

Product Information

MEK Activity Assay Kit

Product Code **CS0490**

Storage Temperature $-20\text{ }^{\circ}\text{C}$

TECHNICAL BULLETIN

Product Description

The MAP kinase kinases (MAPKK, mitogen-activated protein kinase kinase, also termed MEK) are a family of Thr/Tyr dual specificity protein kinases. This family of kinases plays a crucial role in various signal transduction pathways.^{1,2} MEK directs signals of growth factor receptors or G protein-coupled receptors to their intracellular targets³ and thus, regulates several cellular processes including proliferation, differentiation, cellular morphology, and oncogenesis.^{4,5} MEK consists of three different isoforms, MEK1a (45 kDa), MEK1b (41 kDa), and MEK2 (46 kDa) that are highly homologous and are widely expressed.⁶ Activation of MEK1 and MEK2 in mitogen-stimulated cells is directly mediated by MAP kinase kinases (MAPKKs), such as Raf-1 kinase, which phosphorylates two serine residues in the regulatory sites of MEK. Following activation, MEK phosphorylates MAP kinase (MAPK, ERK1, and ERK2) in the MAP kinase cascade.^{1,2,3,5}

The MEK Assay Kit provides an easy method to assay MEK activity and to explore new MEK stimuli, inhibitors, and activators. The kit is based on assaying the activity of immunoprecipitated MEK on its MAP kinase (ERK2) non-activated substrate. The detection of MEK phosphorylation activity is performed by immunoblotting using an antibody that specifically recognizes only the dually phosphorylated form of MAP kinase.

The kit provides all the reagents required for the straightforward detection and measurement of MEK activity in cell lysates, tissue homogenates, and column fractions, or of the purified enzyme. In addition, the kit provides a specific MEK inhibitor to verify the specificity of the kinase activity observed. The kit was tested on rat kidney, brain, and heart tissues, and on NIH3T3 and BHK cells.

Kit Components

The kit contains reagents sufficient for 50 reactions.

- Anti-MAP Kinase Kinase (MEK, MAPKK) Product Code M 5795 0.1 ml
- Monoclonal Anti-MAP Kinase, Activated (Diphosphorylated ERK1&2) Product Code M 9692 Detection Antibody 0.1 ml
- Assay Buffer For Kinase Activity Product Code A 4603 1 ml
75 mM β -glycerophosphate, pH 7.3,
3.75 mM EGTA, 30 mM MgCl_2 , 4.5 mM DTT,
0.15 mM Na orthovanadate, and 0.3 mM ATP
- Wash Buffer 10X Product Code W 3264 8 ml
500 mM β -glycerophosphate, pH 7.3,
15 mM EGTA, 10 mM MgCl_2 , 10 mM DTT,
and 1 mM Na orthovanadate
- EZview™ Red Protein A Affinity Gel Product Code P 6486 1 ml
- MAP Kinase, Non-Activated, (ERK2) from rat, recombinant Product Code M 9426 0.1 mg
- U0126 (MEK inhibitor) Product Code U-120 1 mg

Reagents and Equipment Required But Not Provided

- Microfuge centrifuge - Eppendorf® microcentrifuge 5415 Series (Product Code Z60,406-2) or equivalent.
- Dulbecco's Phosphate Buffered Saline (PBS, Product Code D 8537).
- 4X SDS Sample Buffer - preparation instructions are detailed in the Appendix.
- Materials and equipment required for immunoblot analysis: ProteoQwest™ Chemiluminescent Western Blotting Kit (Product Code PQ0201) or equivalent. For kits without a secondary antibody, Anti-Mouse IgG – Peroxidase Conjugate (Product Code A 9044) is recommended. It is equivalent to the conjugate contained in the ProteoQwest™ Chemiluminescent Western Blotting Kit (Product Code PQ0201). For kits other than the ProteoQwest™ Chemiluminescent Western Blotting Kit, we recommend determining the appropriate titer for the Anti-Mouse IgG – Peroxidase Conjugate (Product Code A 9044) in the immunoblot procedure.

Precautions and Disclaimer

This product is for R&D use only, not for drug, household, or other uses. Please consult the Material Safety Data Sheet for information regarding hazards and safe handling practices.

Preparation Instructions

It is recommended to use ultrapure (17 MΩ•cm or equivalent) water when preparing the reagents.

- 1X Wash Buffer - Dilute the Wash Buffer 10X (Product Code W 3264) 10-fold with ultrapure water. Prepare 1.3 ml of 1X Wash Buffer for each reaction. For each test sample, 4 reactions (2 controls, a sample, and an inhibition reaction) are recommended (see Procedure).
- ERK2 Substrate Solutions - Reconstitute the MAP Kinase, Non-Activated (ERK2, Product Code M 9426) substrate by adding 0.2 ml of ultrapure water to the bottle. Mix well by pipetting up and down. This results in an ERK2 Substrate Stock Solution of ~0.5 mg/ml. Aliquot the ERK2 Substrate Stock Solution and store at –20 °C.

Just before the phosphorylation assay, prepare the ERK2 Substrate Working Solution. For each reaction add 2 µl of the ERK2 Substrate Stock Solution to 13 µl of 1X Wash Buffer.

- U0126 Inhibitor Solutions – Prepare the U0126 Inhibitor Stock Solution by reconstituting the MEK inhibitor, U0126 (Product Code U-120), in 130 µl of DMSO to a concentration of 20 mM. Mix well. For extended storage (up to a month), freeze in aliquots after reconstitution. Do not store in frost-free freezers. Longer storage of the inhibitor in solution may result in a decreased inhibitory activity.

Prepare an U0126 Inhibitor Working Solution for the *in vitro* MEK inhibition reaction. Just before the phosphorylation assay, dilute an aliquot of the U0126 Inhibitor Stock Solution 20-fold in DMSO. Add 2 µl of the U0126 Inhibitor Working Solution to the inhibition reaction.

Note: U0126 is a more effective inhibitor of MEK activity when added to cells in medium than when added to *in vitro* assays. Therefore, we suggest adding the U0126 Inhibitor Stock Solution to cells in medium to a final concentration of 5-10 µM. For the *in vitro* inhibition reaction the final concentration is ~5-fold higher. The suggested concentrations are only guidelines. The optimal concentrations can vary from one cell line to another and need to be optimized for each cell line or sample.

Storage/Stability

The kit is shipped on dry ice and storage at –20 °C is recommended. Upon initial thawing, reconstitute the MAP Kinase, non-activated (ERK2) substrate and the U0126 (MEK inhibitor) according to the Preparation Instructions. Freeze the reconstituted ERK2 Substrate and U0126 Inhibitor Stock Solutions, and the antibodies in working aliquots to avoid multiple freeze-thaw cycles.

Procedure

The MEK is immunoprecipitated from a sample with Anti-MEK antibodies and the EZview Red Protein A Affinity Gel. The immunoprecipitated MEK phosphorylates the MAP Kinase, non-activated (ERK2) substrate and the level of ERK2 phosphorylation is detected by immunoblotting using Anti-diphosphorylated ERK1&2 (Thr¹⁸³ and Tyr¹⁸⁵ in ERK2).

For each sample, two negative control reactions are recommended:

1. One without the addition of the Anti-MEK antibody to detect non-specific protein binding to the agarose (Control 1).
2. The other without the addition of the ERK2 Substrate Working Solution to determine the existence of endogenous phosphorylated products (Control 2).

A MEK inhibition reaction is recommended to determine that the phosphorylation of the ERK2 is performed by MEK and not by any other kinase.

All steps should be performed on ice unless otherwise stated.

Table 1.

Reaction scheme for all three steps of the assay: Immunoprecipitation, Phosphorylation, and Termination.

	Immunoprecipitation			Phosphorylation				Termination
	Cell Lysate	Anti-MEK (M 5795)	EZview Protein A (50% slurry)	Assay Buffer	ERK2 Substrate Working Solution	1X Wash Buffer	U0126 Inhibitor Working Solution	4X SDS Sample buffer
Control 1	250-1,000 μ l	-----	30 μ l	15 μ l	15 μ l	-----	-----	12 μ l
Control 2	250-1,000 μ l	2 μ l	30 μ l	15 μ l	-----	15 μ l	-----	12 μ l
Sample Reaction	250-1,000 μ l	2 μ l	30 μ l	15 μ l	15 μ l	-----	-----	12 μ l
Inhibition Reaction	250-1,000 μ l	2 μ l	30 μ l	15 μ l	15 μ l	-----	2 μ l	12 μ l

The procedure described is for one reaction. Adjust the amounts according to your experiment.

Immunoprecipitation

1. Transfer up to 1 ml of cell lysate (200-500 μ g of protein) into an Eppendorf microcentrifuge tube. The optimal cell lysate volume is \sim 250 μ l.
Note: A cell lysate from U0126 treated cells may be used for MEK inhibition reaction.
2. Add 2 μ l of Anti-MEK antibody (Anti-MAP Kinase Kinase antibody, Product Code M 5795). Mix gently and set the tube on ice while equilibrating the EZview Red Protein A Affinity Gel beads.
3. For equilibrating the EZview Red Protein A Affinity Gel beads:
 - a. Carefully mix the gel beads until uniformly suspended. Aliquot 30 μ l of the 50% slurry into a clean 1.5 ml microcentrifuge tube. For dispensing the beads, use a wide orifice pipette tip or cut \sim 1 mm off the tip to enlarge the opening and allow unrestricted flow of the bead suspension.

- b. Wash/equilibrate beads with PBS. Add 750 μ l of PBS to the tube, vortex, and centrifuge in a microcentrifuge for 30 seconds at \sim 8,000 x g. Carefully remove the supernatant with a micropipette (or carefully aspirate the supernatant).
Note: Because of the enhanced visibility of the red affinity resin beads, it is easy to see if beads were accidentally removed during the wash step. If this happens, transfer the supernatant back into the tube and repeat the centrifugation step to pellet the resin again.
- c. Repeat the wash (step 3b) as indicated above. After removing the supernatant, set the washed bead pellet on ice.

Note: For multiple reactions, it is possible to equilibrate the total volume of resin required for all reactions in one batch, according to the described procedure. Following resin equilibration the resin pellet should be re-suspended in PBS to a 50% slurry. Then, 30 μ l of the 50% slurry should be dispensed into each 1.5 ml microcentrifuge tube according to the number of reactions.

4. Briefly centrifuge the tube containing the cell lysate and the antibody (step 2) for several seconds at $\sim 8,000 \times g$ to collect the liquid at the bottom of the microcentrifuge tube. Carefully remove all the liquid with a 1 ml micropipette and transfer to a tube containing the equilibrated EZview Red Protein A Affinity Gel beads (step 3c).
5. Vortex briefly and incubate with thorough, gentle rotation for 4 hours at 2–8 °C to allow the antibody-antigen complexes to bind to the EZview Red Protein A Affinity Gel beads.
6. Centrifuge the tube in a microcentrifuge for 30 seconds at $\sim 8,000 \times g$. Set the tube on ice.
7. Aspirate the supernatant carefully (or remove with a micropipette) and set the tube containing the red bead pellet on ice.
Note: Because of the enhanced visibility of the red affinity resin beads, it is easy to see if beads were accidentally removed during this step. If this happens, transfer the supernatant back into the tube and repeat the centrifugation step to pellet the resin again.
8. Wash the bead pellet by adding 400 μ l of 1x Wash Buffer. Vortex briefly and incubate with thorough, gentle mixing at 2–8 °C for 1 minute.
9. Centrifuge the tube in a microcentrifuge for 30 seconds at $\sim 8,000 \times g$. Aspirate the supernatant carefully (or remove with a micropipette) and set the tube with the bead pellet on ice.
10. Perform two additional washes (steps 8-9).

Phosphorylation of MAP Kinase, non-activated (ERK2 substrate)

1. Add 15 μ l of Assay Buffer (Product Code A 4603) to the bead pellet and 15 μ l of the prepared ERK2 Substrate Working Solution. Suspend the pellet by gentle pipetting.
Note: For the *in vitro* MEK inhibition test, add 2 μ l of the prepared U0126 Inhibitor Working Solution to the Inhibition Reaction tube. We suggest adding the reagents to the Inhibition Reaction tube in the following order: first suspend the pellet with 15 μ l of Assay Buffer, then add the U0126 Inhibitor Working Solution, and finally add the ERK2 Substrate Working Solution.
2. Incubate for 30 minutes at 30 °C.
3. Terminate the reaction with 12 μ l of 4x SDS Sample Buffer and mix well.
4. Boil the sample for 5 minutes and then centrifuge for 30 seconds. Save the supernatant.

Immunoblot Analysis

A dot blot procedure (Appendix) may be performed in order to preview the results.

1. Load the sample on an SDS-PAGE gel (10-12%), run the gel, and then transfer the proteins to a nitrocellulose membrane. Block the nitrocellulose membrane using an appropriate blocking solution.
2. To detect the phospho-ERK2, follow the procedure for the ProteoQwest™ Chemiluminescent Western Blotting Kit (or equivalent).
Note: The apparent size of the phospho-ERK2 on the gel is ~ 42 kDa. An additional non-specific band ~ 50 kDa may be detected in some preparations, due to the secondary antibody used. Use of the Anti-Mouse IgG - Peroxidase conjugate produced in rabbit (Product Code A 9044) is recommended.
3. The ProteoQwest™ Chemiluminescent Western Blotting Kit requires incubation with a primary antibody. Incubate the membrane with a 1:2,000 dilution of the Detection Antibody - Monoclonal Anti-MAP Kinase, Activated (Diphosphorylated ERK1&2, Product Code M 9692) in blocking solution. During this incubation step the blot should be gently agitated at room temperature for 2 hours (or alternatively, overnight at 4 °C). After washing, incubate the membrane with anti-mouse IgG – peroxidase conjugate antibody (according to the supplier's instructions). Gently agitate for 1 hour.
4. After washing the membrane, incubate it with a chemiluminescent substrate, drain and expose the membrane to X-ray film. An initial 10-second exposure will indicate the need for a different exposure time.

References

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2. Chong, H., et al., Mechanisms of regulating the Raf kinase family. *Cell Signal.*, **15**, 463-469 (2003).
3. Peyssonnaud, C., and Eychene, A., The Raf/MEK/ERK pathway: new concepts of activation. *Biol. Cell*, **93**, 53-62 (2001).
4. Johnson, G.L., and Lapadat, R., Mitogen-activated protein kinase pathways mediated by ERK, JNK, and p38 protein kinases. *Science*, **298**, 1911-1912 (2002).
5. Kolch, W., Meaningful relationships: the regulation of the Ras/Raf/MEK/ERK pathway by protein interactions. *Biochem. J.*, **351**, 289-305 (2000).
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Troubleshooting Guide

Problem	Possible cause	Solution
The signal is very poor or no signal is observed.	The amount of activated MEK in the sample is very low.	Add more than 2 μ l of Anti-MEK antibody to the cell lysate (Immunoprecipitation section, step 2).
		Increase the sample volume - increasing the sample volume up to 1 ml usually has a minor effect on the interaction between the antibody and the activated MEK.
		Use a lower dilution of the Anti-MAP Kinase, activated (diphosphorylated ERK 1&2) antibody.
		Increase the reaction incubation time from 30 minutes up to 90 minutes [Phosphorylation of MAP Kinase, non-activated (ERK2 substrate) section, step 2].
		If possible, load a larger volume of reaction sample on the gel.
	There is no activated MEK in the sample.	Prepare a fresh lysate.
		Add appropriate phosphatase inhibitors to the sample (Product Codes P 2850 and P 5726) or increase their concentration to prevent dephosphorylation of activated MEK.
		Add the appropriate protease inhibitors to the sample (Product Code P 8340) or increase their concentration to prevent degradation of activated MEK.
		Make sure the extraction buffer is not interfering with the kinase activity. Some extraction buffers used for immunoprecipitation are not suitable for activity assays. We highly recommend using the CellLytic™ M Cell Lysis Reagent (Product Code C 2978) for cell extract preparation.
		Verify that the induction procedure for MEK activation is appropriate. Determine the presence of activated MEK in the sample by immunoblotting of the sample using antibodies to the activated MEK Kinase (Product code M 7683 or M 2943) prior to the performance of the immunoprecipitation.
Incubation time is inadequate.	Prolong the incubation duration of the Anti-MEK antibody with the EZview Red Protein A (from several hours to overnight).	
Interfering substance present in sample.	Excessive detergent concentration may interfere with the interaction between the antibody and the activated MEK.	
Signal is too strong or is spread out of the lane.	There is an excess of MAP Kinase, non-activated (ERK2) substrate in the reaction.	Dilute the reconstituted MAP Kinase, non-activated (ERK2) substrate with ultrapure water before adding it to the reaction tube (in the range of 2 to 10-fold dilution).
		Dilute the terminated reaction with more sample buffer or load a smaller volume of reaction sample.
Background is too high or additional bands are observed.	Proteins bind non-specifically to Protein A, the resin beads, or the microcentrifuge tube.	Pre-clear the sample one or more times by pre-incubation with EZview Red Protein A Affinity Gel (without the Anti-MEK 1 antibody) to remove proteins that may bind non-specifically.
		During the final wash (Immunoprecipitation section, step 9), after suspending the resin, transfer the entire sample to a clean microcentrifuge tube before centrifuging the sample.
	Insufficient washes performed in the immunoprecipitation step.	Increase the number of washes.
		Prolong the duration of the washes to up to 15 minutes.
	Detection antibody is in excess.	Centrifuge at a lower speed to avoid non-specific trapping of lysate proteins during the initial centrifugation of Protein A/antigen complexes.

Appendix

I. Preparation of 10 ml of 4X SDS Sample Buffer To 4 ml of 0.5 M Tris-HCl, pH 6.8, (Trizma base, Product Code T 1503, pH adjusted to 6.8) add:

- 1.2 g of SDS (Product Code L 3771)
- 616 mg of DTT (Product Code D 0632)
- 10 mg of Bromophenol blue (Product Code B 0126)
- 4 g of Glycerol (Product Code G 9012).

Bring the final volume to 10 ml with water. Mix well by inversion.

II. Dot Blot

The dot blot procedure may be performed in order to preview the results. This procedure does not replace the requirement for immunoblotting.

1. Cut a nitrocellulose membrane into oblong strips of 5 cm x 2 cm (total of 10 cm²). This is sufficient for 10 samples. Each additional sample (spot) requires an additional membrane area of 1 cm².
2. Spot 1 µl of the sample prepared for gel loading [Phosphorylation of MAP Kinase, non-activated (ERK2 substrate) section, step 4] on the nitrocellulose membrane. Let it dry for a few minutes.
3. Rinse briefly with PBS or equivalent.
4. Incubate the membrane in a blocking solution for 15 minutes.

5. Incubate the membrane with a 1:2,000 dilution of the Detection Antibody - Monoclonal Anti-MAP Kinase, Activated (Diphosphorylated ERK1&2, Product Code M 9692) in blocking solution. Incubate with gentle agitation for 20 minutes at room temperature.
6. Wash the membrane twice for 4 minutes each time with PBS containing 0.2% TWEEN[®] 20 (or an equivalent buffer).
7. Incubate the membrane with anti-mouse IgG – peroxidase conjugate antibody (according to the supplier's instructions). Incubate with gentle agitation for 20 minutes at room temperature.
8. Wash the membrane four times for 4 minutes each time with PBS containing 0.2% TWEEN 20 (or an equivalent buffer).
9. Briefly rinse in PBS or equivalent (with no detergent).
10. Prepare a chemiluminescent peroxidase substrate solution, enough to cover the membrane (based on 0.125 ml/cm² membrane). Incubate the membrane in the reagent, with gentle agitation for 1 minute at room temperature.
11. Drain the membrane of excess reagent solution, wrap in clear plastic wrap, and expose to X-ray film. An initial 10 seconds exposure will indicate the need for different exposure times.

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