Recombinant Human SMAC/Diablo

Catalog Number: 789-SM

DESCRIPTION

Source	*E. coli*-derived human SMAC/Diablo protein Ala56-Asp239, with a C-terminal 6-His tag Accession # Q9NR28
N-terminal Sequence Analysis	Ala56
Predicted Molecular Mass	22 kDa

SPECIFICATIONS

SDS-PAGE	23 kDa, reducing conditions
Activity	Measured by its ability to reverse the inhibition of DEVD-AFC cleavage activity in cell extracts activated by addition of cytochrome c and dATP. The IC_{50} for reversal of XIAP-BIR3 (50 nM) inhibition of DEVD-AFC cleavage in activated cell extracts is <2,000 nM. **Optimal dilutions should be determined by each laboratory for each application.**
Purity	>95%, by SDS-PAGE under reducing conditions and visualized by silver stain.
Formulation	Supplied as a 0.2 µm filtered solution in HEPES and NaCl. See Certificate of Analysis for details.

Activity Assay Protocol

Materials

- Jurkat E6 wild type cell extracts (see supplementary methods for preparation)
- Extraction Buffer: 50 mM HEPES, 10 mM KCl, 5 mM EGTA, 1 mM $MgCl_2$, 0.2% CHAPS, 0.2 mM DTT, pH 7.5
- Assay Buffer: 10 mM HEPES, 0.5 mM EGTA, 5 mM DTT, 10% Glycerol, pH 7.5
- Formulation Buffer: 25 mM HEPES, 0.1 M KCl, pH 7.5
- Recombinant Human SMAC/Diablo (rhSMAC) (Catalog # 789-SM)
- Recombinant Human XIAP BIR3 Domain (rhXIAP) (Catalog # 895-XB)
- Cytochrome C, Bovine heart (Sigma, Catalog # C3131), 2 mg/mL stock in deionized water
- dATP (Sigma, Catalog # D6500), 10 mM stock adjusted to pH 7.5 with NaOH
- Substrate: Ac-Asp-Glu-Val-Asp-AFC (DEVD-AFC) (MP Biomedicals, Catalog # AFC138), 10 mM stock in DMSO
- F16 Black Maxisorp Plate (Nunc, Catalog # 475515)
- Fluorescent Plate Reader (Model: SpectraMax Gemini EM by Molecular Devices) or equivalent

Assay

Note: All reagents and assay components should be kept on ice until use.

1. Thaw cell extracts and centrifuge in a microcentrifuge at 14,000 rpms for 5 minutes at 4 °C. Transfer supernatants to chilled tubes and use within 1 hour.
2. Prepare a 250 nM stock of rhXIAP (BIR3) (MW: 13.0 KDa) in Formulation Buffer.
3. Prepare a curve of rhSMAC (MW: 21.6 KDa) in Formulation Buffer. Make the following serial dilutions: 50,000, 15,000, 7500, 3000, 1500, 600 and 300 nM. Note: High point may not be achievable depending on lot received.
4. Prepare the activator mixture by combining equal volumes of 2 mg/mL Cytochrome C and 10 mM dATP for working concentrations of 1 mg/mL and 5 mM, respectively.
5. Prepare reaction mixtures in tubes by combining 5 µL of each rhSMAC curve dilution, 5 µL of rhXIAP (BIR3), 10 µL of cell extract supernatant, and 5 µL of the cytochrome C/dATP activator mixture. Also, prepare the following controls:
   a. Total Control: 10 µL of Extraction Buffer, 10 µL of cell extract supernatant, and 5 µL of the cytochrome C/dATP activator mixture.
   b. Inactive Control: 15 µL of Extraction Buffer and 10 µL of cell extract supernatant. The total reaction volume is 25 µL.
   c. rhXIAP (BIR3) only Conrol: 5 µL of Extraction Buffer, 5 µL of rhXIAP (BIR3), 10 µL of cell extract supernatant, and 5 µL of the cytochrome C/dATP activator mixture.
6. Incubate for 60 minutes at 30 °C.
7. After incubation, add 100 µL of Assay Buffer to each vial for a five-fold dilution. Mix briefly.
8. Dilute Substrate to 100 µM in Assay Buffer.
9. In a plate load 50 µL of diluted incubated reaction mixtures and start the reaction by adding 50 µL of 100 µM Substrate.
10. Read at excitation and emission wavelengths of 400 nm and 505 nm, respectively, in kinetic mode for 5 minutes.
11. Derive the 50% inhibiting concentration (IC_{50}) of rhSMAC by plotting normalized activity vs. reaction concentration of rhSMAC with 4-PL fitting.
12. Normalized activity may be determined using the following equation:

$$\% \text{ Normalized Activity} = \frac{\text{Sample (RFU/min)} - \text{Inactive Control (RFU/min)}}{\text{Total Control (RFU/min)}} \times 100\%$$

Final Assay Conditions

Per Reaction:

- rhSMAC curve: 10,000, 3000, 1500, 600, 300, 120 and 60 nM

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	**Use a manual defrost freezer and avoid repeated freeze-thaw cycles.** ● 6 months from date of receipt, -20 to -70 °C as supplied. ● 3 months, -20 to -70 °C under sterile conditions after opening.

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BACKGROUND

SMAC (second mitochondria derived activator of caspase)/Diablo promotes caspase activation by interacting with the inhibitor of apoptosis (IAP) proteins in the cytochrome c/Apaf-1/caspase-9 pathway.

SUPPLEMENTARY METHODS

SMAC/Diablo can reverse rhXIAP inhibition of DEVD-AFC cleavage in activated cell lystes.
Recombinant Human XIAP Full Length (Catalog # 822-XF) can be used in the Activity Assay Protocol in place of rhXIAP (BIR3). The IC_{50} for reversal of XIAP (500 nM) inhibition of DEVD-AFC cleavage in activated cell extracts is typically 500-1500 nM.

References:

1. Du, C. *et al.* (2000) Cell **102**:33.
2. Chai, J. *et al.* (2000) Nature **406**:855.
3. Srinivasula, S. *et al.* (2000) J. Biol. Chem. **275**:36152.
4. Ekert, P. *et al.* (2001) J. Cell Biol. **152**:483.
5. Verhagen, A. (2000) Cell **102**:43.

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