

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

- A preparation containing MAP Kinase, such as 10-200 μ g of MAP Kinase immunoprecipitate diluted into ADB or 25-100ng of purified MAP Kinase.
- TBS
- 2X Laemmli sample buffer
- vortex mixer
- 30°C shaking incubator
- timer
- variable volume (5-200 μ l) 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 10 μ l of substrate cocktail, 10 μ l of inhibitor cocktail and 10 μ l of enzyme preparation into the bottom of a microcentrifuge tube. Start the reaction by adding 10 μ l of the Mg²⁺/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 0.5-1 μ g 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 ADB 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 10 μ l of Mg/ATP cocktail to a microcentrifuge tube.
2. Add 10 μ l of ADB.
3. Add 10 μ l (20 μ g) of substrate cocktail (MBP).
4. Add 10 μ l of the inhibitor cocktail (optional control) or ADB.
5. Add 10 μ l of an active MAP Kinase preparation (25-100ng purified enzyme/assay or 10-200 μ g 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 2.5 μ l of the reaction mixture (approximately 1 μ g of phosphorylated-MBP) and place into another centrifuge tube. Add 7.5 μ l of TBS and 10 μ l of 2X Laemmli sample buffer. Load an aliquot of the sample for SDS-PAGE and immunoblot analysis.

Western 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 TBS containing 3% nonfat dry milk (TBS-MLK) for 20 minutes at 20-25°C with constant agitation.
3. Incubate the nitrocellulose with **1mg/ml of anti-Human phospho specific 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 anti-mouse** HRP conjugated IgG, catalog # 12-349, 1:3000 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: 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 (Catalog # 14-173). The immunoblot was probed using 1µg/ml anti-Human Phospho-specific MBP.

