDED:

- **Active PKC**: PKCs must be stored at -70°C. If assaying on separate occasions, once thawed, the kinase may be aliquoted into smaller portions, stored at -70°C and subsequently thawed only once. Refrozen aliquots may result in a reduction in kinase activity.
- Deionized or distilled water
- Disposable pipette tips
- Precision pipettes capable of accurately delivering volumes between 1 µL and 1,000 µL
- Squirt bottle, manifold dispenser, or automated microtiter plate washer
- Beakers for diluting reagents
- Graduated cylinders
- Absorbent paper
- Microtiter plate reader capable of measuring absorbance at 450 nm
- Adhesive plate sealers

RECOMMENDATIONS PRIOR TO USING ASSAY:

Each PKC shows different activity against the substrate (**Table 1**). Therefore, before performing the kinase assay, it is strongly recommended that an initial experiment be performed to determine the appropriate dilution of the active kinase and reaction time to carry out subsequent studies.

- Perform a time course using various kinase concentrations, including a no-enzyme blank, to confirm a linear response of the kinase with respect to phosphorylation.

Select a reaction time and kinase concentration from the results obtained. This will provide a sufficient window of phosphorylation to yield statistically reliable results. For inhibitor/activator screening, the kinase concentration that gives 80% of the maximum signal is recommended.

Unit#253 5489 Byrne Rd.
Burnaby BC Canada
V5J 3J1
Orders@immunechem.com
www.immunechem.com

REAGENT PREPARATION:

NOTE: *All reagents should be freshly prepared prior to use.*
NOTE*: The preparation of the reagents is based on using the complete 1 X 96 well assay, unless otherwise noted. If only a portion of the microtiter plate is to be used, please store all components as previously described (see "***STORAGE OF MATERIALS***" on page 3).*

1. TEMPERATURE OF REAGENTS

Bring the following reagents to room temperature prior to use:

- **Substrate Microtiter Plate** *(Part# ICP0211-1)*
- **Kinase Assay Dilution Buffer** *(Part# ICP0211-2)*
- **Antibody Dilution Buffer (***Part# ICP0211-4)*
- **20X Wash Buffer** *(Part# ICP0211-7)*
- **TMB Substrate** *(Part# ICP0211-8)*
- **Stop Solution (***Part# ICP0211-9)*

2. PREPARATION OF ACTIVE PKC CONTROL

NOTE: *Active PKC are sensitive to temperature variations and freeze/thaw cycles. Thaw kinases on ice.*

a. The active PKC is intended to be used as a positive control and can be serially diluted in **Kinase Assay Dilution Buffer** to a final volume of 60 µL. Keep preparations on ice.
b. 60 µL of **Kinase Assay Dilution Buffer** (with no kinase) can be used as the assay blank.

3. ATP (*Part# ICP0211-6)*

a. Centrifuge the vial before removing the cap to ensure maximum product recovery.
b. Reconstitute the **ATP** with 2 mL of **Kinase Assay Dilution Buffer**.
c. Mix gently by inversion.
d. Reagent is now ready to be used in the **ASSAY PROCEDURE** *(see page 8).*
e. Any remaining diluted **ATP** can be stored at -20°C for up to 6 months or until the kit's expiry date, whichever is earlier.

4. ANTI-RABBIT IgG: HRP CONJUGATE *(Part# ICP0211-5)*

a. Centrifuge the vial before removing the cap to ensure maximum product recovery.

b. Dilute the **Anti-Rabbit IgG:HRP Conjugate** to 1 µg/mL (1:1000) with **Antibody Dilution Buffer**. A minimum of 6 mL of working solution is required for 96-wells (60 µL/well). If only using a portion of the plate, dilute only what is needed for the number of wells used.

c. Mix gently by inversion.

d. Reagent is now ready to be used in the **ASSAY PROCEDURE** *(see page 8).*

e. Do not re-use or store any remaining diluted **Anti-Rabbit IgG:HRP Conjugate**.

5. WASH BUFFER *(Part# ICP0211-7)*

a. Bring the **20X Wash Buffer** to room temperature and swirl gently to dissolve any crystals that may have formed during storage.

b. Dilute the 30 mL of **20X Wash Buffer** with 570 mL of deionized or distilled water. Once diluted, the **1X Wash Buffer** is stable at room temperature for up to 4 weeks. For longer term storage, the **1X Wash Buffer** should be stored at 4°C.

ASSAY PROCEDURE:

1. PREPARATION OF SUBSTRATE MICROTITER PLATE
   a. Determine the number of wells to be used. If less than 96 pre-coated microtiter wells are needed, remove the excess wells from the frame and return them to the foil pouch. Reseal the pouch containing the unused wells with the desiccant and store at 4°C.

2. ADDITION OF STANDARDS AND SAMPLES
   a. Add 60 µL of each of the following to appropriate wells:
      - Purified Active PKC
      - Samples *(previously prepared, see page 6-7)*
      - Blank **(Kinase Assay Dilution Buffer** with no kinase)
      - Negative Control (Inhibitor/Activator Diluent with no inhibitor or activator) *(use for inhibitor or activator screening studies)*
   b. Initiate reaction by adding 10 µL of diluted **ATP** *(previously diluted, see page 6)* to each well, except the blank. To avoid cross contamination, change pipette tips for each well.
   c. Cover wells with an adhesive plate sealer or plastic wrap and incubate at 37°C for 30-60 minutes, preferably with gentle shaking.

      NOTE: *It is recommended that the experiment use the predetermined time point generated during the initial experiment as outlined in the* **RECOMMENDATIONS PRIOR TO USING THE ASSAY SECTION** *on page 5.*
   d. Stop reaction by emptying contents of each well. Invert the plate and carefully pat dry on clean paper towels.

3. WASHING
   a. Remove liquid from all wells.
   b. Add 300 µL of **1X Wash Buffer** to all wells, using a multi-channel pipette, manifold dispenser, automated microplate washer, or a squirt bottle. (To reduce background, it may be necessary to soak the wells for 30-60 seconds between each wash).
   c. Remove liquid from all wells. Repeat the removing and washing 3 more times with **1X Wash Buffer** for a total of 4 washes.
   d. After the 4th wash, remove the liquid from all wells. Invert the plate and carefully pat dry on clean paper towels.

4. ADDITION OF PHOSPHOSPECIFIC SUBSTRATE ANTIBODY
   a. Add 60 µL of the **Phosphospecific Substrate Antibody** to each well, including the blank.
   b. Cover wells with a fresh adhesive plate sealer (or plastic wrap) and incubate at room temperature for 60 minutes, preferably with gentle shaking.
   c. Wash plate as described in Step 3.

5. ADDITION OF ANTI-RABBIT IgG: HRP CONJUGATE *(previously diluted, see page 7)*
   a. Add 60 µL of the previously diluted **Anti-Rabbit IgG:HRP Conjugate** to each well, including the blank.
   b. Cover wells with a fresh adhesive plate sealer (or plastic wrap) and incubate at room temperature for 30 minutes, preferably with gentle shaking.
   c. Wash plate as described in Step 3.

6. ADDITION OF TMB SUBSTRATE AND ACID STOP SOLUTION
   a. Add 60 µL of the **TMB Substrate** to each well.
   b. Incubate the plate at room temperature for 15-30 minutes (incubation time should be monitored by the investigator according to color development).
   c. Add 60 µL of the **Stop Solution** to each well in the same order that the **TMB Substrate** was added.

7. MEASURING ABSORBANCE
   a. Set up the microplate reader according to the manufacturer's instructions.
   b. Set wavelength at 450 nm.
   c. Measure the absorbance.