# p70 S6K activity kit

For screening inhibitors or activators of p70 S6K
and for quantitating p70 S6K activity in
partially purified or purified enzyme preparations.

## Catalog Number: ADI-EKS-470

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

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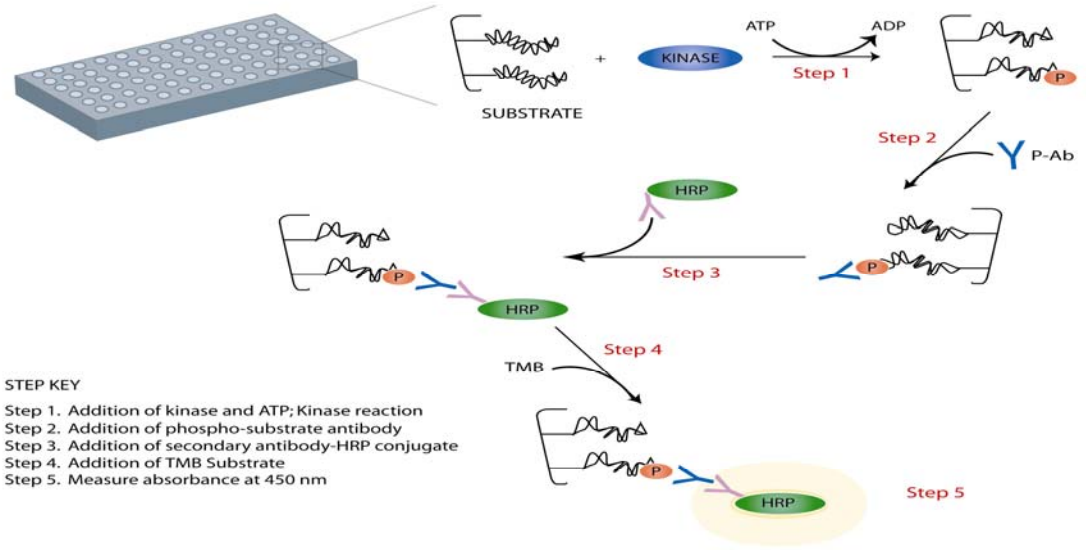
# A. INTRODUCTION

## ASSAY DESIGN

The p70 S6K activity kit provides a safe, simple and reliable method for screening inhibitors or activators of p70 S6K and for quantitating the activity of p70 S6K in partially purified or purified enzyme preparations.
The p70 S6K Activity Assay is based on a solid phase enzyme-linked immuno-absorbent assay (ELISA) that utilizes a specific synthetic peptide as a substrate for p70 S6K and a polyclonal antibody that recognizes the phosphorylated form of the substrate. The assay is designed for analysis of p70 S6K activity in the solution phase.
In the assay, the substrate, which is readily phosphorylated by p70 S6K, is pre-coated on the wells of the provided p70 S6K 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 p70 S6K 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

p70 S6K is a ubiquitously expressed protein serine/threonine kinase that has been reported to be activated by serum stimulation and inhibited by both wortmannin and rapamycin. p70 S6K activity undergoes changes in the cell cycle and increases 20-fold in G1 cells that are released from G0 (2). Throughout G1, S, and G2 the expression of p70 S6K decreases constantly; this parallels changes in enzymatic activity. Interestingly, when cells are released from a nocodazole-induced metaphase block, the kinase activity increases by approximately 10-fold. p70 S6K is an important regulator of cell proliferation. Its activation by growth factors requires phosphorylation by various inputs on multiple sites (4). p70 S6K activation requires sequential phosphorylations at proline-directed residues in the putative autoinhibitory pseudosubstrate domain, as well as threonine 389. Threonine 229, a site in the catalytic loop is phosphorylated by phosphoinositide-dependent kinase 1 (PDK-1). Activation of p70 S6K requires a phosphoinositide 3-kinase (PI3-K)-dependent signal(s).

## ASSAY PROCEDURE SUMMARY

1. Bring to room temperature: **p70 S6K Substrate Microtiter Plate**, **Antibody Dilution Buffer, Kinase Assay Dilution Buffer**, **TMB Substrate** and **Acid Stop Solution.**
2. Soak wells of the **p70 S6K 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 **p70 S6K Substrate Microtiter Plate.**
4. Initiate reaction by adding 10µL of diluted ATP to each well.
5. Incubate for up to 60 minutes at 30°C.
6. Stop reaction by emptying contents of each well.
7. Add 40µL of **Phosphospecific Substrate Antibody** to each well.
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.
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 15-30 minutes. Incubation time should be determined by the investigator according to color development.
15. Add 20µL of **Acid Stop Solution** 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# 470-P3)* is affected by nucleophiles such as azide, cyanide and hydroxylamine.

*Please read the complete kit insert before performing this assay.*

## MATERIALS PROVIDED

The p70 S6K activity kit contains the following components in sufficient quantities for 96 wells.

| PART # | COMPONENT | SIZE | DESCRIPTION |
|---|---|---|---|
| 80-1677 | p70 S6K Substrate Microtiter Plate | 96-well plate | 12x8 removable strips and frame; pre-coated plate with substrate peptide for p70 S6K |
| 80-1678 | Phosphospecific Substrate Antibody | 5mL | Solution of rabbit polyclonal antibody specific for phosphorylated p70 S6K substrate |
| 80-1679 | Anti-Rabbit IgG: HRP Conjugate | 20µL | Solution of horseradish peroxidase conjugated goat anti-rabbit IgG containing 0.01% thimerosal as a preservative |
| 80-1680 | Antibody Dilution Buffer | 10mL | Buffer for the dilution of Anti-Rabbit IgG: HRP Conjugate |
| 80-1681 | Kinase Assay Dilution Buffer | 10mL | Buffer for the dilution of ATP and samples |
| 80-1682 | ATP | 2mg | Adenosine triphosphate |
| 80-1556 | Active p70 S6K | 28µL | Purified recombinant active p70 S6K for use as a positive control |
| 80-1286 | 20X Wash Buffer | 30mL | Concentrated solution of buffer and surfactant |
| 80-0350 | TMB Substrate | 10mL | Stabilized tetramethylbenzidine substrate |
| 80-0377 | Acid Stop Solution | 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 p70 S6K,** which must be stored at –70°C.
- If assaying on separate occasions, once thawed, the purified **Active p70 S6K** 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 **p70 S6K 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

# C. PERFORMING THE ASSAY

## CRITICAL ASSAY PARAMETERS AND NOTES

- The p70 S6K activity kit contains a pre-coated microtiter plate (**p70 S6K 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: **p70 S6K 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 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 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.

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## 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:

- **p70 S6K Substrate Microtiter Plate** *(Part#: 80-1677)*
- **Antibody Dilution Buffer** *(Part#: 80-1680)*
- **Kinase Assay Dilution Buffer** *(Part#: 80-1681)*
- **20X Wash Buffer** *(Part#: 80-1286)*
- **TMB Substrate** *(Part#: 80-0350)*
- **Acid Stop Solution** *(Part#: 80-0377)*

### 2. PREPARATION OF PURIFIED ACTIVE p70 S6K (*Part#: 80-1556)*

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

a. The **Active p70 S6K** (part# 80-1556) is intended to be used as a positive control and can be serially diluted in **Kinase Assay Dilution Buffer** to a final volume of 30µL. Please refer to the lot specific technical data sheet provided for the concentration (ng/µL) of the purified kinase preparation.

b. Keep preparations on ice.

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

3. ATP (*Part#: 80-1682)*

   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 13).*
   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#: 80-1679)*

   a. Centrifuge the vial before removing the cap to ensure maximum product recovery.
   b. Dilute the **Anti-Rabbit IgG: HRP Conjugate** 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 14).*
   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. A general sample protocol may be found in Appendicies I-II (pages 18-19).
    b. Evaluate total protein concentration.
    c. Select desired column and buffers for purification. A sample protocol may be found in Appendix III (page 20).
    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 13).*

    *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 p70 S6K 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 **p70 S6K 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:
      - **Active p70 S6K** control *(previously diluted, see page 10)*
      - Samples *(previously prepared, see page12)*
      - 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. To avoid cross contamination, change pipette tips for each well.
   c. Incubate at 30°C for 60 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 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.
   b. 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 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 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.
b. 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 60µL of the **TMB Substrate** to each well.
b. Incubate at room temperature for 15-30 minutes (incubation time should be monitored by the investigator according to color development).
c. Add 20µL 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 450nm.
c. Measure the absorbance.

## 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*

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

# E. REFERENCES

## PRECISION

*Intra-Assay Precision (Within Run Precision)*
The Intra-Assay Coefficient of variation of the p70 S6K Kinase Assay was determined to be <10%.

*Inter-Assay Precision (Between Run Precision)*
The Inter-Assay Coefficient of variation of the p70 S6K Kinase Assay was determined to be <10%.

## 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


1. Ferrari S, Thomas G. (1994) *Crit Rev Biochem Mol Biol.* **29**(6): 385-413. Review.
2. Edelmann HM, Kuhne C, Petritsch C, Ballou LM**.** (1996) *J Biol Chem.* **271**(2) :963-71.
3. Pearson RB, Dennis PB, Han JW, Williamson NA, Kozma SC, Wettenhall RE, Thomas G. (1995) *EMBO J.* **14**(21):5279-87.
4. Romanelli A, Martin KA, Toker A, Blenis J. (1999) *Mol Cell Biol.* **19**(4): 2921-8.

# 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 (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 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℃. 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 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 1.5mL 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.*
8. 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 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 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°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 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 â-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°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.*

## APPENDIX III

### Sample Protocol for Partially Purified Fractions

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.

Notes

## APPENDIX IV – p70 S6K Substrate Microtiter Plate Template

| | A | B | C | D | E | F | G | H |
|---|---|---|---|---|---|---|---|---|
| 12 | | | | | | | | |
| 11 | | | | | | | | |
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| 1 | | | | | | | | |

## REFERENCE

1. Bring to room temperature: **p70 S6K Substrate Microtiter Plate**, **Antibody Dilution Buffer, Kinase Assay Dilution Buffer**, **TMB Substrate** and **Acid Stop Solution.**
2. Soak wells of the **p70 S6K 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 **p70 S6K Substrate Microtiter Plate.**
4. Initiate reaction by adding 10µL of diluted ATP to each well.
5. Incubate for up to 60 minutes at 30°C.
6. Stop reaction by emptying contents of each well.
7. Add 40µL of **Phosphospecific Substrate Antibody** to each well.
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.
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 15-30 minutes. Incubation time should be determined by the investigator according to color development.
15. Add 20µL of **Acid Stop Solution** to each well.
16. Measure absorbance at 450 nm.

## USE FOR RESEARCH PURPOSES ONLY

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