



## **Technical Information for Kit Components**

### **Anti-MAP Kinase 1/2 (Erk1/2), agarose (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 [CGGPFTFDMELEDDLPKERLKERLKFQETARFQPGAP EAP] 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.

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#### **References:**

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

### **Anti-phospho MBP (mouse monoclonal IgG<sub>2a</sub>)**

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

**Antibody Class:** IgG<sub>2a</sub>, produced by BALB/c mice. Splenocytes were propagated and fused with Sp2 myeloma cells and the resulting hybridoma clone was selected. Clone P12.

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#### **References:**

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

#### **General Kit Reference:**

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

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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 Na<sub>3</sub>VO<sub>4</sub>, 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

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**Safety Warnings and Precautions:** The MAPK 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.

## **MAPK 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 MBP Kinases: p42, p43, p44. These are the abundant MBP kinases after growth factor stimulation in PC-12 cells.

### Stock Solutions:

1. 5X Assay Dilution Buffer (5X ADB): Prepare a 1X ADB (ADB) solution by diluting 1ml of the 5X stock with 4ml of deionized water.
2. Anti-MAP Kinase-1/2 (Erk1/2), agarose: Use 5µl per immunoprecipitation kinase reaction.
3. Buffer A: 50mM Tris, pH 7.5, 1mM EDTA, 1mM EGTA, 0.5mM Na<sub>3</sub>VO<sub>4</sub>, 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:

#### **Step 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 5µl of anti-MAP Kinase 1/2, agarose 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.

### Step II: Kinase Assay of the Enzyme Immunocomplex

To the 5 $\mu$ l of agarose/enzyme immunocomplex from step I.7 above, add the following;

1. Add 10 $\mu$ l of 1X ADB.
2. Add 10 $\mu$ l of MAP Kinase Inhibitor cocktail.
3. Add 10 $\mu$ l of MAP Kinase Substrate Cocktail.
4. Add 10 $\mu$ l of the Mg/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 2.5 $\mu$ l of the reaction mixture (approximately 1 $\mu$ g pMBP) and place into another centrifuge tube. Add 7.5 $\mu$ l of TBS and 10 $\mu$ l of 2X Laemmli sample buffer. 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 nitrocellulose. Wash the blotted nitrocellulose twice with water.
2. Block the blotted nitrocellulose in freshly prepared TBS containing 3% nonfat dry milk (TBS-MLK) for 20 minutes at 20-25°C with constant agitation.
3. Incubate the nitrocellulose with **0.5-1 $\mu$ g/ml of anti-phospho MBP** (Catalog # 12-429-MN) 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, 1:3000 dilution was used, Catalog # 12-349) in TBS-MILK 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).

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### Quality Control Testing

Immunoprecipitation Kinase Assay: 5-10 $\mu$ l of  $\alpha$ -MAP Kinase 1/2 agarose immunoprecipitated MAP Kinase from 50ng/ml NGF-stimulated PC-12 cell extract. The enzyme/immunocomplex was then used to phosphorylate myelin basic protein (MBP). Data is shown at the right, using immunoprecipitated MAP Kinase, MBP as the substrate and 1 $\mu$ g/ml anti-phospho MBP.

**Immunoprecipitation Kinase Assay**  
Representative blot from a previous lot. 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 (1 $\mu$ g) phosphorylation; Lane 2: MBP incubated with immunoprecipitated MAP Kinase.

