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Certificate of Analysis

MKK4/SKK1, unactive

(Recombinant enzyme expressed in *E.coli* cells)
Item # 14-378, 14-378-K, 14-378M
Parent Lot # 30901U

The data presented in this document apply to the parent lot shown above and to all pack sizes derived from subsequent vialling runs of this parent lot. An alphabetical suffix after the parent lot number is used to denote each vialling run.

Product Description: *N*-terminal GST-tagged, recombinant, mouse MKK4, amino acids 34–end expressed in *E.coli* cells. Purified using glutathione-agarose. Purity 50%. MW = 67.8kDa.

Specific Activity (Parent lot# 30901U): As provided, this lot demonstrated <2.5% of maximum activity. Activated by phosphorylation with MEKK (cat# 14-196).

Formulation: 3.945mg/ml of enzyme in 50mM Tris/HCl pH7.5, 150mM NaCl, 50% glycerol, 1mM benzamidine, 0.2mM PMSF, 0.1mM EGTA, 0.1% 2-mercapoethanol, 0.03% Brij-35. Liquid at -20°C.

Storage and Stability: On receipt of material store at -20°C. Unopened reagent is stable for a minimum of 1 year from date of shipment when stored at recommended storage temperature. Avoid repeat freeze/thaw cycles. For maximum recovery of product, centrifuge original vial prior to removing the cap.

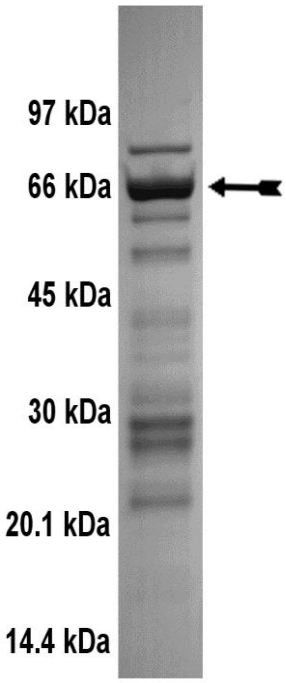
FOR IN VITRO RESEARCH USE ONLY
NOT FOR USE IN HUMANS OR ANIMALS

Quality Control Testing

Activation Assay: 4µM of MKK4, unactive was activated using 0.1mg/ml MEK kinase, then diluted 4-fold, used to activate JNK1α1, and the increased activity of the JNK 1α1 against ATF-2 determined. The activation of MKK4, subsequent activation of JNK 1α1, and assay are described on page two. Results of this assay are shown below.

Active MEKK	Unactive MKK4	Mean cpm	Comment
None	6.8µg	531	MKK4 unactive
2.5µg	6.8µg	22761	Activated MKK4

MS Tryptic Fingerprint: Confirmed identity as MKK4 with the translated native sequence listed on page four.



SDS-PAGE and Coomassie Stain: Representative gel from this lot. Purity was assessed by SDS-PAGE and Coomassie blue staining using 3µg of MKK4, unactive.

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Kinase Assay Protocol

Stock Solutions:

1. **5 x Activation Buffer:** 250mM Tris/HCl pH7.5, 0.5mM EGTA, 0.5% 2-mercaptoethanol.
2. **10 x Assay Buffer:** 500mM Tris/HCl pH7.5, 1mM EGTA, 1mM Na_3VO_4, 1% 2-mercaptoethanol.
3. **Dilution Buffer:** 50mM Tris/HCl pH7.5, 0.1mM EGTA, 0.1mM Na_3VO_4, 0.1% 2-mercaptoethanol 1mg/ml BSA.
4. **Stages One and Two 5 x Mg/ATP:** 50mM magnesium acetate, 0.5mM ATP.
5. **MEKK, active (Catalogue# 14-196):** Use at a final assay concentration of 0.1mg/ml. Dilute with Dilution Buffer to 1.00mg/ml. Add 2.5µl of stock per assay point.
6. **MKK4, unactive:** Use at a final assay concentration of 4µM (0.271mg/ml). Dilute with Dilution Buffer to 2.71mg/ml. Use 2.5µl of stock per assay point.
7. **JNK1α1, unactive (Catalogue#14-328):** Use at a final assay concentration of 2µM (0.09mg/ml). Dilute with Dilution Buffer to 0.45mg/ml. Add 5µl of stock per assay point.
8. **ATF-2 (Catalogue# 12-367):** Use at a final assay concentration of 3µM (0.108mg/ml). Dilute with Dilution Buffer to 1.08mg/ml. Add 2.5µl of stock per assay point.
9. **[γ-^{33}P]ATP:** 2.5 x magnesium acetate/[γ-^{33}P]ATP cocktail: 25mM magnesium acetate and 0.25mM ATP to which is added [γ-^{33}P]ATP (specific activity approximately 500 - 800cpm/pmol as required.)

Assay Procedure:

Stage One: *Activation of MKK4*

1. Add 5µl of 5 x activation buffer to a microcentrifuge tube.
2. Add **2.5µl (6.8µg) of MKK4 unactive**.
3. Add 2.5µl (2.5µg) **MEKK, active**.
4. Add 10µl of dH_2O.
5. Add 5µl stages one and two 5 x Mg/ATP.
6. In appropriate controls, add dilution buffer to a final volume of 25µl.
7. Incubate for 60 minutes at 30°C.
8. Stop the reaction by diluting 4–fold in dilution buffer. Store on ice.

Stage Two: *Activation of JNK 1α1.*

1. Add 5µl 5 x activation buffer to a microcentrifuge tube.
2. Add 5µl (2.25µg) of **JNK 1α1 unactive**.
3. Add 2.5µl of activated MKK4 from **Stage One**.
4. Add 7.5µl of dH_2O.
5. Add 5µl of stages one and two 5 x Mg/ATP.
6. Incubate for 15 minutes at 30°C.
7. Immediately transfer 2.5µl of the reaction product to the **Stage Three** component mixture.

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Stage Three: *Phosphorylation of ATF-2 by Activated JNK 1α1.*

1. Add 2.5µl 10 x assay buffer to a microcentrifuge tube.
2. Add 2.5µl (2.7µg) **ATF-2**.
3. Add 7.5µl of dH_2O.
4. Add 10µl of the 2.5 x magnesium acetate/ [γ-^{33}P]ATP cocktail.
5. Add 2.5µl of JNK 1α1 from **Stage Two** to start the reaction.
6. Incubate for 15 minutes at 30°C.
7. Spot 20µl onto the centre of a 2cm x 2cm **P81** paper.
8. Wash the assay squares twice for 5 minutes with 75mM phosphoric acid.
9. Wash the assay squares once for 2 minutes with acetone.
10. Transfer the assay squares to a scintillation vial and add 1ml of scintillation cocktail.
11. Transfer the filtermat to a sealable plastic bag and add 4ml of scintillation cocktail.
12. Read in a scintillation counter. Compare cpm of enzyme samples with cpm of control samples that contain all assay components plus 1µl of 30% phosphoric acid.

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MKK4 Sequence Information

Protein	Mouse MKK4
Tags	*N*-terminal GST
Native sequence	S226 of the recombinant protein corresponds to S34 of mouse MKK4
Accession number	EMBL U18310

Recombinant MKK4 amino acid sequence:

```
   1 MSPILGYWKI KGLVQPTRLL LEYLEEKYEE HLYERDEGDK WRNKKFELGL EFPNLPYYID
  61 GDVKLTQSMA IIRYIADKHN MLGGCPKERA EISMLEGAVL DIRYGVSRIA YSKDFETLKV
 121 DFLSKLPEML KMFEDRLCHK TYLNGDHVTH PDFMLYDALD VVLYMDPMCL DAFPKLVCFK
 181 KRIEAIPQID KYLKSSKYIA WPLQGWQATF GGGDHPPKSD LVPRGSMQGK RKALKLNFAN
 241 PPVKSTARFT LNPNTTGVQN PHIERLRTHS IESSGKLKIS PEQHWDFTAE DLKDLGEIGR
 301 GAYGSVNKMV HKPSGQIMAV KRIRSTVDEK EQKQLLMDLD VVMRSSDCPY IVQFYGALFR
 361 EGDCWICMEL MSTSFDKFYK YVYSVLDDVI PEEILGKITL ATVKALNHLK ENLKIIHRDI
 421 KPSNILLDRS GNIKLCDFGI SGQLVDSIAK TRDAGCRPYM APERIDPSAS RQGYDVRSDV
 481 WSLGITLYEL ATGRFPYPKW NSVFDQLTQV VKGDPPQLSN SEEREFSPSF INFVNLCLTK
 541 DESKRPKYKE LLKHPFILMY EERTVEVACY VCKILDQMPA TPSSPMYVD
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Recombinant MKK4 nucleotide sequence:

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   1 atgtccccta tactaggtta ttggaaaatt aagggccttg tgcaacccac tcgacttctt
  61 ttggaatatc ttgaagaaaa atatgaagag catttgtatg agcgcgatga aggtgataaa
 121 tggcgaaaca aaaagtttga attgggtttg gagtttccca atcttcctta ttatattgat
 181 ggtgatgtta aattaacaca gtctatggcc atcatacgtt atatagctga caagcacaac
 241 atgttgggtg gttgtccaaa agagcgtgca gagatttcaa tgcttgaagg agcggttttg
 301 gatattagat acggtgtttc gagaattgca tatagtaaag actttgaaac tctcaaagtt
 361 gattttctta gcaagctacc tgaaatgctg aaaatgttcg aagatcgttt atgtcataaa
 421 acatatttaa atggtgatca tgtaacccat cctgacttca tgttgtatga cgctcttgat
 481 gttgttttat acatggaccc aatgtgcctg gatgcgttcc caaaattagt ttgttttaaa
 541 aaacgtattg aagctatccc acaaattgat aagtacttga aatccagcaa gtatatagca
 601 tggcctttgc agggctggca agccacgttt ggtggtggcg accatcctcc aaaatcggat
 661 ctggttccgc gtggatccat gcagggtaag cgcaaagcac tgaagttgaa ttttgcaaat
 721 ccacctgtca aatcgacagc acggtttacc ctgaatccta atactacagg agtccagaac
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1201 gcaactgtga aagcactaaa ccacttaaaa gaaaacttga aaattattca cagagacatc
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1321 agtggacagc ttgtggactc tattgccaag acaagagatg ctgggtgtag gccgtatatg
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1561 tctgaagaaa gggagttctc ccccagtttc atcaactttg tcaacttgtg ccttacgaag
1621 gatgaatcca aaaggccaaa gtataaagag cttctgaaac atccctttat tttgatgtat
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1681 gaagaacgta ctgtagaggt cgcatgctat gtttgtaaaa tcctggatca gatgccagcc
1741 actcccagct cgcccatgta tgtcgactga
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Reviewed and approved by site quality representative.