

## Non Radioactive MAPK Immunoprecipitation Kinase Cascade Assay Kit

Catalog # 17-192

Lot # 16118A

### Kit Components

**5X Assay Dilution Buffer (5X ADB)**, Catalog # 20-145. One vial containing **1ml** of 5X ADB: 100mM MOPS, pH 7.2, 125mM  $\beta$ -glycerol phosphate, 25mM EGTA, 5mM sodium orthovanadate, 5mM dithiothreitol.

**Anti-MAP Kinase 1/2 (Erk1/2), Agarose Conjugate**, Catalog # 16-111, Lot # 17169, see page two for more information. Two vials, each containing **50mg** of immunoaffinity purified rabbit IgG in **100ml** of a 50% gel slurry in PBS, pH 7.4 and 0.05% sodium azide.

**Anti-Phospho MBP**, Catalog # 05-429-MN, Lot # 16177, see page two for more information. One vial containing **25mg** of immunoaffinity purified mouse monoclonal IgG in **35ml** of 0.1M Tris-glycine buffer, pH 7.4, 30% glycerol.

**MAP Kinase Substrate Cocktail**, Catalog # 20-115, Lot # 16203. One vial containing **1ml** of substrate cocktail (2mg/ml myelin basic protein) in 1X ADB.

**MAP Kinase Inhibitor Cocktail**, Catalog # 20-116, Lot # 16204. One vial containing **1ml** of inhibitor cocktail: 20 $\mu$ M PKC inhibitor peptide (Catalog # 12-121), 2 $\mu$ M protein kinase A inhibitor peptide (PKI) (Catalog # 12-151) and 20 $\mu$ M Compound R24571 in 1X ADB.

**Magnesium/ATP Cocktail**, Catalog # 20-113, Lot # 15953. One vial containing **1.0ml** of Mg<sup>2+</sup>/ATP cocktail: 75mM magnesium chloride and 500 $\mu$ M ATP in ADB.

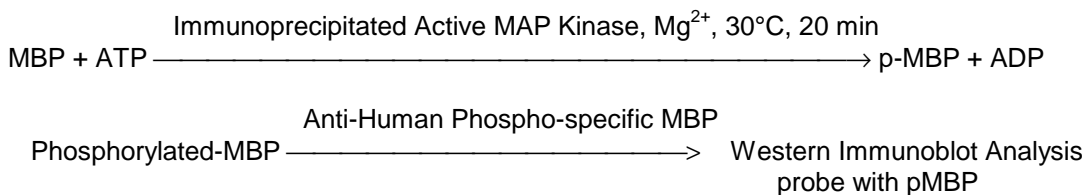
---

### Kit Description

**Quantity:** 20 immunoprecipitation kinase assays per kit.

**Storage and Stability:** Stable for 6 months at -20°C from date of shipment. **Note:** Upon arrival, store the Anti-MAP Kinase 1/2 Agarose Conjugate (Catalog # 16-111) at 4°C. Store the remainder of the kit at -20°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<sup>mpk</sup>). 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.



**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.**

### Technical Information for Kit Components

#### Anti-MAP Kinase 1/2 (Erk1/2), Agarose Conjugate (rabbit polyclonal IgG)

**Immunogen:** Anti-MAP Kinase 1/2 (Erk 1/2) derived from Catalog # 06-182 (immunogen: 38 residue synthetic peptide [CGGPFTFDMEIDDLK-KERLKLIFQETARFQPGAPEAP] corresponding to residues 333-367 of rat 43kDa MAP Kinase-1 and 2) covalently coupled to Protein A agarose by dimethylpimelimidate.

and *mpk* genes, respectively.

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

**Specificity:** Recognizes the 42kDa, 43kDa and 44kDa MAP Kinases encoded by the *mapk*, *erk1*,

---

**Background:** An important convergence point involved in the signal transduction pathways of many different growth factors, hormones, and cytokines is a family of 41-44kDa serine/threonine kinases collectively called either MAPKs (for mitogen-activated protein kinases) or ERKs (for extracellular-regulated kinases). MAPK is activated by sequential phosphorylation on both tyrosine and threonine residues by either the dual tyrosine/threonine kinase MEK (MAPK Kinase) alone or by MEK in conjunction with an as yet undescribed kinase. During intracellular signaling, MEK is phosphorylated and activated by the serine/threonine kinase Raf. Activation of MAPK is directly regulated by a specific MAPK phosphatase and indirectly regulated by protein kinase A activation which results in inhibition of Raf activity in mammalian cells.

**References:**

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

#### Anti-Phospho MBP (mouse monoclonal IgG)

**Immunogen:** Phosphorylated synthetic peptide Thr 98 corresponding to a human myelin basic protein sequence coupled to tuberculin and used to immunize BALB/c mice.

**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).

---

**Background:** Myelin-specific basic protein (MBP, MW = 18,400) is a major component of the myelin sheath that coats neurons of the central nervous system. The exact physiological function of MBP remains unknown, although it has been implicated in the maintenance of the structural integrity of myelin. MBP is phosphorylated at five (and possibly more) sites *in vivo*.

*In vitro* MBP is a substrate for phosphorylation by several different protein kinases such as MAP kinase, cAMP-dependent protein kinase, calmodulin-dependent protein kinase, protein kinase C and phosphorylase kinase. Even highly specific protein kinases such as Raf1, Mek and Mekk can utilize MBP as an alternative substrate.

**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.

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 (5-200 μl) pipet + tips
- Western Immunoblot reagents and materials

---

**Safety Warnings and Precautions:** The MAP Kinase 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 Immunoprecipitation Kinase Cascade Assay Kit Protocol 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*.

**Note:** The **basal level** of phosphorylated MBP can be adjusted by diluting the amount of pMBP loaded on the SDS-PAGE. Dilutions to 100ng per SDS-PAGE lane should be sufficient to decrease the basal level of MBP phosphorylation while obtaining a phosphorylated signal.

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-1 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 solution by diluting 1ml of the 5X stock with 4ml of deionized water.
2. Magnesium/ATP Cocktail: 500μM cold ATP and 75mM magnesium chloride in 1X ADB.
3. MAP Kinase Substrate Cocktail: 2mg/ml myelin basic protein in 1X ADB.
4. Inhibitor Cocktail: 20μM PKC inhibitor peptide (Catalog # 12-121) 2μM PKA inhibitor peptide (PKI) (Catalog # 12-151) and 20μM Compound R24571.
5. Anti-MAP Kinase-1/2 (Erk1/2), agarose conjugate: Use 10μl per immunoprecipitation kinase reaction.
6. 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 Protocol

### 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 10µl of anti-MAP Kinase 1/2, agarose conjugate, to a microcentrifuge tube.
2. Add 300µl of ice-cold Buffer A. (Buffer A is a cell lysis buffer containing protease and phosphatase inhibitors. Microcystin is necessary to ensure complete inactivation of cellular PP1 and PP2A phosphatases which may potentially dephosphorylate the active MAP Kinase.)
3. Incubate for 30 minutes to 1 hour at 4°C on a rotator to thoroughly mix the components during the incubation.
4. Pellet the agarose beads at 14,000rpm for 15 seconds.
5. Remove the supernatant. Wash the agarose beads twice with Buffer A to remove weakly bound antibodies. (This wash is an important step because it removes any form of MAP Kinase 1/2 antibody that does not bind to the agarose, but which may bind to MAP Kinase in a tissue extract or cell lysate.)
6. Resuspend the washed beads pellet in 100µl of Buffer A.
7. Add 1mg of 50ng/ml NGF stimulated PC-12 whole cell/tissue extracts containing active MAP Kinase to the beads, keeping the volume between 200µl and 500µl in the microcentrifuge tube.
8. Incubate for 2 hours on a rotator at 4°C to immunoprecipitate MAP Kinase.
9. Wash the agarose/enzyme immunocomplex two to three times with 500µl of Buffer A.
10. Wash the agarose/enzyme immunocomplex twice with 75µl of 1X ADB. Remove the supernatant, place on ice and proceed to Step II.

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

To the 25µl of agarose/enzyme Immunocomplex from step 10, add the following;

1. Add 10µl of 1X ADB.
2. Add 10µl of MAP Kinase Inhibitor cocktail.
3. Add 10µl of MAP Kinase Substrate Cocktail.
4. Add 10µl 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 2µl of the reaction mixture (approximately 1µg pMBP) and place into another centrifuge tube. Add 8µl of TBS and 10µl of 2X Laemmli sample buffer. Load an aliquot of the sample for SDS-PAGE and western immunoblot analysis.

**Note:** The **basal level** of phosphorylated MBP can be adjusted by diluting the amount of pMBP loaded on the SDS-PAGE. Dilutions to 100ng per SDS-PAGE lane should be sufficient to decrease the basal level of MBP phosphorylation while obtaining a phosphorylated signal.

### 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 **0.5-1ng/ml of  $\alpha$ -Phospho MBP** 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  $\alpha$ -mouse** HRP conjugated IgG, 1:1000 dilution was used) 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).

**NOTE THIS STUFF WAS DELETED FROM COA 77/15/98 due to crummy look.**

**Anti-MAP Kinase Immunoprecipitation Kinase Assay Data:** Immunoprecipitated MAP Kinase was used to phosphorylate myelin basic protein (MBP) *in vitro*. The results of a western immunoblot analysis from an *in vitro* assay are shown below. The basal level of MBP (1 $\mu$ g) phosphorylation is shown in Lane 1. Lane 2 represents MBP incubated with immunoprecipitated MAPK.

