Activated MAP Kinase

INSTRUCTION MANUAL

Catalog #206110
Revision A.02

For In Vitro Use Only
206110-12

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Activated MAP Kinase

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MATERIALS PROVIDED

Materials provided	Concentration	Quantity
Activated MAP Kinase[a]	0.1 µg/µl	50 µl
Reaction buffer[b]	10×	1 ml
PHAS-I (control substrate)	0.5 µg/µl	10 µl

[a] Sufficient Activated MAP Kinase is provided for 250 total reactions.

[b] See *Preparation of Media and Reagents*.

STORAGE CONDITIONS

Activated MAP Kinase: –80°C

PHAS-I: –80°C

10× Reaction Buffer: –80°C

ADDITIONAL MATERIALS REQUIRED

[γ-^{32}P]ATP (>4000 Ci/mmol, ~10 µCi/µl)
85% stock solution (14.9 M) of phosphoric acid (H_3PO_4)
Sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS–PAGE) system
X-ray film

Revision A.02

INTRODUCTION

Mitogen-activated protein kinases (MAPKs), also known as extracellular signal-regulated kinases (ERKs), are activated by MAPK- or ERK-activating kinase (MEK) in response to a variety of extracellular signals. These mitogens may include growth factors (e.g. PDGF, EGF, NGF and insulin), cancer promoting factors (e.g. PMA and okadaic acid) or other extracellular stimuli such as T cell antigens and thrombin.[1, 2, 3, 4] The activated MAPK can phosphorylate many other cellular proteins including transcription factors, protein kinases, receptors and structural proteins.[1, 2]

ERK1 (44 kDa) and ERK2 (42 kDa) are closely related MAPKs that have 90% amino acid sequence identity and exhibit similar biochemical properties (e.g., activator and substrate specificity, and activation and deactivation mechanisms).[3, 5, 6]

Activated MAP Kinase

Our Activated MAP Kinase is a highly active, highly purified kinase produced by phosphorylating a recombinant rat ERK2 protein. *in vitro* with a constitutively active MEK mutant.[6] The recombinant rat ERK2 protein includes a tag of six histidine residues at the N-terminus facilitating the purification of the kinase and the removal of the MAPK from a reaction mixture.

High MAPK activity is required for experimental applications such as phosphorylation of possible substrates, screening for inhibitors, phosphatase studies, microinjections and labeling proteins bearing the MAPK recognition sequence (i.e., the PXPT motif). Activated MAP Kinase is one thousand times more active than nonactivated MAP kinase (~2.0 μmole/min/mg and ~2.0 nmole/min/mg, respectively).[5]

PHAS-I

PHAS-I is included with the Activated MAP Kinase as a control substrate, to facilitate the detection and quantitation of MAPK activity. PHAS-I (phosphorylated heat- and acid- stable protein regulated by insulin) is a 117-amino-acid protein substrate that is highly specific for MAPK.[7] The phosphorylation of PHAS-I increases in response to insulin but not to cAMP stimulation of fat cells. PHAS-I is an excellent substrate for MAPK both *in vivo* and *in vitro* and is the link between insulin and translation initiation control.[7, 8, 9]

PROTOCOLS

Phosphorylating Protein Substrates with Activated MAP Kinase

Activated MAP kinase can be used to phosphorylate proteins in order to identify new MAPK substrates, to generate radiolabeled probes, or to analyze phosphoamino acids.

Notes *All work should be performed behind protective shielding.*

Prepare one control sample lacking the Activated MAP Kinase, one control sample lacking the substrate (PHAS-I), and one control sample lacking both the Activated MAP Kinase and the PHAS-I. Replace the missing components with equal volumes of sterile distilled H_2O (dH_2O).

1. Prepare a 1:5 dilution of the Activated MAP Kinase in dH_2O.

2. Prepare the reaction samples *on ice* by mixing the following components in microcentrifuge tubes:

   Note ***Prepare the experimental sample by replacing the PHAS-I with a protein substrate of interest.***

   4.0 µl of 10× reaction buffer
   2.0 µl of PHAS-I (0.5 µg/µl) (**OR** 1.0 µg of experimental protein substrate)
   2.0 µl of [γ-^{32}P]ATP [1.0 µCi/µl (a 1:10 dilution of the 10 µCi/µl stock in dH_2O)]
   31 µl of dH_20
   1.0 µl of the diluted Activated MAP Kinase (from step 1)

3. Incubate the reaction samples at 30°C for 30 minutes.

The samples may now be qualitatively analyzed as outlined in *Resolving Phosphorylated Proteins by SDS–PAGE* or quantitatively analyzed as outlined in *Filter Binding Assay*.

Resolving Phosphorylated Proteins by SDS–PAGE

1. Add 40 µl of 2× SDS–PAGE sample buffer (see *Preparation of Media and Reagents*) to each reaction sample (from step 3 of *Phosphorylating Protein Substrates with Activated MAP Kinase*) and mix.

2. Load 20 µl of each reaction sample onto a 4–20% denaturing acrylamide gel or a 10% mini denaturing acrylamide gel and resolve the proteins by electrophoresis until the dye front has run within a centimeter of the bottom of the gel.

3. Remove the gel from the electrophoresis apparatus and lay the gel on plastic wrap. Because the dye front contains most of the unincorporated [γ-^{32}P]ATP, the dye front should be removed from the gel by cutting this section off with a razor blade. Dispose of the dye front properly.

4. Rinse the gel for 5 minutes in 200 ml of dH_2O and then dry the gel.

5. Expose the gel to X-ray film for 30 minutes at –80°C.

6. Autoradiography shows the phosphorylated control substrate protein (PHAS-I) as a predominant signal at ~21 kDa.

Filter Binding Assay

1. Label one square (2.5-cm × 2.5-cm) Whatman® P81 chromatography paper for each reaction sample.

2. Prepare 500 ml of 75 mM H_3PO_4 and 100 µl of 0.25 M H_3PO_4.

3. Stop the reactions (from step 3 of *Phosphorylating Protein Substrates with Activated MAP Kinase*) by placing the reaction samples on ice. Add 5 µl of 0.25 M H_3PO_4 to each reaction sample and mix well.

4. Spot 20 µl of each reaction sample onto a prelabeled square of chromatography paper.

5. After 2 minutes, wash the squares for 3 minutes in 100 ml of 75 mM H_3PO_4 with gentle agitation. Discard the H_3PO_4 into an appropriate radioactive waste container.

6. Repeat step 5 three more times.

7. Rinse the squares briefly in 95% (v/v) ethanol and let the squares air dry on a paper towel.

8. Quantitate the γ-^{32}P incorporation of MAPK into the PHAS-I or experimental protein substrate by liquid scintillation counting and calculate the specific activity of the ATP as outlined in *Calculating Phosphotransfer and Specific Activity of ATP*.

CALCULATING PHOSPHOTRANSFER AND SPECIFIC ACTIVITY OF ATP

Direct comparison of liquid scintillation counting results (in counts per minute) between samples may be adequate for some experimental purposes. However, converting the liquid scintillation counting results into units of specific activity eliminates possible effects that variances in laboratory equipment may have on the resulting data. Before the phosphotransfer can be calculated, the specific activity of the ATP must be determined using the method outlined below.

Note *Specific activity is an indication of the fraction of the ATP in the reaction that is radioactive. If only 10% of the ATP used by the kinase to label the protein is radioactive, then the number of counts on the square of chromatography paper is only 10% of the total phosphotransfer by the kinase.*

1. Calculate the specific activity (S_A) of the ATP in the reaction using the following equation (see, also, *Sample Calculation* for an example):

$$S_A = \frac{\text{The number of counts added to each reaction [in counts per minute (cpm)]}}{\text{The amount of ATP in the reaction [in picomoles (pmol)]}}$$

   To determine the number of counts added to each reaction, dilute the [γ-^{32}P]ATP used in the experiment to 1:100 and 1:1000 and quantitate the diluted sample in a liquid scintillation counter. For example, if 1 µl of the 1:100 dilution of [γ-^{32}P]ATP produces 1,129 cpm in the liquid scintillation counter, and 1 µl of [γ-^{32}P]ATP is added to each reaction, then each reaction contains 112,900 cpm.

   To determine the amount of ATP in the reaction, multiply the buffer volume of each reaction (40 µl) by the final concentration of ATP in the reaction (50 µM):

$$(40 \times 10^{-6} \text{ liters})(50 \text{ µmol / liter}) = 2.0 \times 10^{-3} \text{ µmol}$$

   or, on unit conversion to picomoles, 2,000 pmol.

2. Calculate the phosphotransfer (P) from the ATP into the protein substrate by dividing the number of counts incorporated by the MAPK in the experimental samples (i.e., the average cpm of the three samples minus the average cpm of the three negative controls) by the specific activity (S_A) (see the following equation and the *Sample Calculation* for an example):

$$P = \frac{\text{Average cpm of triplicate samples - average cpm of triplicate negative controls}}{S_A \text{ (in cpm / pmol of ATP)}}$$

where P is equal to the picomoles of phosphate transferred per assay in 10 minutes by the MAPK used in the assay.

Sample Calculation

In a 10-minute triplicate assay, the average cpm of the triplicate samples is 101,200 and the average cpm of the triplicate negative controls is 1,200.

The specific activity (S_A) can be calculated by dividing the cpm added to each sample by the amount of ATP in each sample.

$$S_A = \frac{110{,}000 \text{ cpm}}{2{,}000 \text{ pmol}}$$

$$S_A = 55 \text{ cpm / pmol}$$

The phosphotransfer (P) can be calculated by subtracting the average cpm of the negative controls from the average cpm of triplicate MAPK samples and dividing this number by the specific activity of the ATP.

$$P = \frac{101{,}200 \text{ cpm} - 1{,}200 \text{ cpm}}{55 \text{ cpm / pmol}}$$

$$P = \frac{100{,}000 \text{ cpm}}{55 \text{ cpm / pmol}}$$

$$P = 1{,}818 \text{ pmol or } 1.82 \text{ nmol}$$

$$P = \frac{1.82 \text{ nmol}}{10 \text{ minutes}}$$

$$P = 0.18 \text{ nmol / minute}$$

$$P = 180 \text{ pmol / minute}$$

TROUBLESHOOTING

Observation	Suggestion(s)
The exposed X-ray film is blank or nearly blank	Because the specific activity and purity of the [γ-^{32}P]ATP is essential for optimal labeling of protein substrates, use of high-performance liquid chromatography (HPLC)- purified [γ-^{32}P]ATP with a minimum specific activity of 4000 Ci/mmol and a concentration of ~10 µCi/µl is required. In addition, because the half-life of ^{32}P is ~14.3 days, check the expiration or reference date on the manufacturer's label to ensure that only fresh [γ-^{32}P]ATP is used
	Increase the autoradiograph exposure time
	Improperly stored enzyme will lose activity. Ensure that the enzyme is stored at the proper temperature
High background is produced on the X-ray film	Unincorporated [γ-^{32}P]ATP may be nonspecifically bound to the gel. Wash the gel several times in deionized water or transfer the proteins to a nitrocellulose membrane and expose the membrane to the X-ray film
	Remove the dye front from the gel by cutting the section off with a razor blade

PREPARATION OF MEDIA AND REAGENTS

10× Reaction Buffer	2× SDS–PAGE Sample Buffer
250 mM HEPES (pH 7.5) 100 mM magnesium acetate 500 µM ATP	125 mM Tris-HCl (pH 6.8) 4% (w/v) SDS 20% (v/v) glycerol 10% (w/v) β-mercaptoethanol 0.004% (w/v) bromophenol blue

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ENDNOTE

MSDS INFORMATION

The Material Safety Data Sheet (MSDS) information for Stratagene products is provided on the web at *http://www.stratagene.com/MSDS/*. Simply enter the catalog number to retrieve any associated MSDS's in a print-ready format. MSDS documents are not included with product shipments.

STRATAGENE

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Activated MAP Kinase

Catalog #206110

QUICK-REFERENCE PROTOCOL

Phosphorylating Protein Substrates with Activated MAP Kinase

- Prepare a reaction on ice by mixing 4 µl of 10× reaction buffer, 2 µl of PHAS-I **or** protein substrate of interest, 1 µl of diluted (1:5) Activated MAP Kinase, 2 µl of [γ-^{32}P]ATP (1 µCi/µl) and 31 µl of dH_20 in a microcentrifuge tube. Incubate the reaction at 30°C for 30 minutes

- The samples may now be qualitatively analyzed by SDS-PAGE as outlined in *Resolving Phosphorylated Proteins by Polyacrylamide Gel Electrophoresis* or quantitatively analyzed as outlined in *Filter Binding Assay*

Resolving Phosphorylated Proteins by SDS–PAGE

- Add 40 µl of SDS–PAGE sample buffer to each phosphorylation reaction and mix

- Resolve 20 µl of each reaction by SDS–PAGE until the dye front has run within a centimeter of the bottom of the gel

- Remove the dye front from the gel by cutting this section off with a razor blade

- Rinse the gel for 5 minutes in 200 ml of dH_2O and then dry the gel

- Expose the gel to X-ray film for 30 minutes at –80°C

- Autoradiography shows the phosphorylated control substrate protein (PHAS-I) as a predominant signal at ~21 kDa

Filter Binding Assay

- Place the phosphorylation reactions on ice, add 5 µl of 0.25 M H_3PO_4 to each reaction and mix

- Spot 20 µl of each reaction mixture onto a square of chromatography paper, wash the squares with 75 mM H_3PO_4 and then rinse the squares in 95% (v/v) ethanol

- Quantitate the ^{32}P incorporation in a liquid scintillation counter