Akt Kinase activity kit
(Non-Radioactive)

For the screening of inhibitors or activators of Akt/PKB and for quantitating the activity of Akt/PKB in purified or partially purified enzyme preparations.

Catalog Number: ADI-EKS-400A

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

TABLE OF CONTENTS

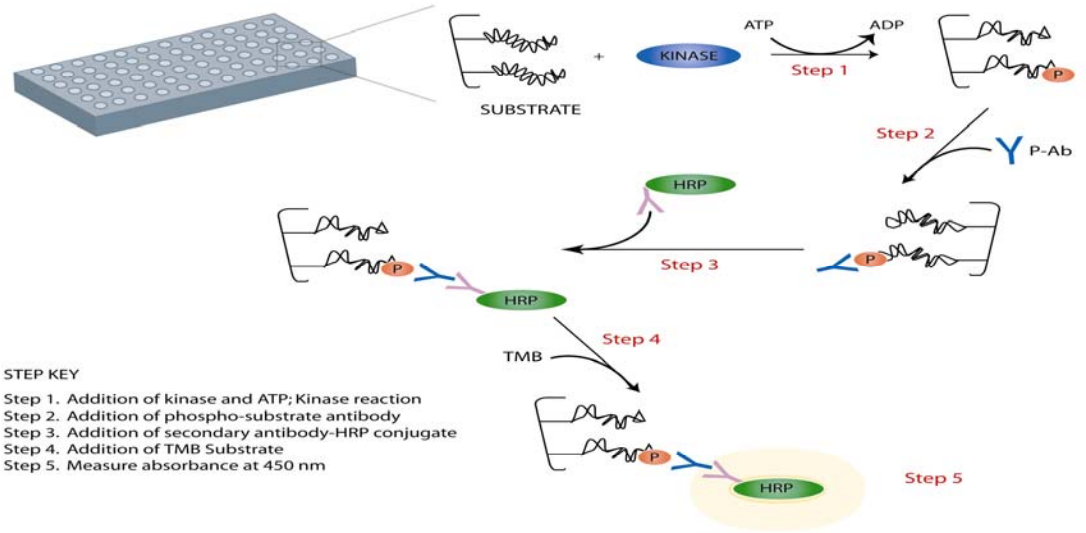
A. INTRODUCTION

ASSAY DESIGN

The Akt Kinase activity kit provides a safe, rapid and reliable method for screening of inhibitors or activators of Akt/PKB and for quantitating the activity of Akt/PKB in purified or partially purified enzyme preparations.

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

In the assay, the substrate, which is readily phosphorylated by PKB, is pre-coated on the wells of the provided PKB 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 PKB phosphotransferase activity. The color development is stopped with acid stop solution and the intensity of the color is measured in a microplate reader at 450nm.



SCIENTIFIC OVERVIEW

Protein Kinase B (PKB) also known as Akt is a 57kDa serine/threonine kinase belonging to a subfamily termed the AGC protein kinases that include PKB isoforms, the cyclic-AMP-dependent PKA, SGK and p90RSK. There are three widely expressed isoforms of PKB (PKBα, PKBβ and PKBγ, also known as Akt1, Akt2 and Akt3, respectively) that mediate many of the downstream events controlled by PI3-kinase. Each isoform is composed of an N-terminal PI(3,4,5)P_3- and PI(3,4)P_2-binding Pleckstrin Homology (PH) domain and a C-terminal kinase catalytic domain.

Activation of Akt/PKB involves a complex series of events. First, PI3-kinase-generated lipid products, PI(3,4,5)P_3 and PI(3,4,)P_2, recruit PKB to the plasma membrane through their affinity for the PH domain of PKB. At the plasma membrane, PKB is thought to undergo a conformational change and becomes activated by the phosphorylation of two residues, Thr-308 within the P-loop of the protein kinase domain and Ser-473 (1-6).

PKB has been the subject of intense study due to its role in transducing signals from PI3-kinase that regulates cell survival and intermediary metabolism. PKB plays a key role in cancer progression by stimulating cell proliferation and inhibiting apoptosis and is suggested to be a key mediator of insulin signaling. These findings indicate that PKB is likely to be a hot drug target for the treatment of cancer, diabetes and stroke (7).

ASSAY PROCEDURE SUMMARY

1. Bring to room temperature: **PKB Substrate Microtiter Plate**, **Antibody Dilution Buffer, Kinase Assay Dilution Buffer**, **TMB Substrate** and **Stop Solution 2**.
2. Soak wells of the **PKB Substrate Microtiter Plate** with 50µL **Kinase Assay Dilution Buffer** at room temperature for 10 minutes. Carefully aspirate liquid from each well.
3. Add samples to appropriate wells of the **PKB Substrate Microtiter Plate.**
4. Initiate reaction by adding 10µL of diluted ATP to each well, except the blank.
5. Incubate for up to 90 minutes at 30°C.
6. Stop reaction by emptying contents of each well.
7. Add 40µL of **Phosphospecific Substrate Antibody** to each well, except the blank.
8. Incubate at room temperature for 60 minutes.
9. Wash wells 4 times with 100µL 1X Wash Buffer.
10. Add 40µL of diluted **Anti-Rabbit IgG: HRP Conjugate** to each well, except the blank.
11. Incubate at room temperature for 30 minutes.
12. Wash wells 4 times with 100µL 1X Wash Buffer.
13. Add 60µL 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 20µL of **Stop Solution 2** to each well.
16. Measure absorbance at 450 nm.

B. MATERIALS

PRECAUTIONS

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

- The activity of the **Anti-Rabbit IgG: HRP Conjugate** *(part# 80-1534)* is affected by nucleophiles such as azide, cyanide and hydroxylamine.

Please read the complete kit insert before performing this assay.

MATERIALS PROVIDED

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

PART #	COMPONENT	SIZE	DESCRIPTION
80-1532	PKB Substrate Microtiter Plate	96-well plate	12x8 removable strips and frame; pre-coated plate with substrate peptide for PKB
80-1533	Phosphospecific Substrate Antibody	5mL	1µg/mL solution of rabbit polyclonal antibody specific for phosphorylated PKB substrate
80-1534	Anti-Rabbit IgG: HRP Conjugate	20µL	1mg/mL solution of horseradish peroxidase conjugated goat anti-rabbit IgG containing 0.01% thimerosal as a preservative
80-1535	Antibody Dilution Buffer	10mL	Buffer for the dilution of Anti-Rabbit IgG: HRP Conjugate
80-1536	Kinase Assay Dilution Buffer	10mL	Buffer for the dilution of ATP, standards and samples
80-1537	ATP	2mg	Adenosine triphosphate
80-1517	Active PKB	28µL	Purified recombinant active Protein Kinase B (Akt)
80-1286	20X Wash Buffer	30mL	Concentrated solution of buffer and surfactant
80-0350	TMB Substrate	10mL	Stabilized tetramethylbenzidine substrate
80-0377	Stop Solution 2	10mL	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 PKB**, which must be stored at –70°C.
- If assaying on separate occasions, once thawed, the **Active PKB** may be aliquotted 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 **PKB 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 1µL and 1,000µL
- 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 450nm
- Adhesive plate sealers

C. PERFORMING THE ASSAY

CRITICAL ASSAY PARAMETERS AND NOTES

- The Akt Kinase activity kit contains a pre-coated microtiter plate (**PKB 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: **PKB Substrate Microtiter Plate**, **Antibody Dilution Buffer, Kinase Assay Dilution Buffer, 20X Wash Buffer, TMB Substrate** and **Stop Solution 2**.

- 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 pipettors 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 and carefully remove liquid from the wells.

- 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 Step#1. 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 7).*

1. TEMPERATURE OF REAGENTS

Bring the following reagents to room temperature prior to use:

- **PKB Substrate Microtiter Plate** *(Part#: 80-1532)*
- **Antibody Dilution Buffer** *(Part#: 80-1535)*
- **Kinase Assay Dilution Buffer** *(Part#: 80-1536)*
- **20X Wash Buffer** *(Part#: 80-1286)*
- **TMB Substrate** *(Part#: 80-0350)*
- **Stop Solution 2** *(Part#: 80-0377)*

2. PREPARATION OF PURIFIED ACTIVE PKB

(*Part#: 80-1517)*

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

NOTE: *For the concentration of the purified kinase preparation included in the kit, please refer to the lot specific technical data sheet provided.*

a. Dilute **Active PKB** into **Kinase Assay Dilution Buffer** to a final volume of 30µL *(refer to data presented on page 17 as an example only).* Keep on ice.

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

3. ATP (*Part#: 80-1537)*

a. Centrifuge the vial before removing the cap to ensure maximum product recovery.
b. Reconstitute the **ATP** with 2mL of **Kinase Assay Dilution Buffer**.
c. Mix gently by inversion.
d. Reagent is now ready to be used in the Assay Procedure *(see page 14).*
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#: 80-1534)*

a. Centrifuge the vial before removing the cap to ensure maximum product recovery.
b. Dilute the **Anti-Rabbit IgG: HRP Conjugate** to 1µg/mL (1:1000) with **Antibody Dilution Buffer** in a polypropylene tube. A minimum of 4mL of working solution is required for 96-wells (40µL/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 16).*
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 30mL of 20X Wash Buffer with 570mL 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

NOTE: *Crude sample preparations have not been used directly with this assay. It is suggested that purified or partially purified kinase preparations be used.*

1. PREPARATION OF PARTIALLY PURIFIED FRACTIONS

a. Prepare cell lysates or tissue extracts according to desired protocol. Sample protocols may be found in Appendices I-II (page 20).
b. Evaluate total protein concentration.
c. Select desired column and buffers for purification. A sample protocol may be found in Appendix III (page 22) (refer to reference 8 if more detail of purification is required).
d. Load sample and run desired purification protocol.
e. If necessary, dilute fractionated sample accordingly in **Kinase Assay Dilution Buffer** *(please see page 9 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 14)*.

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 9.*

ASSAY PROCEDURE

1. PREPARATION OF PKB 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 **PKB Substrate Microtiter Plate** with 50µL 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 30µL of each of the following to appropriate wells:
      - Purified **Active PKB** *(previously prepared, see page 10)*
      - Samples *(previously prepared, see page 12)*
      - 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 10µL of diluted ATP *(previously diluted, see page 11)* to each well, except the blank. 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 during as outlined in the Recommendations Prior To Using the Assay section on page 9.*

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 40µL of the **Phosphospecific Substrate Antibody** to each well, except the blank.
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 100µL 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 4^{th} 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 11)

a. Add 40µL of the previously diluted **Anti-Rabbit IgG:HRP Conjugate** to each well, except the blank.
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 STOP SOLUTION 2

a. Add 60µL 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 20µL of the **Stop Solution 2** to each well in the same order that the **TMB Substrate** was added.

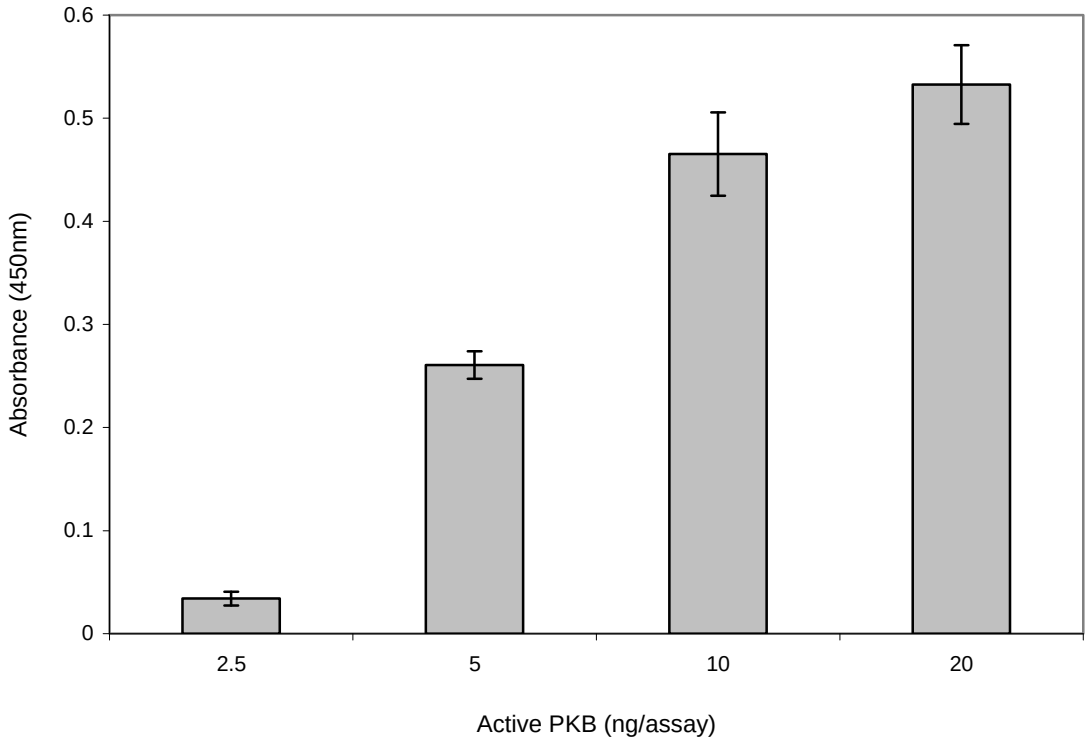
7. MEASURING ABSORBANCE

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

D. ASSAY PERFORMANCE CHARACTERISTICS

EXAMPLE OF PKB ACTIVITY ASSAY

The graph below illustrates results using purified recombinant active PKB included with this kit. Varying quantities of purified active PKB were added the PKB 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 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 PKB (Akt) Kinase Activity Assay was determined to be <10%.

Inter-Assay Precision (Between Run Precision)
The Inter-Assay Coefficient of variation of the PKB (Akt) Kinase Activity Assay was determined to be ≤12%.

SPECIFICITY

The Akt Kinase activity kit is specific for Akt 1,2,3/ PKB α, β, γ.

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.

E. REFERENCES

REFERENCES

1. Leevers, S.J., Vanhaesebroeck, B. and Waterfield, M.D. (1999) *Curr. Opin. Cell. Biol.* **11**: 219-225.
2. Rameh, L.E. and Cantley, L.C. (1999) *J. Biol. Chem.* **274**: 8347-8350.
3. Coffer, P.J., Jin, J. and Woodgett, J.R. (1998) *Biochem. J.* **335**: 1-13.
4. Vanhaesebroeck, B. and Alessi, D.R. (2000) *Biochem. J.* **346**: 561-576.
5. Scheid, M.P., Marignani, P.A. and Woodgett, J.R. (2002) *Mol. Cell. Biol.* **22**: 6247-6260.
6. Lawlor, M.A. and Alessi, D.R., (2001) *J. Cell Sciences* **114**: 2903-2910.
7. Hill, M.H. and Hemmings, B.A. (2002) *Pharmacol. Therap.* **93**: 243-251.
8. Bhanot, S., Salh, B.S., Verma, S., McNeill, J.H., Pelech, S.L. (1999) *Am. J. Physiol.* **277**: E299-E307.

F. APPENDICES

APPENDIX I

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 (1M, pH 7.4).
4. Add 1mL of lysis buffer, pH 7.2 [20mM MOPS, 50mM β-glycerolphosphate, 50mM sodium fluoride, 1mM sodium vanadate, 5mM EGTA, 2mM EDTA, 1% NP40, 1mM dithiothreitol (DTT), 1mM benzamidine, 1mM phenylmethanesulphonylfluoride (PMSF) and 10 µg/mL leupeptin and aprotinin] to 100mm 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 1.5mL 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 1.5mL microcentrifuge tube. This is the cytosolic fraction. *Note: samples may be stored at –70°C. 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 Lowry, Bradford, or BCA methods.

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 5mL of 1X with ice-cold PBS (1M, pH 7.4).*
4. Add 1 mL of lysis buffer, pH 7.2 [20mM MOPS, 50 mM β-glycerolphosphate, 50Mm sodium fluoride, 1mM sodium vanadate, 5mM EGTA, 2mM EDTA, 1% NP40, 1mM dithiothreitol (DTT), 1mM benzamidine, 1mM 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 1.5mL microcentrifuge tube and store at –70°C. This is the cytosolic fraction. *Note: samples may be stored at –70°C. 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 Lowry, Bradford, or BCA methods.

APPENDIX II
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 5mL of lysis buffer, pH 7.2 [20mM MOPS, 50mM β-glycerolphosphate, 50mM sodium fluoride, 1mM sodium vanadate, 5mM EGTA, 2mM EDTA, 1% NP40, 1mM dithiothreitol (DTT), 1mM benzamidine, 1mM phenylmethane- sulphonylfluoride (PMSF) and 10µg/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 15mL 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 1.5mL 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 Lowry, Bradford, or BCA methods.

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, pH 7.2 [20mM MOPS, 50mM β-glycerolphosphate, 50mM sodium fluoride, 1mM sodium vanadate, 5mM EGTA, 2mM EDTA, 1% NP40, 1mM 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 1.5mL microcentrifuge tube and store at –70°C. This is the cytosolic fraction. Transfer clear supernatant to a pre-chilled 1.5mL 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 Lowry or Bradford, or BCA methods. *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.

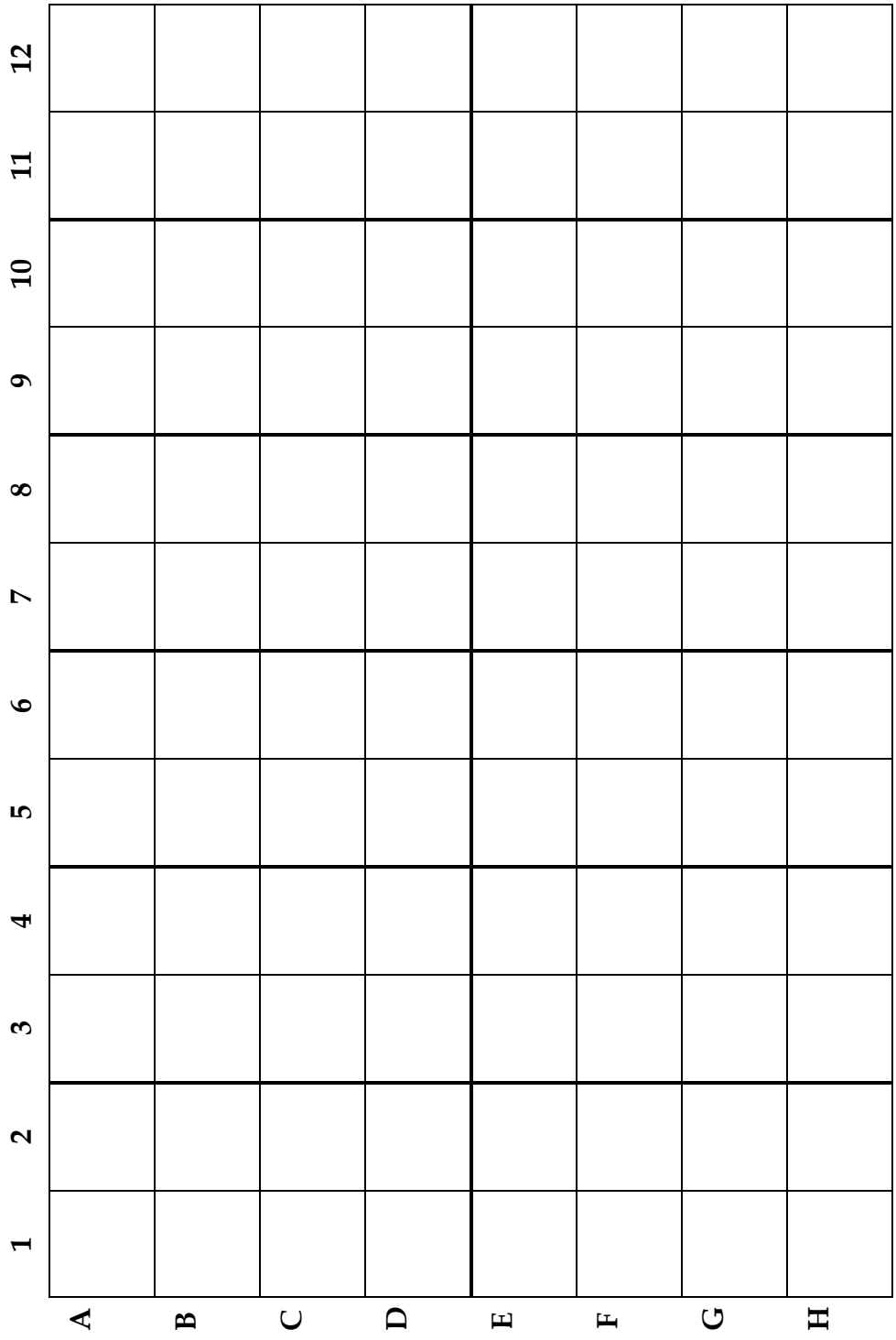
2. Equilibrate Mono Q anion exchange column (1 mL column) with Buffer A (containing 10mM MOPS, pH 7.2, 25mM β-glycerolphosphate, 5mM EGTA, 2 mM EDTA, 2mM sodium orthovanadate and 2mM DTT).

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

4. Collect between 0.25 – 0.5mL fractions.

5. Assay fractions as outlined in manual.

APPENDIX III – PKB Substrate Microtiter Plate Template



Notes

REFERENCE

1. Bring to room temperature: **PKB Substrate Microtiter Plate**, **Antibody Dilution Buffer, Kinase Assay Dilution Buffer**, **TMB Substrate** and **Acid Stop Solution 2**.
2. Soak wells of the **PKB Substrate Microtiter Plate** with 50µL **Kinase Assay Dilution Buffer** at room temperature for 10 minutes. Carefully aspirate liquid from each well.
3. Add samples to appropriate wells of the **PKB Substrate Microtiter Plate.**
4. Initiate reaction by adding 10µL of diluted ATP to each well, except the blank.
5. Incubate for up to 90 minutes at 30°C.
6. Stop reaction by emptying contents of each well.
7. Add 40µL of **Phosphospecific Substrate Antibody** to each well, except the blank.
8. Incubate at room temperature for 60 minutes.
9. Wash wells 4 times with 100µL 1X Wash Buffer.
10. Add 40µL of diluted **Anti-Rabbit IgG: HRP Conjugate** to each well, except the blank.
11. Incubate at room temperature for 30 minutes.
12. Wash wells 4 times with 100µL 1X Wash Buffer.
13. Add 60µL 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 20µL of **Stop Solution 2** to each well.
16. Measure absorbance at 450 nm.

Use of Product

This product contains research chemicals. As such, they should be used and handled only by or under the supervision of technically qualified individuals. This product is not intended for diagnostic or human use.

Warranty

Enzo Life Sciences International, Inc. makes no warranty of any kind, expressed or implied, which extends beyond the description of the product in this brochure, except that the material will meet our specifications at the time of delivery. Enzo Life Sciences International, Inc. makes no guarantee of results and assumes no liability for injuries, damages or penalties resulting from product use, since the conditions of handling and use are beyond our control.

www.enzolifesciences.com

Enabling Discovery in Life Science®

Global Headquarters
Enzo Life Sciences Inc.
10 Executive Blvd
Farmingdale, NY 11735
(p) 1-800-942-0430
(f) 1-631-694-7501
(e) info-usa@enzolifesciences.com

Enzo Life Sciences (ELS) AG
Industriestrasse 17, Postfach
CH-4415 Lause / Switzerland
(p) +41/0 61 926 89 89
(f) +41/0 61 926 89 79
(e) info-ch@enzolifesciences.com

Please visit our website at www.enzolifesciences.com for additional contact information.

01/31/2015