

Certificate of Analysis

MAP Kinase/Erk Immunoprecipitation Kinase Assay Kit

(non-radioactive)

Catalog # 17-192

Lot # DAM1723427

Kit Components

Assay Dilution Buffer, 5X (5X ADB), Catalog # 20-145. One vial containing 1ml of 5X ADB: 100mM MOPS, pH 7.2, 125mM β -glycerol phosphate, 25mM EGTA, 5mM sodium orthovanadate, 5mM dithiothreitol. Frozen at -20°C.

Anti-MAP Kinase/Erk 1/2, agarose conjugate, Catalog # 16-111, see page two for more information. Two vials, each containing 50 μ g of MAP Kinase/Erk 1/2 antibody bound to 50 μ l of packed Protein A agarose beads in PBS, pH 7.4 containing 0.05% sodium azide. Provided as a 50% slurry for a total volume of 100 μ l. Liquid suspension.

Anti-phospho-MBP, clone P12, Catalog # 05-429-MN, see page two for more information. One vial containing 25 μ g of protein A purified IgG_{2a} in 25 μ l of 70% storage buffer (PBS, pH 7.4, 0.1% sodium azide) and 30% glycerol. Store at -20°C.

MAP Kinase/Erk Substrate Cocktail II, Catalog # 20-166. One vial containing 1ml of substrate cocktail: 2mg/ml dephosphorylated myelin basic protein in 1X ADB (20mM MOPS, pH 7.2, 25mM β -glycerophosphate, 5mM EGTA, 1mM sodium orthovanadate, 1mM dithiothreitol).

Inhibitor Cocktail, Catalog # 20-116. One vial containing 1ml of inhibitor cocktail: 20 μ M PKC inhibitor peptide (Catalog # 12-121), 2 μ M PKA inhibitor peptide (PKI) (Catalog # 12-151) and 20 μ M Compound R24571 in 1X ADB.

Magnesium/ATP Cocktail, Catalog # 20-113. One vial containing 1ml of Mg²⁺/ATP cocktail: 75mM magnesium chloride and 500 μ M ATP in 1X ADB.

**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**

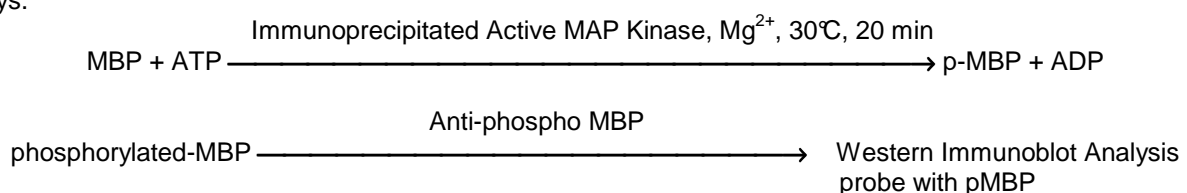
Kit Description

Quantity: 20 immunoprecipitation kinase assays per kit.

Storage and Stability: Stable for 1 year at -20°C from date of shipment.

Note: Upon arrival, store Anti-MAP Kinase/Erk 1/2, agarose conjugate (Catalog # 16-111) at 4°C.

Use: The assay kit is designed to measure phosphotransferase activity in an immunocomplex formed between the MAP Kinase R2 antibody and MAP Kinase (p44^{mapk}). This precipitated enzyme is used to phosphorylate a specific substrate, myelin basic protein (MBP). The phosphorylated substrate is then analyzed by western immunoblot using an antibody specific for phosphorylated MBP. The measurement of MAPK activity in most cell lysates is not accurate, due to the phosphorylation of MBP by other kinases. This enzyme assay is rapid, convenient and specific for MAP Kinase and contains sufficient reagents for 20 immunoprecipitation kinase assays.



Technical Information for Kit Components

Anti-MAP Kinase/Erk 1/2, agarose conjugate (rabbit polyclonal IgG)

Product Description: Anti-MAP Kinase 1/2 (Erk 1/2-CT) derived from Catalog # 06-182, immunogen: 38 residue, KLH-coupled, synthetic peptide [CGGPFTFDMELDDLPKERLKERLIFQETARFQPGA PEAP] of the C-terminal 35 amino acids of the rat 44kDa MAP Kinase 2/Erk2, covalently conjugated to protein A agarose by dimethyl-pimelimidate.

Species Cross-reactivity: Human, mouse, chicken and starfish; other species cross-reactivity unknown.

Note: It is recommended to wash the agarose beads with appropriate buffer prior to use to remove sodium azide.

References:

Boulton, T.G., *et al.*, Science **249**: 64-67, 1990.

Anti-phospho MBP, clone P12 (mouse monoclonal IgG_{2a})

Immunogen: Synthetic peptide containing phospho-Thr98 from a human myelin basic protein sequence and coupled to tuberculin. Clone P12.

References:

1. Yon, M., *et al.*, J. Neuroimmunol. **58**: 121-129, 1995.
2. Yon, M., *et al.*, J. Neuroimmunol. **65**: 55-59, 1996.

General Kit Reference:

Alessi, D.R., *et al.*, Methods Enzymol. **255**: 279-289, 1995.

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

- Enzyme preparation or stimulated cell extract containing active MAP Kinase
- Buffer A: 50mM Tris, pH7.5, 1mM EDTA, 1mM EGTA, 0.5mM Na₃VO₄, 0.1% 2-mercaptoethanol, 1% Triton X-100, 50mM sodium fluoride, 5mM sodium pyrophosphate, 10mM sodium β-glycerol phosphate, 0.1mM PMSF, 1μg/ml of aprotinin, pepstatin, leupeptin, and 1μM Microcystin
- vortex mixer
- 30°C shaking incubator
- timer
- variable volume (5-200μl) pipet + tips
- Western Immunoblot reagents and materials

Safety Warnings and Precautions: The MAP Kinase/Erk Immunoprecipitation 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/Erk Immunoprecipitation Kinase Assay Kit Overview

The kit components should be thawed and mixed by vortexing before proceeding with the assay. Do not use extended thawing time. In particular, 5X ADB and Magnesium/ATP cocktail must be rapidly thawed and mixed completely. 1X Assay Dilution Buffer can be obtained by diluting 1ml of the 5X stock with 4ml of deionized water. The assay components can be refrozen at -20°C for extended periods. Perform all pre-incubation steps at 1°C over an ice bath. The kinase assay may be performed at room temperature but linear results are more easily achieved at 30°C. After formation of the enzyme-antibody immunocomplex, MAP Kinase activity is determined using the phosphorylation assay protocol described below. The active enzyme-immunocomplex will phosphorylate the MBP substrate *in vitro*.

Suitable blanks should always be performed to correct for non-specific binding of active kinase to the protein A Agarose immunocomplex. Controls for endogenous phosphorylation of proteins in the sample extract can be performed by substituting 1X ADB for substrate cocktail. PC-12 cell lysate stimulated with 50ng/ml NGF is a good model control for the immunoprecipitation of the active MAP Kinases: p42, p43, p44. These are the abundant MAP kinases after growth factor stimulation in PC-12 cells.

Stock Solutions:

1. Assay Dilution Buffer, 5X (5X ADB): Prepare a 1X ADB (ADB) solution by diluting 1ml of the 5X stock with 4ml of deionized water.
2. Anti-MAP Kinase/Erk 1/2, agarose conjugate: Use 5µl per immunoprecipitation kinase reaction.
3. Buffer A: 50mM Tris, pH 7.5, 1mM EDTA, 1mM EGTA, 0.5mM Na₃VO₄, 0.1% 2-mercaptoethanol, 1% Triton X-100, 50mM sodium fluoride, 5mM sodium pyrophosphate, 10mM sodium β-glycero phosphate, 0.1mM PMSF, 1µg/ml of aprotinin, pepstatin, leupeptin, and 1µM Microcystin.

Immunoprecipitation Kinase Assay Procedure:

Stage I: Immunoprecipitation of MAP Kinase

Note: To maximize MAP Kinase activity, carry out all reactions on ice and pulse spin in a centrifuge that is equilibrated at 4°C.

1. Add 10µl of anti-MAP Kinase/Erk 1/2, agarose conjugate to a microcentrifuge tube.
2. Wash the agarose beads twice with 500µl per wash of Buffer A.
3. Resuspend the washed beads in 25µl of Buffer A.
4. Add 1mg of 50ng/ml NGF-stimulated PC-12 or serum-stimulated 3T3 whole cell/tissue extracts containing active MAP Kinase to the beads, keeping the volume between 200µl and 500µl in the microcentrifuge tube.
5. Incubate for 2 hours on a rotator at 4°C to immunoprecipitate MAP Kinase.
6. Wash the agarose/enzyme immunocomplex two to three times with 500µl of Buffer A.
7. Wash the agarose/enzyme immunocomplex twice with 75µl of 1X ADB. Remove the supernatant, place agarose/enzyme immunocomplex on ice and proceed to Step II.

Stage II: Kinase Assay of the Enzyme Immunocomplex

To the 5µl of agarose/enzyme immunocomplex from Stage I, Step 7 above, add the following:

1. Add 10µl of 1X ADB.
2. Add 10µl of Inhibitor cocktail.
3. Add 10µl of MAP Kinase/Erk Substrate Cocktail II.
4. Add 10µl of the Mg²⁺/ATP Cocktail.
5. Incubate for 20 minutes in a 30°C shaking incubator. Pulse spin to pellet the agarose/enzyme immunocomplex.

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

6. Remove 2.5µl of the reaction mixture (approximately 1µg pMBP) and place into another centrifuge tube. Add 7.5µl of TBS and 10µl of 2X Laemmli sample buffer. Boil 5 minutes and load an aliquot of the sample for SDS-PAGE and western immunoblot analysis.

Immunoblot Protocol

1. Perform SDS-polyacrylamide gel electrophoresis (SDS-PAGE) on the experimental sample and transfer the MBP to PVDF. Wash the blotted PVDF twice with water.
2. Block the blotted PVDF in freshly prepared TBS containing 3% nonfat dry milk (Catalog # 20-200), (TBS-MLK) for 20 minutes at room temperature with constant agitation.
3. Incubate the PVDF with **0.5-1µg/ml of anti-phospho MBP** (Catalog # 05-429-MN) diluted in freshly prepared TBS-MILK overnight with agitation at 4°C.
4. Wash the PVDF twice with water.
5. Incubate the PVDF in the secondary reagent of choice (a **goat anti-mouse** HRP conjugated IgG, Catalog # 12-349, 1:5000 dilution was used) in TBS-MILK for 1.5 hours at room temperature with agitation.
6. Wash the PVDF with water twice.
7. Wash the PVDF in TBS-0.05% Tween[®]-20 for 3-5 minutes.
8. Rinse the PVDF in 4-5 changes of water.
9. Use detection method of choice (enhanced chemiluminescence was used).

Quality Control Testing

Immunoprecipitation Kinase Assay: 5-10µl of anti-MAP Kinase/Erk 1/2, agarose conjugate immunoprecipitated MAP Kinase from 50ng/ml NGF-stimulated PC-12 cell extract. The enzyme/immunocomplex was then used to phosphorylate myelin basic protein (MBP). Representative data is shown at the right, using immunoprecipitated MAP Kinase, MBP as the substrate and 1µg/ml anti-phospho MBP.

Immunoprecipitation Kinase Assay

Representative lot data. Immunoprecipitated MAP Kinase was used to phosphorylate myelin basic protein (MBP) *in vitro*. The results of 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 immunoprecipitated MAP Kinase.



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