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Certificate of Analysis

A-Raf, active
(Recombinant enzyme expressed in Sf21 insect cells)

Item # 14-956, 14-956-K, 14-956M

Parent Lot # D14SP002N

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 A-Raf, amino acids 273-end, containing the mutations Y301D and Y302D, expressed by baculovirus in Sf21 insect cells. Purified using glutathione agarose.
Purity 58% by SDS-PAGE and Coomassie blue staining. MW = 65kDa.

Specific Activity (Parent lot# D14SP002N): 749U/mg, where one unit of A-Raf, active activity is defined as 1nmol phosphate incorporated into 0.5mg/ml myelin basic protein per minute at 30°C with a final ATP concentration of 100µM.

Formulation: 0.14mg/ml of enzyme in 40mM Tris/HCl pH8.0, 200mM NaCl, 0.08mM EGTA, 0.024% Brij-35, 4mM glutathione, 20% (v/v) glycerol, 0.8mM benzamidine, 0.16mM PMSF, 0.08% 2-mercaptoethanol. Frozen solution.

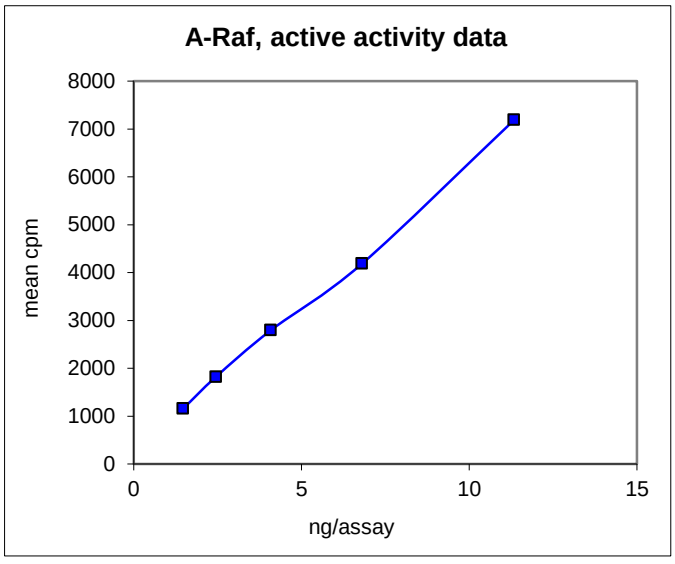
Storage and Stability: On receipt of material store at -70°C. Unopened reagent is stable for a minimum of 1 year from date of shipment when stored at recommended storage temperature. Avoid repeat freeze/thaw cycles. For maximum recovery of product, centrifuge original vial prior to removing the cap.

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.

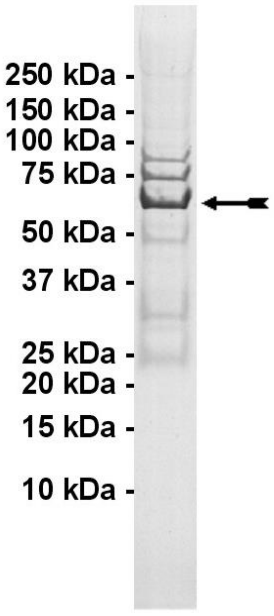
FOR IN VITRO RESEARCH USE ONLY
NOT FOR USE IN HUMANS OR ANIMALS

Quality Control Testing

Kinase Assay: 1.5–11.3ng of this lot of enzyme phosphorylated 0.5mg/ml myelin basic protein in the assay described on page two. Assay background was subtracted from the actual counts to yield the results shown below.



MS Tryptic Fingerprint: Confirmed identity as A-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 A-Raf, active.

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Kinase Assay Protocol

Stock Solutions:

1. **2 x Reaction Buffer:** 50mM Tris/HCl pH7.5, 0.4mM EGTA, 1mM sodium orthovanadate, 1mM sodium β-glycerophosphate, 0.02% Triton X-100, 2% glycerol, and 20mM DTT.
2. **Myelin Basic Protein**: Use at a final assay concentration of 0.5mg/ml. Prepare a 10mg/ml stock and add 1.25µl of stock per assay point.
3. **MEK1, unactive (cat# 14-420):** Use at a final assay concentration of 2.5µg/ml. Prepare a 10x stock in 2 x Reaction buffer and use 2.5µl per assay point.
4. **MAPK2, unactive (cat# 14-198**): Use at a final assay concentration of 4.7µg/ml. Prepare a 10x stock in 2 x Reaction buffer and use 2.5µl per assay point.
5. **A-Raf, active:** Dilute with 50mM Tris/HCl pH7.5, 0.4mM EGTA, 1mM sodium orthovanadate, 1mM sodium β-glycerophosphate, 0.02% Triton X-100, 2% glycerol, 20mM DTT, 30mM sodium chloride, and 2mg/ml BSA. Use 1.5–11.3ng per assay point.
6. **[γ-^{33}P]ATP:** 2.5 x MgAc/[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 5µl of 2 x reaction buffer per assay to wells.
2. Add 1.25µl of dH_2O.
3. Add 2.5µl of MEK1, unactive.
4. Add 2.5µl of MAPK2, unactive
5. Add 1.25µl of myelin basic protein.
6. Add **2.5µl (1.5–11.3ng) A-Raf, active.**
7. Add 10µl of diluted [γ-^{33}P]ATP mixture.
8. Incubate for 10 minutes at 30°C.
9. Stop the reaction by adding 5µl of 3% phosphoric acid.
10. Transfer a 10µl aliquot onto the appropriate area of a **P30 Filtermat.**
11. Wash the filtermat three times for 5 minutes with 75mM phosphoric acid.
12. Wash the filtermat once for 2 minutes with methanol.
13. Transfer the filtermat to a sealable plastic bag and add 4ml of scintillation cocktail.
14. 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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A-Raf Sequence Information

Protein human A-Raf

Tags *N*-terminal GST

Native sequence K231 of the recombinant protein is equivalent to K273 of human A-Raf

Accession number GenBank U01337.1
The recombinant protein contains the amino acid substitution Y301D and Y302D with reference to GenBank U01337.1. These mutations provide constitutive activation of the enzyme.

Recombinant A-Raf 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 LEVLFQGPEF KSPAEQRERK
241 SLADDKKKVK NLGYRDSGDD WEVPPSEVQL LKRIGTGSFG TVFRGRWHGD VAVKVLKVSQ
301 PTAEQAQAFK NEMQVLRKTR HVNILLFMGF MTRPGFAIIT QWCEGSSLYH HLHVADTRFD
361 MVQLIDVARQ TAQGMDYLHA KNIIHRDLKS NNIFLHEGLT VKIGDFGLAT VKTRWSGAQP
421 LEQPSGSVLW MAAEVIRMQD PNPYSFQSDV YAYGVVLYEL MTGSLPYSHI GCRDQIIFMV
481 GRGYLSPDLS KISSNCPKAM RRLLSDCLKF QREERPLFPQ ILATIELLQR SLPKIERSAS
541 EPSLHRTQAD ELPACLLSAA RLVP
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Recombinant A-Raf 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 aagtcaccag cagagcagcg cgagcggaag
 721 tccttggccg atgacaagaa gaaagtgaag aacctggggt accgggactc aggcgatgac
 781 tgggaggtac cacccagtga ggtgcagctg ctgaagagga tcgggacggg ctcgtttggc
 841 accgtgtttc gagggcggtg gcatggcgat gtggccgtga aggtgctcaa ggtgtcccag
 901 cccacagctg agcaggccca ggctttcaag aatgagatgc aggtgctcag gaagacgcga
 961 catgtcaaca tcttgctgtt tatgggcttc atgacccggc cgggatttgc catcatcaca
1021 cagtggtgtg agggctccag cctctaccat cacctgcatg tggccgacac acgcttcgac
1081 atggtccagc tcatcgacgt ggcccggcag actgcccagg gcatggacta cctccatgcc
1141 aagaacatca tccaccgaga tctcaagtct aacaacatct tcctacatga ggggctcacg
1201 gtgaagatcg gtgactttgg cttggccaca gtgaagactc gatggagcgg ggcccagccc
1261 ttggagcagc cctcaggatc tgtgctgtgg atggcagctg aggtgatccg tatgcaggac
1321 ccgaacccct acagcttcca gtcagacgtc tatgcctacg gggttgtgct ctacgagctt
1381 atgactggct cactgcctta cagccacatt ggctgccgtg accagattat ctttatggtg
1441 ggccgtggct atctgtcccc ggacctcagc aaaatctcca gcaactgccc caaggccatg
1501 cggcgcctgc tgtctgactg cctcaagttc cagcgggagg agcggccccc cttcccccag
1561 atcctggcca caattgagct gctgcaacgg tcactcccca agattgagcg gagtgcctcg
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1621 gaaccctcct tgcaccgcac ccaggccgat gagttgcctg cctgcctact cagcgcagcc
1681 cgccttgtgc cttag
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Reviewed and approved by site quality representative.