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Recombinant Human Active Raf-1 (Y340E Y341E), aa 306-648

Catalog Number: 4540-KS

DESCRIPTION

Source	*Spodoptera frugiperda*, *Sf* 9 (baculovirus)-derived aa 306-648 Accession # NM_002880
N-terminal Sequence Analysis	Using an N-terminal GST tag
Predicted Molecular Mass	63 kDa

SPECIFICATIONS

Activity	The activity of Raf-1 is typically 5100-6900 nmol/min/mg using a myelin basic protein (MBP) substrate (see Activity Assay Protocol).
Purity	>70%, by SDS-PAGE under reducing conditions and visualized by Colloidal Coomassie® Blue stain at 5 µg per lane.
Formulation	Supplied in 50 mM Tris-HCl, pH 7.5, 150 mM NaCl, 0.25 mM DTT, 10 mM glutathione, 0.1 mM EDTA, 0.1 mM PMSF, and 25% glycerol. See Certificate of Analysis for details.

Activity Assay Protocol

Materials

- Active Kinase - Active Raf-1(EE) (0.1 µg/µL) diluted with Kinase Dilution Buffer III and assayed as outlined in Figure 2. Note: *These are suggested working dilutions. Optimal dilutions should be determined by each laboratory for each application*.
- Kinase Assay Buffer I, pH 7.2 - 25 mM MOPS, 12.5 mM β-glycerolphosphate, 25 mM $MgCl_2$, 5 mM EGTA, 2 mM EDTA. Add 0.25 mM DTT to the Kinase Assay Buffer prior to use.
- Kinase Dilution Buffer III, pH 7.2 - Kinase Assay Buffer I diluted at a 1:4 ratio (5-fold dilution) with 50 ng/µL BSA solution.
- 10 mM ATP Stock Solution - Prepare the ATP Stock Solution by dissolving 55 mg of ATP in 10 mL of Kinase Assay Buffer I. Store 200 µL aliquots at ≤ -20 °C.
- [^{33}P]-ATP Assay Cocktail - Prepare 250 µM [^{33}P]-ATP Assay Cocktail in a designated radioactive work area by combining 150 µL of 10 mM ATP Stock Solution, 100 µL of [^{33}P]-ATP (1 mCi/100 µL), and 5.75 mL of Kinase Assay Buffer I. Store 1 mL aliquots at ≤ -20 °C.
- Substrate - Inactive MEK1 and ERK1 were activated in a coupled reaction. Myelin Basic Protein (MBP) substrate diluted in distilled or deionized water to a final concentration of 1 mg/mL was subsequently used as a substrate for the activated ERK1.

Assay

1. Thaw the [^{33}P]-ATP Assay Cocktail in a shielded container in a designated radioactive work area.
2. Thaw the Active Raf-1 (EE), Kinase Assay Buffer I, inactive ERK1, and inactive MEK1 on ice.
3. In a pre-cooled microfuge tube, add the following reaction components bringing the initial reaction volume up to 20 µL.
   a. Diluted Active Raf-1(EE): 10 µL
   b. Inactive ERK1 (0.2 µg/mL): 1 µL
   c. Inactive MEK1 (0.2 µg/mL): 1 µL
   d. Kinase Dilution Buffer: 8 µL
4. Start the reaction with the addition of 5 µL ATP (250 µM) and incubate in a water bath at 30 °C for 15 minutes.
5. After the 15 minute incubation, remove 5 µL and add it to the following reaction components on ice, bringing the initial reaction volume up to 20 µL.
   a. Reaction Mixture: 5 µL
   b. Distilled or deionized water (on ice): 10 µL
   c. MBP Substrate (1 mg/mL; on ice): 5 µL
6. Set up the blank control as outlined in step 5, excluding the addition of the substrate. Replace the substrate with an equal volume of distilled or deionized water.
7. Initiate the reaction with the addition of 5 µL [^{33}P]-ATP Assay Cocktail, bringing the final volume up to 25 µL. Incubate the mixture in a water bath at 30 °C for 15 minutes.
8. After the 15 minute incubation, terminate the reaction by spotting 20 µL of the reaction mixture onto individual pre-cut strips of phosphocellulose P81 paper.
9. Air dry the pre-cut P81 strip and sequentially wash in a 1% phosphoric acid solution (dilute 10 mL of phosphoric acid and make a 1 liter solution with distilled or deionized water) with constant gentle stirring. It is recommended that the strips be washed a total of three times for approximately 10 minutes each.
10. Count the radioactivity on the P81 paper in the presence of scintillation fluid in a scintillation counter.
11. Determine the corrected cpm by removing the blank control value (see step 6) for each sample and calculate the kinase specific activity as outlined below:

Calculation of [^{33}P]-ATP Specific Activity (SA) (cpm/pmol)
Specific Activity (SA) = cpm for 5 µL [^{33}P]-ATP/pmol of ATP (in 5 µL of a 250 µM ATP stock solution; *i.e.* 1250 pmol)

Calculation of Kinase Specific Activity (SA) (pmol/minutes/µg or nmol/minutes/mg)
Corrected cpm from reaction / [(SA of 33P-ATP in cpm/pmol) x (Reaction time in minutes) x (Enzyme amount in µg or mg)] x [(Reaction volume) / (Spot Volume)]

PREPARATION AND STORAGE

Shipping	The product is shipped with dry ice or equivalent. Upon receipt, store it immediately at the temperature recommended below.
Stability & Storage	This product is stable at ≤ -70° C for up to 1 year from the date of receipt. For optimal storage, aliquot into smaller quantities after centrifugation and store at recommended temperature. **Avoid repeated freeze-thaw cycles.**

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USA & CANADA Tel: (800) 343-7475 EUROPE Tel: +44 (0)1235 529449 CHINA Tel: +86 (21) 52380373
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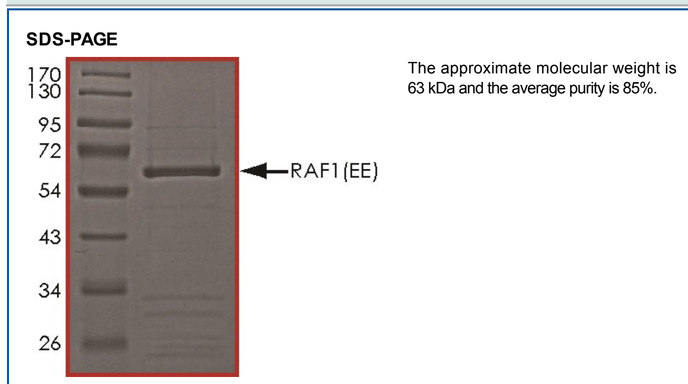
DATA



The approximate molecular weight is 63 kDa and the average purity is 85%.

BACKGROUND

Raf-1 is a MAP kinase kinase kinase (MAP3K), which functions downstream of the Ras family of membrane associated GTPases to which it binds directly (1). The activated Raf-1 can phosphorylate and activate the dual specificity protein kinases MEK1 and MEK2, which in turn phosphorylate to activate the Serine/Threonine specific protein kinases ERK1 and ERK2. Activated ERKs are pleiotropic effectors of cell physiology and play an important role in the control of gene expression involved in the cell division cycle, apoptosis, cell differentiation, and cell migration (2).

References:

1. Rapp, U. *et al.* (1983) Proc. Natl. Acad. Sci. USA **80**:4218.
2. Li, P. *et al.* (1991) Cell **64**:479.

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