

PKA kinase activity kit

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

Catalog Number: ADI-EKS-390A

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

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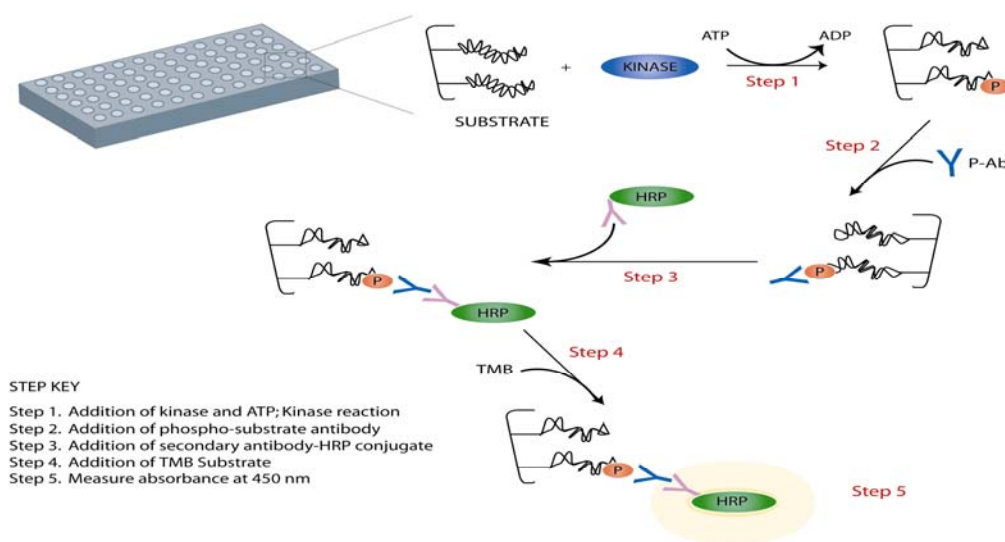
A. INTRODUCTION

ENZO LIFE SCIENCES

The non-radioactive PKA Kinase Activity Assay provides a safe, rapid and reliable method for screening of inhibitors or activators of PKA and for quantitating the activity of PKA in partially purified, purified or crude enzyme preparations.

The PKA 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 PKA and a polyclonal antibody that recognizes the phosphorylated form of the substrate. The assay is designed for the analysis of PKA activity in the solution phase.

In the assay, the substrate, which is readily phosphorylated by PKA, is pre-coated on the wells of the provided PKA 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 PKA 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 A (PKA) is the primary mediator of cAMP action and a key regulatory enzyme responsible for many key cellular processes such as cell growth and metabolism, DNA replication, cell division and actin cytoskeleton rearrangements by catalyzing phosphorylation in response to hormonal stimuli. The PKA holoenzyme is a heterotetramer composed of a regulatory (R) subunit dimer and two monomeric catalytic (C) subunits. Four genes have been identified that encode the regulatory subunits RI α , RI β , RII α and RII β . The RI α subunit has a ubiquitous tissue distribution while RI β is localized mainly in brain, testes as well as B and T lymphocytes. RII α is also ubiquitous in its distribution whereas RII β is found mainly in brain, adipose and some endocrine tissues. There are at least two PKA isoforms, type I and type II. PKA type I is found predominantly in the cytoplasm and PKA type II is associated mainly with membranes, subcellular organelles and the cytoskeleton.

cAMP-dependent PKA is the principle intracellular target for cAMP in mammalian cells. The binding of cAMP to tandem sites in each R subunit causes dissociation of the holoenzyme and alleviates an autoinhibitory contact that releases the active C subunit. The active kinase is then free to phosphorylate substrates on serine or threonine residues that are presented in a sequence context of Arg-Arg-Xaa-Ser/Thr or Lys-Arg-Xaa-Xaa-Ser/Thr. The entrance of the active C subunit into the cell nucleus and subsequent phosphorylation of transcriptions factors such as CREB, CREM, NF- κ B and nuclear receptors form the basis of PKA regulation of transcriptional activation. Several regulatory mechanisms are in place to ensure that cAMP levels and kinase activity are tightly controlled. Cellular adenylyl cyclase and phosphodiesterase activities determine total cAMP levels and additional factors besides access to cAMP are responsible for returning the kinase to the inactive state. PKA has become one of the important drug targets because of its key role in cellular functions (1-4).

INTRODUCTION

ASSAY PROCEDURE SUMMARY

1. Bring to room temperature: **PKA Substrate Microtiter Plate, Antibody Dilution Buffer, Kinase Assay Dilution Buffer, TMB Substrate** and **Stop Solution 2**.
2. Soak wells of the **PKA Substrate Microtiter Plate** with 50 μ L **Kinase Assay Dilution Buffer** at room temperature for 10 minutes. Carefully aspirate liquid from each well.
3. Add samples to appropriate wells of the **PKA Substrate Microtiter Plate**.
4. Initiate reaction by adding 10 μ L 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 40 μ L of **Phosphospecific Substrate Antibody** to each well, except the blank.
8. Incubate at room temperature for 60 minutes.
9. Wash wells 4 times with 100 μ L 1X Wash Buffer.
10. Add 40 μ L 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 100 μ L 1X Wash Buffer.
13. Add 60 μ L 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 μ L of **Stop Solution 2** to each well.
16. Measure absorbance at 450 nm.

B. MATERIALS

PRECAUTIONS

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- The activity of the **Anti-Rabbit IgG: HRP Conjugate** (*part# 80-1553*) is affected by nucleophiles such as azide, cyanide and hydroxylamine.

Please read the complete kit insert before performing this assay.

MATERIALS

MATERIALS PROVIDED

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

| PART # | COMPONENT | SIZE | DESCRIPTION |
|---------|------------------------------------|---------------|---|
| 80-1550 | PKA Substrate Microtiter Plate | 96-well plate | 12x8 removable strips and frame; pre-coated plate with substrate peptide for PKA |
| 80-1554 | Phosphospecific Substrate Antibody | 5mL | 1 μ g/mL solution of rabbit polyclonal antibody specific for phosphorylated PKA substrate |
| 80-1553 | Anti-Rabbit IgG: HRP Conjugate | 20 μ L | 1mg/mL solution of horseradish peroxidase conjugated goat anti-rabbit IgG containing 0.01% thimerosal as a preservative |
| 80-1552 | Antibody Dilution Buffer | 10mL | Buffer for the dilution of Anti-Rabbit IgG: HRP Conjugate |
| 80-1551 | Kinase Assay Dilution Buffer | 10mL | Buffer for the dilution of ATP, standards and samples |
| 80-1549 | ATP | 2mg | Adenosine triphosphate |
| 80-1518 | Active PKA | 30 μ L | Purified active Protein Kinase A |
| 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 |

MATERIALS

STORAGE OF MATERIALS

- All reagents are stable as supplied at 4°C until the kit's expiry date, except the purified **Active PKA**, which must be stored at –70°C.
- If assaying on separate occasions, once thawed, the purified **Active PKA** 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 **PKA 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
- A disposable beaker for diluting reagents
- 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 PKA Kinase Activity Assay Kit contains a pre-coated microtiter plate (**PKA 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: **PKA 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.

PERFORMING THE ASSAY

- 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.

PERFORMING THE ASSAY

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:

- **PKA Substrate Microtiter Plate** (Part#: 80-1550)
- **Antibody Dilution Buffer** (Part#: 80-1552)
- **Kinase Assay Dilution Buffer** (Part#: 80-1551)
- **20X Wash Buffer** (Part#: 80-1286)
- **TMB Substrate** (Part#: 80-0350)
- **Stop Solution 2** (Part#: 80-0377)

2. PREPARATION OF PURIFIED ACTIVE PKA CONTROL (Part#: 80-1518)

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

- a. The **Active PKA** (part# 80-1518) 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 μ L. Please refer to the vial for the concentration (ng/ μ L) of the purified kinase preparation. Keep preparations on ice.
- b. 30 μ L of **Kinase Assay Dilution Buffer** (without kinase) can be used as the assay blank.

PERFORMING THE ASSAY

3. **ATP** (*Part#: 80-1549*)
 - 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#: 80-1553*)
 - 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**.

PERFORMING THE ASSAY

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

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 (page 20).
- b. Evaluate total protein concentration.

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

PERFORMING THE ASSAY

Partially Purified Sample Preparation

- a. Select desired column and buffers for purification. A sample protocol may be found in Appendix III (page 22) (refer to references 5-6 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 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 15*).

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.*

PERFORMING THE ASSAY

ASSAY PROCEDURE

1. PREPARATION OF PKA 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 **PKA Substrate Microtiter Plate** with 50µL of **Kinase Assay Dilution Buffer** at room temperature for 10 minutes.

Carefully aspirate liquid from all wells.

2. ADDITION OF STANDARDS AND SAMPLES

- a. Add 30µL of each of the following to appropriate wells:
 - Purified **Active PKA** (*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.

PERFORMING THE ASSAY

- c. Cover wells with an adhesive plate sealer or plastic wrap 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 use 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 (or plastic wrap) 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 4th wash, aspirate the liquid from all wells. Invert the plate and carefully pat dry on clean paper towels.

PERFORMING THE ASSAY

5. ADDITION OF ANTI-RABBIT IgG: HRP CONJUGATE
(previously diluted, see page 11)
 - a. Add 40 μ L of the previously diluted **Anti-Rabbit IgG:HRP Conjugate** to each well, except 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, thorough shaking every 10 minutes.
 - c. Wash plate as described in Step #4.

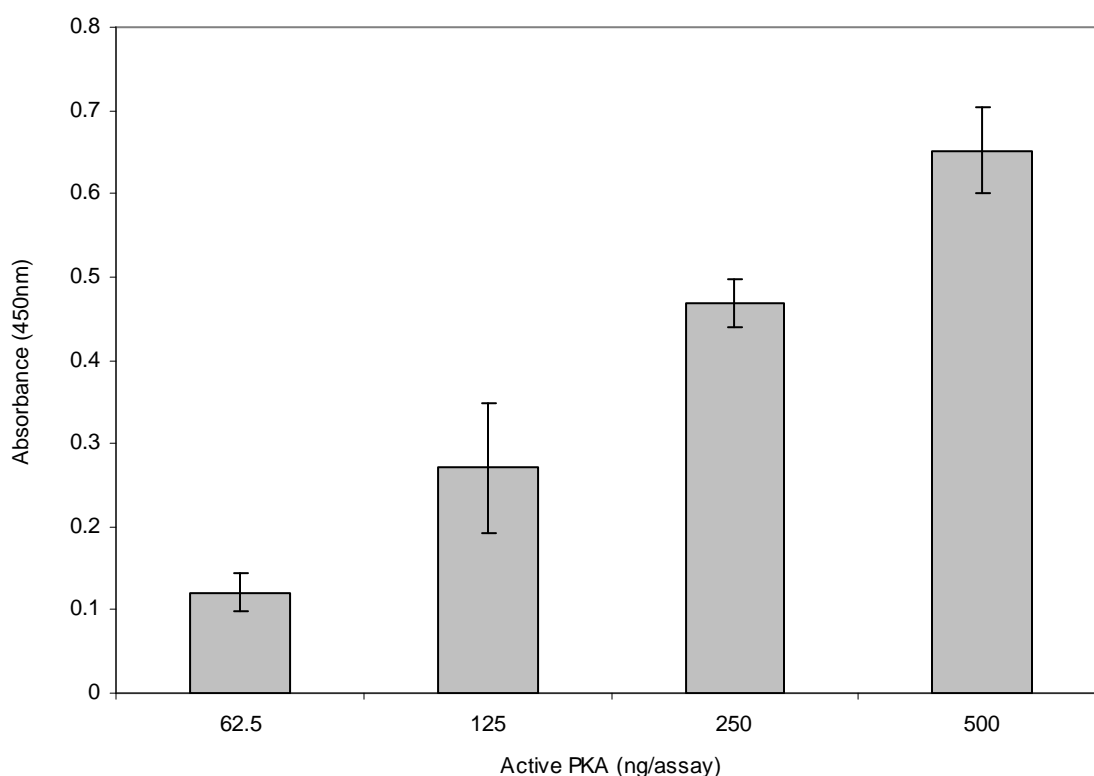
6. ADDITION OF TMB SUBSTRATE AND ACID STOP SOLUTION
 - a. Add 60 μ L of the **TMB Substrate** to each well.
 - b. Incubate the plate at room temperature for 30-60 minutes (incubation time should be monitored by the investigator according to color development).
 - c. Add 20 μ L of the **Stop Solution 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 PKA ACTIVITY ASSAY

The graph below illustrates results using purified active PKA included with this kit. Varying quantities of purified active PKA were added the PKA Substrate Microtiter 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.



ASSAY PERFORMANCE CHARACTERISTICS

Calculations

Calculating Kinase Activity in Column Fractions

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

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

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 PKA Kinase Assay was determined to be <10%.

Inter-Assay Precision (Between Run Precision)

The Inter-Assay Coefficient of variation of the PKA Kinase Assay was determined to be ≤12%.

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

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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 [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 μ g/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 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 5mL of 1X with ice-cold PBS (1M, pH 7.4).*
4. Add 1 mL of lysis buffer [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 μ g/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 1.5mL 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.*
8. Determine protein concentration using BCA method.

APPENDICES

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 [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 10 μ g/mL leupeptin and aprotinin].
3. *Note: Add PMSF, Leupeptin and Aprotinin prior to preparation of lysate.*
4. 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).
5. Allow to stand on ice for 10 minutes
6. Centrifuge at 150,000 g for 30minutes at 4°C.
7. Transfer clear supernatant to 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 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 [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 μ g/mL leupeptin and aprotinin].

Note: Add PMSF, Leupeptin and Aprotinin prior to preparation of lysate.

3. Using a hand-held 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 °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.

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.*

APPENDICES

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.

APPENDICES

APPENDIX III – PKA Substrate Microtiter Plate Template

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Notes

Notes

REFERENCE

- Bring to room temperature: **PKA Substrate Microtiter Plate**, **Antibody Dilution Buffer**, **Kinase Assay Dilution Buffer**, **TMB Substrate** and **Stop Solution 2**.
- Soak wells of the **PKA Substrate Microtiter Plate** with 50µL **Kinase Assay Dilution Buffer** at room temperature for 10 minutes. Carefully aspirate liquid from each well.
- Add samples to appropriate wells of the **PKA Substrate Microtiter Plate**.
- Initiate reaction by adding 10µL of diluted ATP to each well, except the blank.
- Incubate for up to 90 minutes at 30°C.
- Stop reaction by emptying contents of each well.
- Add 40µL of **Phosphospecific Substrate Antibody** to each well, except the blank.
- Incubate at room temperature for 60 minutes.
- Wash wells 4 times with 100µL 1X Wash Buffer.
- Add 40µL of diluted **Anti-Rabbit IgG:HRP Conjugate** to each well, except the blank.
- Incubate at room temperature for 30 minutes.
- Wash wells 4 times with 100µL 1X Wash Buffer.
- Add 60µL of **TMB Substrate** to each well.
- Incubate at room temperature for 30-60 minutes. Incubation time should be determined by the investigator according to color development.
- Add 20µL of **Stop Solution 2** to each well.
- Measure absorbance at 450 nm.



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