PKC kinase activity kit

For the screening of inhibitors or activators of PKC and for quantitating the activity of PKC in partially purified, purified or crude enzyme preparations.

Catalog Number: ADI-EKS-420A

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

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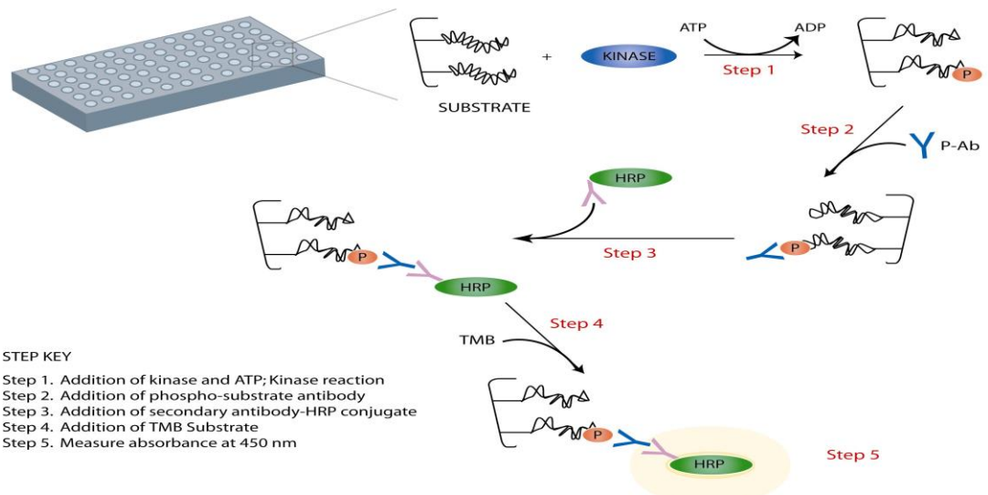
A. INTRODUCTION

ASSAY DESIGN

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 bind 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.



SCIENTIFIC OVERVIEW

Protein Kinase C (PKC) is a large superfamily of serine/threonine kinases that mediate essential cellular signals required for activation, proliferation, differentiation and survival. There are at least ten PKC isotypes that are closely related in structure but that have distinct patterns of tissue distribution and function. The PKC isotypes can be subdivided into three classes based on primary structure and biochemical properties. These are: classical or conventional PKC isotypes (cPKC), novel PKC isotypes (nPKC) and atypical PKC isotypes (aPKC). All PKC isotypes share a characteristic sequence motif C1 in addition to a serine/threonine-protein kinase domain.

INTRODUCTION

The cPKC isotypes include PKCα, βI, βII and γ and contain two conserved modules, C1 and C2 domains in the cPKC regulatory domain. The cPKC-C1 domains contain a repeat of a cysteine-rich sequence, C1A and C1B, each of which tightly bind two zinc ions and constitute a binding site for diacylglycerols (DAGs) and phorbol esters. The cPKC-C2 domains are Ca^{2+}-dependent phospholipid binding domains that show specificity to acidic phospholipids such as phosphatidylserine. The nPKC isotypes include PKCδ, ε, η, and θ. The nPKC-C1 domains, like cPKCs, bind DAG and phorbol ester but unlike cPKCs, the C2 domain is missing. The aPKC isotypes which include PKCζ and λ/τ, lack the entire C2 domain and one cysteine-rich loop in the C1 domain. aPKCs lack the critical residues required for the interaction of DAG and phorbol esters.

Studies indicate that the isotype-specific physiological function of PKC is regulated by three events: maturation, catalytic activation and targeting. PKC has become one of the important drug targets because of its key role in cellular functions (1-5).

ASSAY PROCEDURE SUMMARY

1. Bring to room temperature: **PKC Substrate Microtiter Plate**, **Antibody Dilution Buffer, Kinase Assay Dilution Buffer**, **TMB Substrate** and **Acid Stop Solution.**
2. Soak wells of the **PKC Substrate Microtiter Plate** with 50 µL **Kinase Assay Dilution Buffer** at room temperature for 10 minutes. Carefully aspirate liquid from each well.
3. Add samples to appropriate wells of the **PKC Substrate Microtiter Plate.**
4. Initiate reaction by adding 10 µL of diluted ATP to each well.
5. Incubate for up to 90 minutes at 30°C.
6. Stop reaction by emptying contents of each well.
7. Add 40 µL of **Phosphospecific Substrate Antibody** to each well.
8. Incubate at room temperature for 60 minutes.
9. Wash wells 4 times with 100 µL 1X Wash Buffer.
10. Add 40 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 100 µL 1X Wash Buffer.
13. Add 60 µL of **TMB Substrate** to each well.
14. Incubate at room temperature for 30-60 minutes. Incubation time should be determined by the investigator according to color development.

15. Add 20 µL of **Acid Stop Solution** to each well.
16. Measure absorbance at 450 nm.

PRECAUTIONS

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- The activity of the **Anti-Rabbit IgG: HRP Conjugate** *(part# 80-1488)* is affected by nucleophiles such as azide, cyanide and hydroxylamine.

Please read the complete kit insert before performing this assay.

MATERIALS PROVIDED

The PKC Kinase Activity Assay Kit contains the following components in sufficient quantities for 96 wells.

PART #	COMPONENT	SIZE	DESCRIPTION
80-1490	PKC Substrate Microtiter Plate	96-well plate	12x8 removable strips and frame; pre-coated plate with substrate peptide for PKC
80-1489	Phosphospecific Substrate Antibody	5 mL	1µg/mL solution of rabbit polyclonal antibody specific for phosphorylated PKC substrate
80-1488	Anti-Rabbit IgG: HRP Conjugate	20 µL	1mg/mL solution of horseradish peroxidase conjugated goat anti-rabbit IgG containing 0.01% thimerosal as a preservative
80-1487	Antibody Dilution Buffer	10 mL	Buffer for the dilution of Anti-Rabbit IgG: HRP Conjugate
80-1486	Kinase Assay Dilution Buffer	10 mL	Buffer for the dilution of ATP, standards and samples
80-1485	ATP	2 mg	Adenosine triphosphate
80-1484	Active PKC	28 µL	Purified recombinant active Protein Kinase C for use as a positive control
80-1286	20X Wash Buffer	30 mL	Concentrated solution of buffer and surfactant
80-0350	TMB Substrate	10 mL	Stabilized tetramethylbenzidine substrate
80-0377	Stop Solution 2	10 mL	Acid solution to stop color reaction

STORAGE OF MATERIALS

- All reagents are stable as supplied at 4°C until the kit's expiry date, except the **Active PKC,** which must be stored at –70°C.
- If assaying on separate occasions, once thawed, the purified **Active PKC** 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.
- Unused wells of the **PKC 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

- Deionized or distilled water
- Disposable pipette tips
- Precision pipettes capable of accurately delivering volumes between 1 µL and 1,000 µL
- Repeater pipettes
- Squirt bottle, manifold dispenser, or automated microtiter plate washer
- Graduated cylinders
- Adsorbent paper for blotting
- Microtiter plate reader capable of measuring absorbance at 450 nm
- Adhesive plate sealers

C. PERFORMING THE ASSAY

CRITICAL ASSAY PARAMETERS AND NOTES

- The PKC Kinase Activity Assay Kit contains a pre-coated microtiter plate (**PKC Substrate Microtiter Plate**) with removable wells to allow assaying on separate occasions. Unused wells should be kept at 4°C in the sealed foil bag with the desiccant. The wells should be used in the frame provided.

- The following kit components should be brought to room temperature prior to use: **PKC Substrate Microtiter Plate**, **Antibody Dilution Buffer, Kinase Assay Dilution Buffer, 20X Wash Buffer, TMB Substrate and Acid Stop Solution**.

- For statistical results, it is recommended that assays be run in triplicate.

- To avoid cross contamination, change disposable pipette tips between the addition of each standard, sample, and reagent and add to the side of the wells. Use separate troughs/reservoirs for each reagent.

- This assay requires pipetting of small volumes. To minimize imprecision caused by pipetting, ensure that pipettes are calibrated and pipette tips are pre-rinsed with the reagent.

- Mix all reagents and samples gently, yet thoroughly, prior to use. Avoid foaming of reagents.

- Consistent, thorough washing of each well is critical. If using an automatic washer, check washing head before use. If washing manually, ensure all wells are completely filled at each wash. Air bubbles should be avoided.

- When aspirating, tilt plate slightly to carefully remove liquid from the well.

- Absorbance is a function of the incubation time. Therefore, prior to starting the assay it is recommended that all reagents are ready to use and all required strip-wells secured in the microtiter frame. This will ensure equal elapsed time for each pipetting step, without interruption.

- For each step in the procedure, total dispensing time for addition of reagents to the assay plate should not exceed 15 minutes.

- Exercise appropriate laboratory safety precautions when performing this assay.

NOTE: *The components in each kit lot # have been quality assured and warranted in this specific combination only; please do not mix them with components from other kit lot #s.*

RECOMMENDATIONS PRIOR TO USING ASSAY

Before performing the kinase assay, it is strongly recommended that an initial experiment be performed to determine an appropriate dilution of the purified sample 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 in the previous step. This will provide a sufficient window of phosphorylation to yield statistically reliable results.

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 page 6).*

1. TEMPERATURE OF REAGENTS

   Bring the following reagents to room temperature prior to use:

   - **PKC Substrate Microtiter Plate** *(Part#: 80-1490)*
   - **Antibody Dilution Buffer** *(Part#: 80-1487)*
   - **Kinase Assay Dilution Buffer** *(Part#: 80-1486)*
   - **20X Wash Buffer** *(Part#: 80-1286)*
   - **TMB Substrate** *(Part#: 80-0350)*
   - **Acid Stop Solution** *(Part#: 80-0377)*

2. PREPARATION OF PURIFIED ACTIVE PKC CONTROL (*Part#: 80-1484)*

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

   a. The **Active PKC** (part# 80-1484) is intended to be used as a positive control and can be serially diluted in **Kinase Assay Dilution Buffer** to a final volume of 30 µL. Please refer to the vial for the concentration (ng/µL) of the purified kinase preparation. Keep preparations on ice.

   b. 30 µL of **Kinase Assay Dilution Buffer** (without kinase) can be used as the assay blank.

3. ATP (*Part#: 80-1485)*

   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 11).*
   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#: 80-1488)*

   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** in a polypropylene tube. A minimum of 4mL of working solution is required for 96-wells (40 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 12).*
   e. Do not re-use or store any remaining diluted **Anti-Rabbit IgG:HRP Conjugate**.

5. WASH BUFFER

   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 Wash Buffer should be stored at 4°C.

SAMPLE PREPARATION

1. PREPARATION OF CRUDE OR PARTIALLY PURIFIED FRACTIONS

Crude Sample Preparations

NOTE: Crude sample preparations may be used with the assay, however, crude preparations may contain other kinases which could phosphorylate the substrate, thus it is recommended that purified or partially purified kinase preparations be used.

a. Prepare cell lysates or tissue extracts according to desired protocol. Sample protocols may be found in Appendices I-II (pages 15-16).
b. Evaluate total protein concentration.

Note: In experiments using mouse brain lysates, it was found that 0.02 -2μg of crude protein per assay was sufficient to measure kinase activity. However, each user should determine the optimal protein concentration for their particular experiments.

Partially Purified Sample Preparation

a. Select desired column and buffers for purification. A sample protocol may be found in Appendix III (page 17) (refer to references 6-7 on page 14 if more detail of purification is required).
b. Load sample and run desired purification protocol.
c. If necessary, dilute fractionated sample accordingly in **Kinase Assay Dilution Buffer** *(please see page 8 for recommendations prior to using the assay).*

NOTE: It is suggested that the fractionated sample be serially diluted (i.e. start with 30μL and dilute 1:2, etc or use 5, 10, 15, 20 and 30μL of the fractionated sample. Remember the final volume should be adjusted to 30μL as this is what the reaction calls for per well).

2. FOR INHIBITOR OR ACTIVATOR SCREENING

a. Dilute the inhibitor appropriately. It is recommended that the inhibitor diluent by itself be used as a negative control.
b. Incubate the kinase in the presence of the inhibitor prior to initiating the kinase reaction *(Step#2c in the Assay Procedure, page 11).*

***Note**: The reaction time should be pre-determined by the investigator prior to use in the assay as outlined in the Recommendations Prior To Using the Assay section on page 8.*

ASSAY PROCEDURE

1. PREPARATION OF PKC 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.
   b. Soak each well of the **PKC Substrate Microtiter Plate** with 50 µL of **Kinase Assay Dilution Buffer** at room temperature for 10 minutes.
   c. Carefully aspirate liquid from all wells.

2. ADDITION OF CONTROLS AND SAMPLES

   a. Add 30 µL of each of the following to appropriate wells:
      - Purified **Active PKC** control *(previously prepared, see page 8)*
      - Samples *(previously prepared, see page10)*
      - Blank **(Kinase Assay Dilution Buffer** with no kinase)
      - Negative Control (Inhibitor Diluent with no inhibitor) *(use for inhibitor screening studies)*
   b. Initiate reaction by adding 10 µL of diluted ATP *(previously diluted, see page 9)* to each well. To avoid cross contamination, change pipette tips for each well.

   c. Cover wells with an adhesive plate sealer and incubate at 30°C for up to 90 minutes, preferably with gentle, thorough shaking every 20 minutes by hand or a shaker with rotate angle at 60 rpm. Thorough mixing is recommended to yield optimal results

      Note: It is recommended that the experiment uses the predetermined time point generated as outlined in the Recommendations Prior To Using the Assay section on page 8.
   d. Stop reaction by emptying contents of each well. Invert the plate and carefully pat dry on clean paper towels.

3. ADDITION OF PHOSPHOSPECIFIC SUBSTRATE ANTIBODY

   a. Add 40 µL of the **Phosphospecific Substrate Antibody** to each well.
   b. Cover wells with a fresh adhesive plate sealer and incubate at room temperature for 60 minutes, preferably with gentle, thorough shaking every 20 minutes.

4. WASHING

a. Aspirate liquid from all wells.
b. Add 100 µL of 1X Wash Buffer all wells, using a multi-channel pipette, manifold dispenser, automated microplate washer, or a squirt bottle. (To reduce background, it may be necessary to wait 1-2 minutes between each wash).
c. Repeat the aspirating and washing 3 more times with 1X Wash Buffer for a total of 4 washes.
d. After the 4th wash, aspirate the liquid from all wells. Invert the plate and carefully pat dry on clean paper towels.

5. ADDITION OF ANTI-RABBIT IgG: HRP CONJUGATE
(Previously diluted, see page 9)

a. Add 40 µL of the previously diluted **Anti-Rabbit IgG:HRP Conjugate** to each well.
b. Cover wells with a fresh adhesive plate sealer and incubate at room temperature for 30 minutes, preferably with gentle, thorough shaking every 10 minutes.
c. Wash plate as described in Step #4.

6. ADDITION OF TMB SUBSTRATE AND ACID STOP SOLUTION

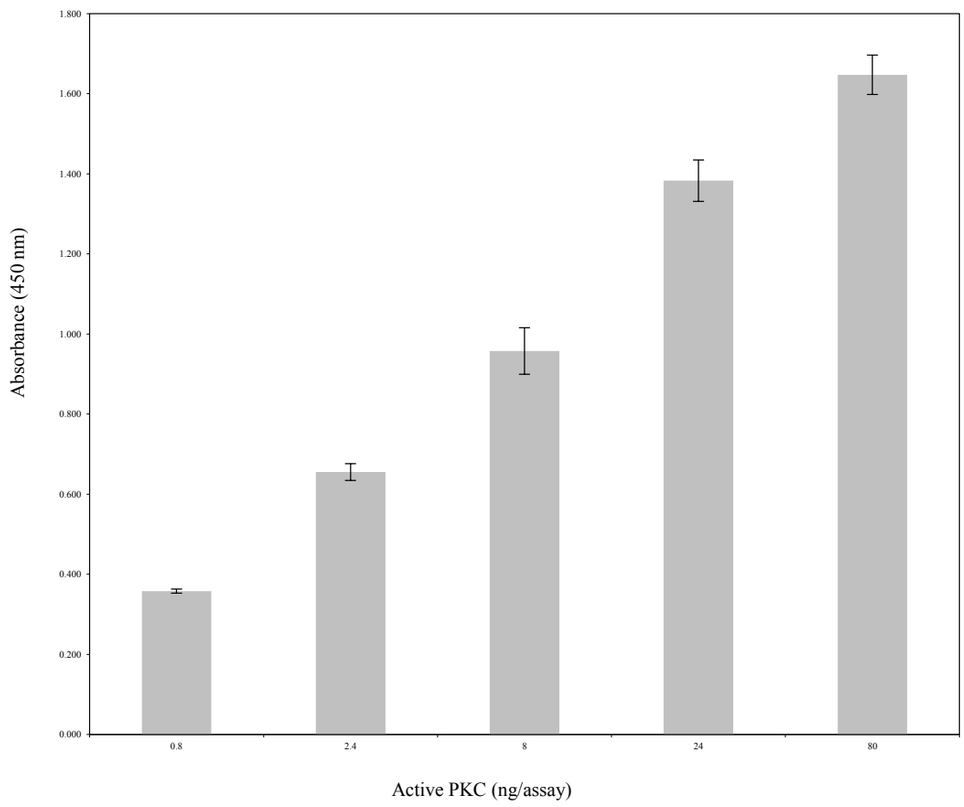
a. Add 60 µL of the **TMB Substrate** to each well.
b. Incubate the plate at room temperature for 30-60 minutes (incubation time should be monitored by the investigator according to color development).
c. Add 20 µL of the **Acid Stop Solution** to each well in the same order that the **TMB Substrate** 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.

EXAMPLE OF PKC ACTIVITY ASSAY

The graph below illustrates results using purified recombinant active PKC included with this kit. Varying quantities of purified active PKC were added the PKC Substrate Mictotiter Plate and incubated for 60 minutes at 30°C. Activity was detected as described in the Assay Procedure. The data represented below is an example only and should not be used to calculate actual assay results.



Calculations

Calculating Kinase Activity in Column Fractions

$$\text{Relative kinase activity in fractionated sample} = \frac{(\text{Average Absorbance}_{(\text{sample})} - \text{Average Absorbance}_{(\text{blank})})}{\text{Volume used in assay}} \times \text{Dilution factor}$$

Calculating Kinase Activity in Cell Lysates

$$\text{Relative kinase activity in the cell lysate} = \frac{(\text{Average Absorbance}_{(\text{sample})} - \text{Average Absorbance}_{(\text{blank})})}{\text{Quantity of crude protein used per assay}}$$

Calculating Kinase Activity of Purified Kinase

$$\text{Relative kinase activity} = \frac{(\text{Average Absorbance}_{(\text{sample})} - \text{Average Absorbance}_{(\text{blank})})}{\text{Quantity of purified kinase used per assay}}$$

PRECISION

Intra-Assay Precision (Within Run Precision)
The Intra-Assay Coefficient of variation of the PKC Kinase Assay was determined to be <10%.

Inter-Assay Precision (Between Run Precision)
The Inter-Assay Coefficient of variation of the PKC Kinase Assay was determined to be <10%.

SPECIFICITY

The PKC Kinase Assay is specific for all isoforms PKC (PKCα, β, γ, δ, ε, μ, θ, ζ).

LIMITATIONS OF THE ASSAY

- Amendments to the protocol can compromise the performance of this assay.
- The components in each kit lot number have been quality assured and warranted in this specific combination only; please do not mix them with components from other kit lot numbers.

REFERENCES

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F. APPENDICES

APPENDIX I
Sample Preparation of Cell Lysates

Adherent Cells

1. Treat cells according to desired protocol (i.e. agonist/inhibitor). Note: Desired confluence of plate is determined by individual researcher. *Recommended: 90% confluency/100 mm dish.*
2. Remove media from plate using suction filtration.
3. Wash plate 1X with ice-cold PBS (pH 7.4).
4. Add 1 mL of lysis buffer [20mM MOPS, 50 mM β-glycerolphosphate, 50 mM sodium fluoride, 1 mM sodium orthovanadate, 5mM EGTA, 2 mM EDTA, 1% NP40, 1 mM dithiothreitol (DTT), 1 mM benzamidine, 1 mM phenylmethanesulphonylfluoride (PMSF) and 10 µg/mL leupeptin and aprotinin] to 100 mm tissue culture plate and allow to stand for 10 min on ice. *Note: Add PMSF, Leupeptin and Aprotinin to tissue culture plate prior to preparation of lysate.*
5. After 10 min incubation period, scrape cells using a rubber policeman/cell scraper and collect cell lysate in a pre-chilled 1.5 mL microcentrifuge tube. Keep on ice. *Optional: Sonicate lysate, 3 x 20 sec intervals.*
6. Centrifuge at 13,000 rpm for 15 min.
7. Transfer clear supernatant to a pre-chilled 1.5 mL microcentrifuge tube. This is the cytosolic fraction. *Note: samples may be stored at –70℃. For optimal results, it is recommended that fresh lysates be used for each experiment as the kinase activity decreases with each subsequent freeze thaw.*
8. Determine protein concentration using BCA method.

Suspension Cells

1. Treat cells according to desired protocol (i.e. agonist/inhibitor).
2. Transfer cells to 15 mL conical tube.
3. Spin cells at 1200 rpm for 5-10 min to pellet. *Optional: Wash cells with 5 mL of 1X with ice-cold PBS (pH 7.4).*
4. Add 1 mL of lysis buffer [20 mM MOPS, 50 mM β-glycerolphosphate, 50 Mm sodium fluoride, 1 mM sodium orthovanadate, 5mM EGTA, 2 mM EDTA, 1% NP40, 1 mM dithiothreitol (DTT), 1 mM benzamidine, 1 mM phenylmethanesulphonylfluoride (PMSF) and 10 µg/mL leupeptin and aprotinin] to 100 mm tissue culture plate and allow to stand for 10 min on ice. *Note: Add PMSF, Leupeptin and Aprotinin to tissue culture plate prior to preparation of lysate. Optional: Sonicate lysate, 3 x 20 sec intervals.*
5. Centrifuge at 13,000 rpm for 15 min.
6. Transfer clear supernatant to a pre-chilled 1.5 mL microcentrifuge tube and store at –70°C. This is the cytosolic fraction. *Note: samples may be stored at –70℃. For optimal results, it is recommended that fresh lysates be used for each experiment as the kinase activity decreases with each subsequent freeze thaw.*
7. Determine protein concentration using BCA method.

APPENDIX II
Sample Preparation of Tissue Extracts

Protocol #1

1. Weigh ~ 1g of tissue, place in a petri dish on ice and slice tissue into tiny pieces.
2. Add 5 mL of lysis buffer [20 mM MOPS, 50mM β-glycerolphosphate, 50 mM sodium fluoride, 1mM sodiumorthovanadate, 5 mM EGTA, 2mM EDTA, 1% NP40, 1 mM dithiothreitol (DTT), 1 mM benzamidine, 1mM phenylmethane- sulphonylfluoride (PMSF) and 10 µg/mL leupeptin and aprotinin].
   Note: Add PMSF, Leupeptin and Aprotinin prior to preparation of lysate.
3. Transfer sample in lysis buffer to a pre-chilled 15 mL conical tube and process the tissue using a polytron at setting of 10, 000 rpm (3 x 20 sec bursts).
4. Allow to stand on ice for 10 minutes
5. Centrifuge at 150,000 g for 30minutes at 4°C.
6. Transfer clear supernatant to pre-chilled 1.5 mL microcentrifuge tube. This is the cytosolic fraction.
   Note: samples may be stored at –70℃. For optimal results, it is recommended that fresh lysates be used for each experiment as the kinase activity decreases with each subsequent freeze thaw.
7. Determine protein concentration using BCA method.

Protocol #2

1. Slice tissue into thin sections using a cryostat (3-5 micron sections).
2. Place sections into a pre-chilled microcentrifuge tube containing 1mL of lysis buffer [20 mM MOPS, 50mM β-glycerolphosphate, 50 mM sodium fluoride, 1 mM sodiumorthovanadate, 5 mM EGTA, 2 mM EDTA, 1% NP40, 1 mM dithiothreitol (DTT), 1mM benzamidine, 1mM phenylmethanesulphonylfluoride (PMSF) and 10 µg/mL leupeptin and aprotinin].
   Note: Add PMSF, Leupeptin and Aprotinin prior to preparation of lysate.
3. Using a handheld homogenizer, perform 3 x 20 sec bursts.
4. Allow to stand on ice for 10 minutes.
5. Sonicate lysate 3 x 20 sec intervals.
6. Allow to stand on ice for 10 minutes.
7. Centrifuge at 13,000 rpm for 15 minutes.
8. Transfer clear supernatant to a pre-chilled 1.5 mL microcentrifuge tube and store at –70°C. This is the cytosolic fraction. Transfer clear supernatant to a pre-chilled 1.5 mL microcentrifuge tube. This is the cytosolic fraction.
   Note: samples may be stored at –70℃. For optimal results, it is recommended that fresh lysates be used for each experiment as the kinase activity decreases with each subsequent freeze thaw.
9. Determine protein concentration using BCA method. *Note: There are several acceptable methods for preparing tissue lysates that have been published in the literature. The preceding protocols are provided as examples of suitable methods.*

APPENDIX III

Sample Mono Q Anion Exchange Protocol

1. Prepare cell/tissue extracts according to desired protocol.

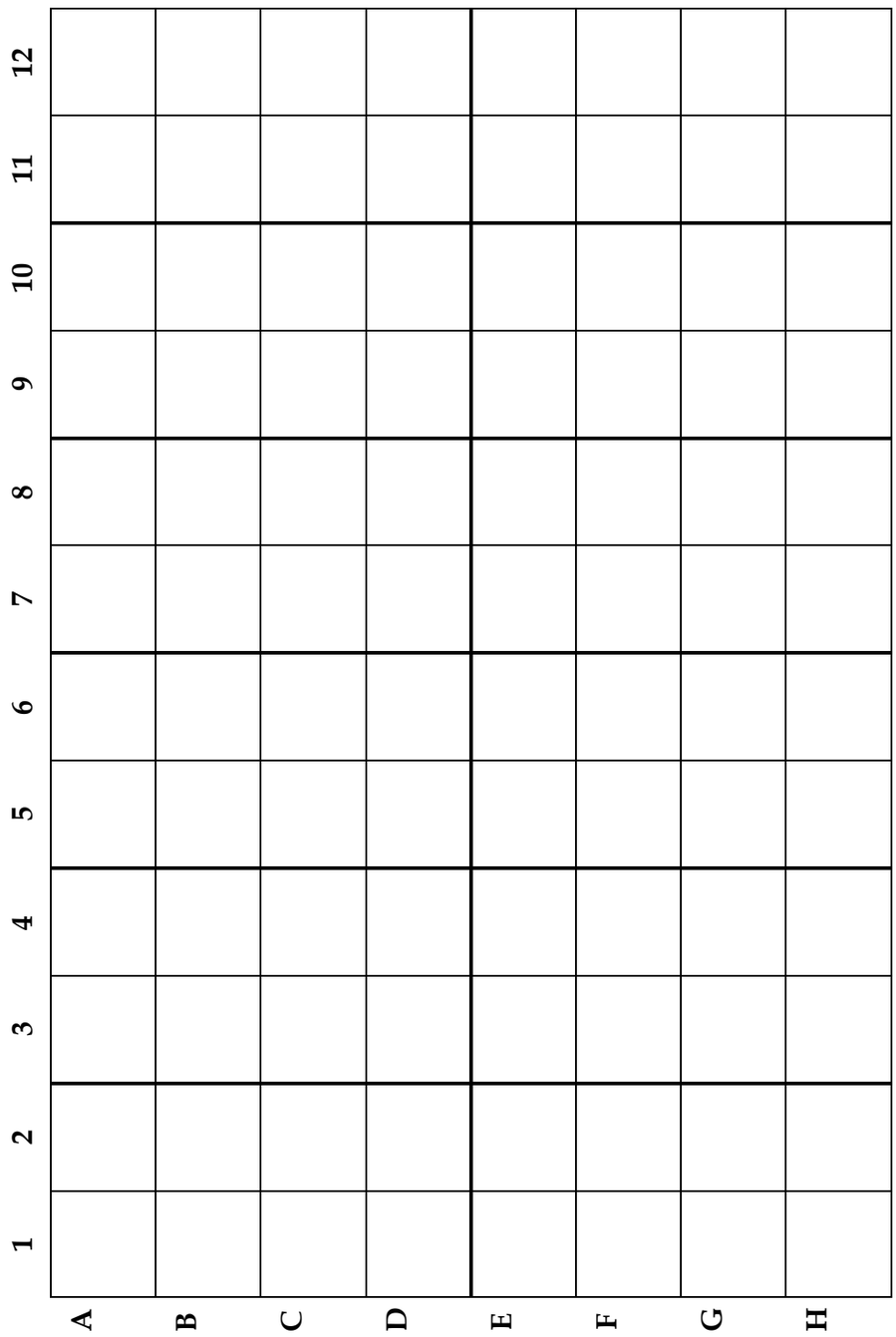
2. Equilibrate Mono Q anion exchange column (1 mL column) with Buffer A (containing 10 mM MOPS, pH 7.2, 25 mM β-glycerolphosphate, 5 mM EGTA, 2 mM EDTA, 2mM sodium orthovanadate and 2 mM DTT).

3. Load 1-2 mg of protein onto Mono Q anion-exchange column and run at a flow-rate of 0.5 mL/min using a 12 mL linear NaCl gradient (0 – 0.8 M NaCl).

4. Collect between 0.25 – 0.5 mL fractions.

5. Assay fractions as outlined in manual.

APPENDIX IV – PKC Substrate Microtiter Plate Template



REFERENCE

1. Bring to room temperature: **PKC Substrate Microtiter Plate**, **Antibody Dilution Buffer, Kinase Assay Dilution Buffer**, **TMB Substrate** and **Acid Stop Solution.**
2. Soak wells of the **PKC Substrate Microtiter Plate** with 50 µL **Kinase Assay Dilution Buffer** at room temperature for 10 minutes. Carefully aspirate liquid from each well.
3. Add samples to appropriate wells of the **PKC Substrate Microtiter Plate.**
4. Initiate reaction by adding 10 µL of diluted ATP to each well.
5. Incubate for up to 90 minutes at 30°C.
6. Stop reaction by emptying contents of each well.
7. Add 40 µL of **Phosphospecific Substrate Antibody** to each well.
8. Incubate at room temperature for 60 minutes.
9. Wash wells 4 times with 100 µL 1X Wash Buffer.
10. Add 40 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 100 µL 1X Wash Buffer.
13. Add 60 µL of **TMB Substrate** to each well.
14. Incubate at room temperature for 30-60 minutes. Incubation time should be determined by the investigator according to color development.
15. Add 20 µL of **Acid Stop Solution** to each well.
16. Measure absorbance at 450 nm.

USE FOR RESEARCH PURPOSES ONLY

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Revision: 11/09/16