



cell signaling solutions

Certificate of Analysis

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B-Raf Kinase Cascade Assay Kit

Catalog # 17-358

Lot # 26724

Kit Components

B-Raf ($\Delta 1-415$), active, Catalog # 14-530, Lot # 26145U, see page two for more information. One vial containing **5 μ g** of active B-Raf 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-mercaptoethanol.

MEK1, unactive, Catalog # 14-205, Lot # 23925U, see page two for more information. Two vials, each vial containing **12.5 μ g** of MEK1 in **50 μ l** of 50mM Tris-HCl, pH 7.5, 0.1mM EGTA, 0.03% Brij-35, 0.1% 2-mercaptoethanol, 150mM NaCl, 0.2mM PMSF, 1mM benzamidine, 5% glycerol.

Myelin Basic Protein, Catalog # 13-104B, Lot # 27413, **1mg** MBP in **200 μ l** of 10mM MOPS, pH 7.0, with 0.05% sodium azide. Final concentration: 5mg/ml. Frozen solution.

MAP Kinase 2/Erk2, unactive, Catalog # 14-198, Lot # 26367U, see page two for more information. One vial containing **50 μ g** of recombinant enzyme in **200 μ l** of 50mM Tris-HCl, pH 7.5, 150mM NaCl, 0.1mM EGTA, 0.03% Brij-35, 0.1% 2-mercaptoethanol, 1mM benzamidine, 0.1mM PMSF and 50% glycerol.

Assay Dilution Buffer I (ADBI), Catalog # 20-108. Two vials, each 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.

P81 Phosphocellulose Squares, Catalog # 20-134. One pouch containing 200 pre-labeled squares.

Kit Description

Quantity: 50 kinase assays per kit.

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

Use: The assay kit is designed to measure B-Raf dependent phosphotransferase activity in a kinase cascade reaction using recombinant MEK1, unactive as a B-Raf substrate. Recombinant MAPK2/Erk2, unactive is phosphorylated and activated by the activated MEK1 leading to phosphorylation of a MAP Kinase substrate, myelin basic protein (MBP). *Whole cell and tissue extracts expressing B-Raf may be used with this kit but there may be varying levels of activated MEK1, activated MAPK2 and kinases that have phosphotransferase activity towards MBP, which will interfere with this assay. Other activators such as MEK Kinase and Mos may be present. Therefore, for best results B-Raf should be immunoprecipitated from whole cell and tissue extracts.* The assay kit is based on the phosphorylation of MBP by a B-Raf activated kinase cascade using $[\gamma\text{-}^{32}\text{P}]\text{ATP}$ as the final phosphate donor. Phosphorylated MBP is then separated from the residual $[\gamma\text{-}^{32}\text{P}]\text{ATP}$ by differential binding to P81 phosphocellulose paper followed by extensive washing with phosphoric acid. The level of incorporation is subsequently determined by liquid scintillation counting. The assay is linear for incubation times of up to 30 minutes and incorporation of up to 20% of total ATP. Longer incubation times or higher levels of incorporation may not be linear and therefore may not be a true indication of B-Raf activity in the sample extract. The enzyme assay is rapid, convenient and specific for B-Raf. Each kit contains sufficient reagents for 50 individual B-Raf assays. Enough B-Raf is supplied for 25 reactions of the kinase cascade summarized on page two.

**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 Cascade Reaction

MEK1, unactive + ATP $\xrightarrow{\text{Active B-Raf, 30}^\circ\text{C, 30 min}}$ Active MEK1* +ADP

MAP Kinase 2/Erk2, unactive + ATP $\xrightarrow{\text{Active MEK1, 30}^\circ\text{ for 30 min}}$ Active MAPK2* + ADP

MBP + [γ - ^{32}P]ATP $\xrightarrow{\text{Active MAPK2, 30}^\circ\text{C, 10 min}}$ [^{32}P]-MBP + ADP

*MEK1 and MAP Kinase 2/Erk2 are activated simultaneously.

Technical Information for Kit Components

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

Product Description: N-terminal GST-tagged recombinant human B-Raf residues 416-end. Expressed by baculovirus in Sf21 insect cells. Purified using glutathione-agarose. Purity 32% by SDS-PAGE and Coomassie blue staining. MW = 67.2kDa.

Specific Activity (lot # 23887): 128,586U/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 0.67mg/ml myelin basic protein per minute at 30°C with a final ATP concentration of 167 μ M.

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

Product Description: Recombinant rabbit MEK1 fused with GST at the N terminus and His₆ at the C terminus (71kDa fusion protein), expressed in *E. coli*. The enzyme has been purified by glutathione-agarose chromatography and nickel agarose. Purity 90% by SDS-PAGE.

Specific Activity (lot # 23925U): As provided, this lot demonstrated <10% of maximum activity as assayed by Raf-1. Activated by phosphorylation with B-Raf, Raf-1 or MEKK.

MAP Kinase 2/Erk2, unactive (recombinant protein expressed in *E. coli*)

Product Description: Recombinant full-length mouse p42 MAP Kinase 2/Erk2 fused with GST at the N terminus and expressed in *E. coli*. Purified using glutathione-agarose. Purity 94% by SDS-PAGE. MW = 67.8kDa.

Specificity: Recognizes the phosphorylation site motif: Pro-X-(Ser/Thr)-Pro, where X is ideally a basic or neutral amino acid residue. MAP Kinase 2/Erk2 can phosphorylate a range of proteins *in vitro* including p90^{rsk}, p70 S6 kinase, p74^{raf1}, EGF receptor, Myc, and Jun.

References:

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Rossomando, A.J., *et al.*, Mol. Cell. Biol. **14**: 1594-1602, 1994.
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Wu, J., *et al.*, Science **262**: 1065-1069, 1993.
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Other components required but not included as part of kit:

- cell or tissue extract containing B-Raf
- acetone
- vortex mixer
- plexiglass shielding
- shaking incubator
- timer
- variable volume (5-200ml) pipet and tips
- phosphoric acid
- microcentrifuge tube
- scintillation vials
- scintillation fluid
- scintillation counter
- [γ -³²P]ATP - ~3000Ci/mmol, obtained from PerkinElmer, Catalog # BLU002A

Safety Warnings and Precautions: The B-Raf kinase cascade kit is designed for research use only and not recommended for internal use in humans or animals. Since the kit involves the use of radioactive [γ -³²P]ATP, please follow your institutional instructions for handling, use, storage and disposal of such materials. All chemicals should be considered potentially hazardous and the principles of good laboratory practice should be followed.

B-Raf Kinase Cascade 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 -70°C for extended periods.

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

Suitable blanks should always be performed to correct for non-specific binding of [γ -³²P]ATP and its breakdown products to the phosphocellulose paper. Controls for endogenous phosphorylation of proteins in the sample extract can be performed by substituting ADBI for substrate 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): Dilute to 0.4ng/ μ l with ADBI containing 1mg/ml of BSA. Use 2.5 μ l per assay point.
4. MEK1, unactive (Catalog # 14-205): Use at 0.4 μ g per assay point.
5. MAP Kinase 2/Erk2, unactive (Catalog # 14-198): Use at 1.0 μ g per assay point.
6. MBP Substrate: Dilute MBP, bovine, purified (Catalog # 13-104) with ADBI to prepare a 2mg/ml stock solution. Use 10 μ l per assay point.
7. [γ -³²P]ATP: Stock 1mCi/100 μ l (3000Ci/mmol, obtained from PerkinElmer, Catalog # BLU002A). Make 10 μ l aliquots (100 μ Ci/vial). Before starting the assay, dilute an aliquot to 1 μ Ci/ μ l with 90 μ l of Magnesium/ATP Cocktail.

B-Raf Kinase Cascade Assay Protocol

First Stage: B-Raf Dependent Activation of MAP Kinase 2/Erk2, unactive

1. Add 10 μ l of Magnesium/ATP cocktail.
2. Add either 2.5 μ l (1ng) of diluted B-Raf (Δ 1-415), or a cell or tissue extract containing B-Raf, in conjunction with 1.6 μ l (0.4 μ g) MEK1, unactive.
3. Add 4 μ l (1.0 μ g) of MAP Kinase 2/Erk2, unactive.
4. Add ADBI to bring the volume up to 38 μ l per assay tube.
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. Remove 4 μ l of this mixture and add to Second Stage component mixture.

Technical Note: As an alternative to scintillation counting, the first stage phosphorylation reaction may be performed with the diluted [γ -³²P] ATP. A sample of the radiolabeled reaction mixture may then be analyzed by a combination of SDS-PAGE and autoradiography to determine B-Raf activity qualitatively by the visualization of phosphorylated MEK1 and MAPK.

Second Stage: Phosphorylation of MBP

1. Add 10 μ l of ADBI to a microcentrifuge tube containing 4 μ l of First Stage reaction mixture.
2. Add 10 μ l of diluted MBP substrate.
3. Add 10 μ l of the diluted [γ -³²P] ATP.
4. Use a microcentrifuge pulse to collect all of the components into the bottom of the tube and vortex gently.
5. Incubate for 10 minutes at 30°C in a shaking incubator. **Make sure the sample is mixed thoroughly.**
6. Slowly spot 25 μ l onto the center of a 2cm x 2cm P81 paper.
7. Wash assay squares three times with 0.75% phosphoric acid for 10 minutes per wash.
8. Wash assay squares once with acetone for five minutes.
9. Transfer assay squares to a scintillation vial and add 5ml scintillation cocktail.
10. Read in scintillation counter. Compare CPM of enzyme samples to CPM of control samples that contain no enzyme (background control).

Technical Note: Allow the radiolabeled substrate to bind to the filter paper for 30 seconds before immersing the paper into a 50ml conical tube containing 40ml 0.75% phosphoric acid. Gently shake the assay squares for 5 minutes on a rotator. Discard the wash in a liquid radioisotope waste container, (dispose of per institutional regulations) and repeat the wash step twice. Wash the squares in 20ml of acetone for 5 minutes. Drain and add scintillation cocktail.

Quality Control Testing

B-Raf Kinase Cascade Assay: This lot of enzyme was tested by using active B-Raf and MEK1, unactive to activate MAP Kinase 2/Erk2 (Catalog # 14-198). Activation of MAP Kinase 2/Erk2 was demonstrated by phosphorylation of MBP (Catalog # 13-104) substrate *in vitro*. MAP Kinase activity was determined using the B-Raf Kinase Cascade Assay Kit. Test results are shown below.

B-Raf (Δ 1-415)	MEK1	MAPK 2/Erk2	MBP	Mean CPM	Comments
1ng	none	1 μ g	20 μ g	6,602	B-Raf Background
none	0.4 μ g	1 μ g	20 μ g	8,683	MEK1 Background
none	None	none	20 μ g	15,216	Substrate Background
1ng	0.4 μ g	1 μ g	20 μ g	422,547	MEK1-dependent MAPK activity