
Certificate of Analysis

B-Raf Kinase Assay Kit, Chemiluminescence Detection

Catalog # 17-359

Lot # JBC1371377

Kit Components

B-Raf (Δ 1-415), active, Catalog # 14-530, see page two for more information. One vial containing **5 μ g** of recombinant protein in **50 μ l** of 50mM Tris-HCl, pH 7.5, 150mM NaCl, 0.1mM EGTA, 0.03% Brij 35, 270mM sucrose, 1mM benzamidine, 0.2mM PMSF, 0.1% 2-mercapto-ethanol.

MEK1, unactive, Catalog # 14-420, see page two for more information. One vials, each vial containing **50 μ g** recombinant enzyme in **50 μ l** of 50mM Tris-HCl, pH 7.5, 150mM NaCl, 0.1mM EGTA, 0.03% Brij-35, 5% glycerol, 0.1% 2-mercaptoethanol, 0.2mM PMSF, 1mM benzamidine.

Anti-phospho-MEK1 (Ser218/222)/MEK2 (Ser222/226). Catalog # 07-461. One vial containing **200 μ g** of protein A purified rabbit IgG in **200 μ l** of 0.1M Tris-glycine, pH 7.4, 0.15M NaCl, 0.05% sodium azide before the addition of glycerol to 30%.

Assay Dilution Buffer I (ADBI), Catalog # 20-108. One vial containing **1.0ml** of ADBI: 20mM MOPS, pH 7.2, 25mM β -glycerol phosphate, 5mM EGTA, 1mM sodium orthovanadate, 1mM dithiothreitol.

Magnesium/ATP Cocktail, Catalog # 20-113. One vial containing **1.0ml** of Mg^{2+} /ATP cocktail: 75mM magnesium chloride and 500 μ M ATP in ADBI.

Kit Description

Quantity: 50 kinase assays per kit.

Storage and Stability: Stable 6 months at -70°C from date of shipment.

Use: Intended for the assay of recombinant B-Raf (supplied) kinase activity, with experimental variables as determined by the end user. The assay kit is designed to measure B-Raf dependent phosphotransferase activity in a kinase reaction using recombinant MEK1, unactive as a B-Raf substrate. The enzyme assay is rapid, convenient and specific for the Raf family of kinases. Each kit contains sufficient reagents for 50 individual B-Raf 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.**

B-Raf Kinase Reaction

Active B-Raf, 30°C, 30 min

MEK1, unactive + ATP $\xrightarrow{\hspace{10em}}$ Active MEK1* + ADP

Technical Information for Kit Components

B-Raf (Δ 1-415), active (recombinant protein expressed in Sf21)

Product Description: Raf residues 416-end. Expressed by baculovirus in Sf21 insect cells. Purified using glutathione agarose. Purity 46% by SDS-PAGE and Coomassie blue staining. MW = 67.2kDa.

Specific Activity (lot # 25491AU): 74,150U/mg, where one unit of B-Raf equals 1 unit of MAP Kinase 2/Erk2 activity which in turn is equivalent to 1nmol phosphate incorporated into 0.33mg/ml myelin basic protein per minute at 30°C with a final ATP concentration of 100 μ M.

MEK1, unactive (recombinant protein expressed in *E. coli*)

Product Description: N-terminal GST, and C-terminal His₆-tagged, recombinant full-length human MEK1 expressed in *E. coli*. Purified using glutathione-agarose followed by Ni²⁺/NTA-agarose. Purity 71% by SDS PAGE and Coomassie blue staining. MW = 71kDa. Maximally activated with B-Raf, Raf-1 or MEKK.

Specific Activity (lot # 25557AU): As provided, this lot demonstrated <2% of maximum activity.

Anti-phospho-MEK1 (Ser218/222)/MEK2 (Ser222/226) (rabbit polyclonal IgG)

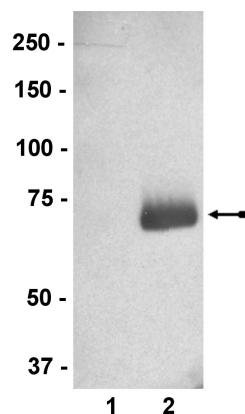
Immunogen: KLH-conjugated, synthetic peptide corresponding to amino acids surrounding 218 and 222 of human MEK1. The immunizing sequence is highly conserved.

Specificity: Recognizes MEK1/MEK2, Mr 45kDa. A non-specific protein may also be detected in HEK293 cells, Mr 50kDa.

Species Cross-reactivity: Human, mouse and rat.

Quality Control Testing

B-Raf Kinase Assay: This lot was tested by using 100ng of active B-Raf (Catalog # 14-530) to activate 500ng of MEK1, unactive (Catalog # 14-420). Magnesium/ATP Cocktail (Catalog # 20-113) was used at a final assay concentration of 100 μ M. The reaction was incubated at 30°C for 30 minutes in a shaker-incubator. Anti-phospho-MEK1 (Catalog # 07-461) was used at 0.5-2 μ g/ml to detect the phosphorylated MEK1.



B-Raf Kinase Assay
Representative blot from a previous lot. MEK1, unactive was untreated (lane 1) or treated with 10ng of B-Raf using the protocol described on page four (lane 2). The reaction was resolved by electrophoresis, transferred to nitrocellulose, and probed with anti-phospho-MEK1 (1 μ g/ml). Proteins were visualized using a goat anti-rabbit secondary antibody and a chemiluminescence detection system. Arrow indicates phospho-MEK1 (~71kDa).

References:

1. Alessi, D.R., *et al.*, *Methods Enzymol.* **255**: 279-289, 1995.
2. Alessi, D.R., *et al.*, *EMBO J.* **13**: 1610-1619, 1994.
3. Van Aelst, L., *et al.*, *Proc. Natl. Acad. Sci. USA* **90**: 6213-6217, 1993.
4. Jelinek, T., *et al.*, *Mol. Cell. Biol.* **14**: 8212-8218, 1994.
5. Rossomando, A.J., *et al.*, *Mol. Cell. Biol.* **14**: 1594-1602, 1994.
6. Reuter, C.W., *et al.*, *J. Biol. Chem.* **270**: 7645-7655, 1995.
7. Barnier, J.V., *et al.*, *J. Biol. Chem.* **270**: 23381-23389, 1995.

B-Raf Kinase Assay Kit Protocol Overview

ADBI and Magnesium/ATP cocktail must be rapidly thawed and mixed completely before proceeding with assay.

Store all components on ice. Do not use extended thawing time. The assay components can be refrozen at -20°C for extended periods.

Perform all pre-incubation reactions at 1°C over an ice bath.

Controls for endogenous phosphorylation of proteins in the sample extract can be performed by substituting ADBI for MEK1, inactive cocktail.

Stock Solutions

1. Assay Dilution Buffer I (ADBI, Catalog # 20-108)
20mM MOPS, pH 7.2, 25mM β -glycerol phosphate, 5mM EGTA, 1mM sodium orthovanadate, 1mM dithiothreitol.
2. Magnesium/ATP Cocktail (Catalog # 20-113):
500 μ M cold ATP and 75mM magnesium chloride in ADBI.
3. B-Raf (Δ 1-415), active (Catalog # 14-530): Use 1 μ l per assay point, as provided.
4. MEK1, inactive (Catalog # 14-420): Use 1 μ l per assay point, as provided.
5. Anti-phospho-MEK1 (Ser218/222)/MEK2 (Ser222/226), (Catalog # 07-461): Use at 1 μ g/ml.

B-Raf Kinase Assay Protocol

1. Add 20 μ l of Magnesium/ATP cocktail to a microcentrifuge tube.
2. Add either **1.0 μ l (0.1 μ g) of B-Raf (Δ 1-415), active** (Catalog # 14-530), or a cell or tissue extract containing B-Raf.
3. Add 1 μ l (1 μ g) of **MEK1, inactive**.
4. Add 22 μ l of ADBI.
5. Use a microcentrifuge pulse to collect all of the components into the bottom of the tube and gently vortex.
6. Incubate for 30 minutes at 30°C in a shaking incubator. **Make sure the sample is mixed thoroughly.**
7. Add an equal volume (40 μ l) of reducing sample buffer and boil for 5 minutes.
8. Perform SDS-polyacrylamide gel electrophoresis (SDS-PAGE) using 10 μ l per well and transfer the proteins to nitrocellulose. Wash the blotted nitrocellulose twice with water.
9. Block the blotted nitrocellulose in freshly prepared 5% nonfat dry milk (Catalog # 20-200) in TBS with 0.05% Tween 20 (TBST-MLK) for 1 hour at room temperature with constant agitation.
10. Incubate the nitrocellulose with **1 μ g/ml of anti-phospho-MEK1 (Ser218/222)/MEK2 (Ser222/226)** diluted in freshly prepared TBST-MLK overnight with agitation at 4°C.
11. Wash the nitrocellulose twice with water.
12. Incubate the nitrocellulose in the secondary reagent of choice (a goat anti-rabbit HRP conjugated IgG, Catalog # 12-348, 1:5000 dilution was used) in TBST-MLK for 1 hour with agitation at room temperature.
13. Wash the nitrocellulose twice with water.
14. Wash the nitrocellulose in TBS-0.05% Tween 20 for 3-5 minutes.
15. Rinse the nitrocellulose in 4-5 changes of water.
16. Use detection method of choice (enhanced chemiluminescence was used).