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Certificate of Analysis

B-Raf (V599E), active
Also designated B-Raf (V600E)
(Recombinant enzyme expressed in Sf21 insect cells)
Item # 14-557, 14-557-K, 14-557M
Parent Lot # 28439U

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 human B-Raf residues 416–end, containing a V599E mutation. Expressed by baculovirus in Sf21 insect cells. Purified using glutathione agarose. Purity 44% by SDS-PAGE and Coomassie blue staining. MW = 67.3kDa.

Specific Activity (Parent lot# 28439U): 167914U/mg, where one unit of B-Raf, active = 1 unit of MAPK2(m) 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 100µM. Note the activity is determined by a triple linked assay which involves the activation of MEK1(h) by B-Raf, followed by the subsequent activation of MAPK2(m) by the activated MEK1(h).

Formulation: 0.823mg/ml of enzyme in 50mM Tris/HCl pH7.5, 150mM NaCl, 0.1mM EGTA, 0.03% Brij-35, 270mM sucrose, 1mM benzamidine, 0.2mM PMSF, 0.1% 2-mercaptoethanol. Frozen solution.

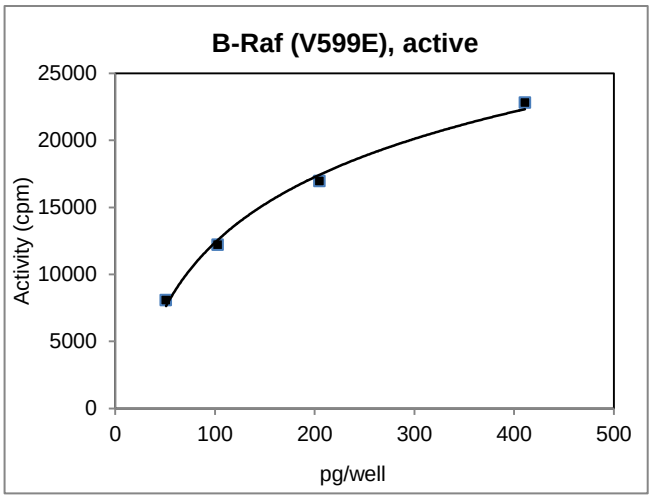
Storage and Stability: On receipt of material store at -70°C. Unopened reagent is stable for a minimum of 1 year from date of shipment when stored at recommended storage temperature. Avoid repeat freeze/thaw cycles. For maximum recovery of product, centrifuge original vial prior to removing the cap.

Handling Recommendations: Rapidly thaw the vial under cold water and immediately place on ice. Aliquot unused material into pre-chilled micro-centrifuge tubes and immediately snap-freeze the vials in liquid nitrogen prior to re-storage at -70°C.

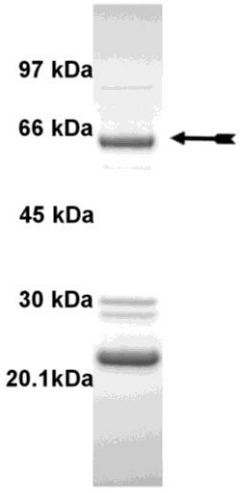
FOR IN VITRO RESEARCH USE ONLY
NOT FOR USE IN HUMANS OR ANIMALS

Quality Control Testing

Kinase Assay: 51-411pg of this lot of enzyme was used to activate 0.2µM MEK1(h) in the assay described on page two. Assay background was subtracted from the actual counts to yield the results.



MS Tryptic Fingerprint: Confirmed product identity as B-Raf with the translated sequence listed on page three.



SDS-PAGE and Coomassie Stain: Purity was assessed by SDS-PAGE and Coomassie blue staining using 3µg of B-Raf, active.

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Kinase Assay Protocol

Stock Solutions:

1. **10 x Reaction Buffer:** 500mM Tris/HCl pH7.5, 1mM EGTA, 1mM Na_3VO_4, 1% 2-mercaptoethanol, 0.1% Brij-35.
2. **MEK1(h), unactive:** Use at a final assay concentration of 0.2µM (0.0126mg/ml). Prepare a 0.126mg/ml stock and add 2.5µl stock per assay point.
3. **MAPK2(m), unactive:** Use at a final assay concentration of 2µM (0.14mg/ml). Prepare a 0.7mg/ml stock and add 5µl of stock per assay point.
4. **Myelin Basic Protein (MBP):** Use at a final assay concentration of 0.33mg/ml. Prepare a 3.33mg/ml stock and add 2.5µl stock per assay point.
5. **B-Raf, active:** Dilute with 25mM Tris/HCl pH7.5, 0.1mM EGTA, 1mg/ml BSA. Use 51-411pg per assay point.
6. **[γ-^{33}P]ATP:** 2.5 x magnesium acetate/[γ-^{33}P]ATP cocktail: 25mM MgAc and 0.25mM ATP to which is added [γ-^{33}P]ATP (specific activity approximately 500 - 800cpm/pmol as required.)

Assay Procedure (96 well plate format):

1. Add 2.5µl of 10 x reaction buffer to wells.
2. Add 2.5µl of **MEK1(h), unactive**.
3. Add 2.5µl of **MAPK2 (m), unactive**.
4. Add 2.5µl of **MBP**.
5. Add **5µl (51–411pg) B-Raf, active**.
6. Add 10µl of diluted [γ-^{33}P] ATP mixture.
7. Incubate for 10 minutes at 30°C.
8. Stop the reaction by adding 5µl 3% phosphoric acid to each well.
9. Using a multichannel pipette, spot 10µl onto the appropriate area of a **P30 Filtermat**.
10. Wash the filtermat twice for 5 minutes with 75mM phosphoric acid.
11. Wash the filtermat once for 2 minutes with methanol.
12. Once dry, transfer the filtermat to a sealable plastic bag and add 4ml of scintillation cocktail.
13. 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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B-Raf (V599E) Sequence Information

Protein Human B-Raf (V599E)

Tags *N*-Terminal GST

Native sequence Q237 of the recombinant protein is equivalent to Q416 of human B-Raf

Accession number GenBank NM_004333.
The V599E mutation is thought to mimic phosphorylation of the native enzyme, resulting in a protein with high activity and leading to constitutive ERK activation. As B-Raf is commonly activated by somatic point mutation in human cancer, it may provide a new therapeutic approach to malignant melanoma. (Davies H., *et al., Nature*, 2002. **417**: 949-54, and Mercer KE., & Pritchard CA., *Biochim Biophys Acta.* 2003. **1653**: 25-40)

Recombinant B-RAF (V599E) amino acid sequence:

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  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 LEVLFQGPEF KGLRRQQKSP
241 GPQRERKSSS SSEDRNRMKT LGRRDSSDDW EIPDGQITVG QRIGSGSFGT VYKGKWHGDV
301 AVKMLNVTAP TPQQLQAFKN EVGVLRKTRH VNILLFMGYS TKPQLAIVTQ WCEGSSLYHH
361 LHIIETKFEM IKLIDIARQT AQGMDYLHAK SIIHRDLKSN NIFLHEDLTV KIGDFGLATE
421 KSRWSGSHQF EQLSGSILWM APEVIRMQDK NPYSFQSDVY AFGIVLYELM TGQLPYSNIN
481 NRDQIIFMVG RGYLSPDLSK VRSNCPKAMK RLMAECLKKK RDERPLFPQI LASIELLARS
541 LPKIHRSASE PSLNRAGFQT EDFSLYACAS PKTPIQAGGY GAFPVH
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Recombinant B-RAF (V599E) 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 ctggaagttc tgttccaggg gcccgaattc aaaggcctac gtcgacaaca gaaatctcca
 721 ggacctcagc gtgaaaggaa gtcatcttca tcctcagaag acaggaatcg aatgaaaaca
 781 cttggtagac gggactcgag tgatgattgg gagattcctg atgggcagat tacagtggga
 841 caaagaattg gatctggatc atttggaaca gtctacaagg gaaagtggca tggtgatgtg
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1081 ctccatatca ttgagaccaa atttgagatg atcaaactta tagatattgc acgacagact
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1201 aatatatttc ttcatgaaga cctcacagta aaaataggtg attttggtct agctacagag
1261 aaatctcgat ggagtgggtc ccatcagttt gaacagttgt ctggatccat tttgtggatg
1321 gcaccagaag tcatcagaat gcaagataaa aatccataca gctttcagtc agatgtatat
1381 gcatttggaa ttgttctgta tgaattgatg actggacagt taccttattc aaacatcaac
1441 aacagggacc agataatttt tatggtggga cgaggatacc tgtctccaga tctcagtaag
1501 gtacggagta actgtccaaa agccatgaag agattaatgg cagagtgcct caaaaagaaa
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1561 agagatgaga gaccactctt tccccaaatt ctcgcctcta ttgagctgct ggcccgctca
1621 ttgccaaaaa ttcaccgcag tgcatcagaa ccctccttga atcgggctgg tttccaaaca
1681 gaggatttta gtctatatgc ttgtgcttct ccaaaaacac ccatccaggc agggggatat
1741 ggtgcgtttc ctgtccactg a
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Reviewed and approved by site quality representative.