10 Old Barn Road • Lake Placid, NY 12946
Technical Support: T: 800 548-7853 • F: 518 523-4513
email: techserv@upstate.com
Sales Department: T: 800 233-3991 • F: 781 890-7738
Licensing Dept.: 800 310-4659
www.upstate.com

Certificate of Analysis

MAP Kinase Assay Kit

(Non-Radioactive)
Catalog # 17-191
Lot # 24894

Kit Components

Assay Dilution Buffer I (ADBI), Catalog # 20-108. Two vials, each containing **1ml** of ADBI: 20mM MOPS, pH 7.2, 25mM β-glycerol phosphate, 5mM EGTA, 1mM sodium orthovanadate, 1mM dithiothreitol.

Anti-phospho-MBP, clone P12, Catalog # 05-429, Lot # 23402. One vial containing **100µg** of protein A purified IgG_{2a} in **100µl** of PBS, pH 7.4, 0.1% sodium azide and 30% glycerol.

Magnesium/ATP Cocktail, Catalog # 20-113. Two vials, each containing **1ml** of Mg^{2+}/ATP cocktail: 75mM magnesium chloride and 500µM ATP in ADBI.

MAP Kinase Substrate Cocktail, Catalog # 20-166. Two vials, each containing **1ml** of substrate cocktail: 2mg/ml dephosphorylated myelin basic protein in 20mM MOPS, pH 7.2, 25mM β-glycerophosphate, 5mM EGTA, 0.4mM $MnCl_2$, 0.4mM $CaCl_2$, 1mM sodium orthovanadate, 1mM dithiothreitol.

MAP Kinase Inhibitor Cocktail, Catalog # 20-116. One vial containing **1ml** of inhibitor cocktail: 20µM PKC inhibitor peptide (Catalog # 12-121) 2µM PKA inhibitor peptide (PKI) (Catalog # 12-151), and 20µM Compound R24571 in ADBI.

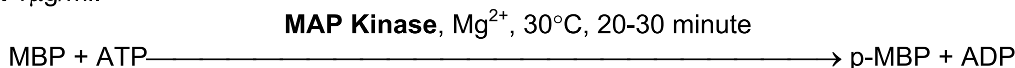
FOR IN VITRO RESEARCH USE ONLY
NOT RECOMMENDED OR INTENDED FOR DIAGNOSIS OF DISEASE IN HUMANS OR ANIMALS
DO NOT USE IN HUMANS OR IN ANIMALS

Kit Description

Quantity: 100 kinase assays per kit, if analyzed as 10 samples per western immunoblot at 1µg/ml.

Storage and Stability: Stable for 1 year at -20°C from date of shipment. Aliquot to avoid repeated freezing and thawing. For maximum recovery of the components, centrifuge the original vial after thawing and prior to removing the cap.

Use: The assay kit is designed to measure the phosphotransferase activity of MAP (mitogen-activated protein) Kinase in immunoprecipitates and column fractions. Crude cell lysates may also be used but detergents/biochemicals contained in the cell lysis buffer may inhibit MAP Kinase activity. Furthermore, although three inhibitors are included with the kit, editors may suggest other unknown kinases found in crude lysates are responsible for MBP phosphorylation. The assay kit is based on phosphorylation of a specific substrate (myelin basic protein, MBP). The phosphorylated substrate is then analyzed by immunoblot analysis, probing with a monoclonal Phospho-specific MBP antibody. The enzyme assay is rapid, convenient and specific for MAP Kinase and contains sufficient reagents for 100 individual MAP Kinase assays, if analyzed as 10 samples per western immunoblot at 1µg/ml.



Other components required but not included as part of kit are:

- A preparation containing MAP Kinase, such as 10-200μg of MAP Kinase immunoprecipitate diluted into ADBI or 25-100ng of purified MAP Kinase.
- TBS
- 2X Laemmli sample buffer
- vortex mixer
- 30°C shaking incubator
- timer
- variable volume (5-200μl) pipet + tips
- Western Immunoblot reagents and materials

Safety Warnings and Precautions: The Non-radioactive MAP Kinase assay kit is designed for research only and not recommended for internal use in humans or animals. All chemicals should be considered potentially hazardous and principles of good laboratory practice should be followed.

MAP Kinase Assay Kit Overview

All assay components must be rapidly thawed and mixed completely before proceeding with assay. Do not use extended thawing time. Once the assay components are thawed, mix thoroughly by vortexing. The assay components can be refrozen at -20°C for extended periods.

Perform all preincubation reactions at 1°C over an ice bath. The kinase assay can also be performed at room temperature but will not give linear results. Aliquot 10μl of substrate cocktail, 10μl of inhibitor cocktail and 10μl of enzyme preparation into the bottom of a microcentrifuge tube. Start the reaction by adding 10μl of the Mg^{2+}/ATP cocktail, vortex gently and incubate the microcentrifuge tube shaking at 30°C for 20-30 minutes. Remove and dilute an aliquot so that approximately 0.5-1μg of phosphorylated MBP is analyzed by western immunoblot analysis.

Suitable blanks should always be performed to correct for non-specific binding. Controls for endogenous phosphorylation of proteins in the sample extract can be performed by substituting ADBI for substrate cocktail. Controls for the basal level of phosphorylated MBP should be run to compare the background basal level (noise) of the MBP substrate and the phosphorylated MBP signal on the western immunoblot.

Assay Protocol:

1. Add 10μl of Mg^{2+}/ATP cocktail to a microcentrifuge tube.
2. Add 10μl of ADBI.
3. Add 10μl (20μg) of Map Kinase substrate cocktail.
4. Add 10μl of the inhibitor cocktail (optional control) or ADBI.
5. Add 10μl of an active MAP Kinase preparation (25-100ng purified enzyme/assay or 10-200μg immunoprecipitated protein).
6. Incubate for 20-30 minutes in a 30°C shaking incubator.

   Note: Components must be thoroughly mixed throughout the reaction time to ensure that the MBP, and the enzyme achieve maximum interaction.

7. Remove 2.5μl of the reaction mixture (approximately 1μg of phosphorylated-MBP) and place into another centrifuge tube. Add 7.5μl of TBS and 10μl of 2X Laemmli sample buffer. Load an aliquot of the sample for SDS-PAGE and immunoblot analysis.

Immunoblot Protocol

1. Perform SDS-polyacrylamide gel electrophoresis (SDS-PAGE) on the experimental samples and transfer the phosphorylated MBP to nitrocellulose. Wash the blotted nitrocellulose twice with water.
2. Block the blotted nitrocellulose in freshly prepared 3% nonfat dry milk (Catalog # 20-200) in TBS (TBS-MLK) for 20 minutes at room temperature with constant agitation.
3. Incubate the nitrocellulose with **0.5-2μg/ml of anti-phospho MBP, clone P12,** diluted in freshly prepared TBS-MILK overnight with agitation at 4°C.
4. Wash the nitrocellulose twice with water.
5. Incubate the nitrocellulose in the secondary reagent of choice (a **goat anti-mouse** HRP conjugated IgG, catalog # 12-349, 1:2000 dilution was used) in TBS-MLK for 1.5 hours at room temperature with agitation.
6. Wash the nitrocellulose with water twice.
7. Wash the nitrocellulose in TBS-0.05% Tween-20 for 3-5 minutes.
8. Rinse the nitrocellulose in 4-5 changes of water.
9. Use detection method of choice (enhanced chemiluminescence was used).

Quality Control Testing

Immunoblot Analysis:
Representative blot from a previous lot. MAP Kinase was used to phosphorylate myelin basic protein (MBP) *in vitro*. The results of an immunoblot analysis from an *in vitro* assay are shown to the right. Lane 1: basal level of MBP (1μg) phosphorylation; Lane 2: MBP incubated with MAP Kinase 2/Erk2, active (Catalog # 14-173). The immunoblot was probed using 1μg/ml anti-phospho-MBP, clone P12.