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Product Information

Caspase 9, human
C-terminal histidine-tagged protein
recombinant, expressed in *E. coli*

Catalog Number **C8726**
Storage Temperature –70 °C

EC 3.4.22.62
Synonyms: Mch6, ICE-Lap6

Product Description

Caspase 9 (Mch6, ICE-Lap6)[2,3] is a member of the CED-3 subfamily of the caspase family of cysteine proteases that play an essential role in the execution phase of apoptosis. These enzymes share a dominant primary specificity for cleaving bonds following aspartic acid residues. "Initiator" caspases, such as caspase 8, activate "effector" caspases, such as caspases 3 and 7. The effector caspases then cleave cellular substrates, ultimately leading to the morphological changes of apoptosis.[4-6]

Caspases are synthesized as inactive proenzymes. The precursor proteins contain N-terminal prosequences of various lengths followed by the p20 and p10 subunits. Caspases are activated by cleavage at specific Asp residues to produce two subunits of approximately 20 kDa (p20) and 10 kDa (p10), which together form the heterodimeric active protease.[5,6] In some cases, these subunits are separated by a linker that may be involved in regulation of the activation of the caspase. All caspases contain an active-site pentapeptide of general structure QACXG (where X is R, Q or G). The amino acids Cys^{285} and His^{237} involved in catalysis, and those involved in forming the P1 carboxylate binding pocket (Arg^{179}, Gln^{283}, Arg^{341}, and Ser^{347}) are conserved in all caspases, except for the substitution of Thr for Ser^{347} in caspase 8. This explains the absolute requirement for an Asp in the P1 position. Residues forming the P2–P4 binding pocket are not well conserved. This suggests they may determine the substrate specificities of the caspases. Evidence suggests that not all caspases are required for cell death, and some caspases appear to be more important than others.[5]

Caspase 9 is highly expressed in the heart, testis, and ovary.

When cells receive apoptotic stimuli, such as upon activation of the TNFα/Fas cell surface receptor, the receptor activation leads to caspase 8 activation, Bid processing, and its translocation to the mitochondria.[7] As a result, mitochondria release cytochrome c, which then binds to Apaf-1 together with dATP. The resulting complex then recruits caspase 9, leading to its activation. *In vitro* mutagenesis demonstrated that procaspase 9 may be activated by both caspase 3 and granzyme B.[2,3] Once activated, caspase 9 cleaves downstream caspases such as caspases 3, 6, and 7.[7,8]

The product is supplied as a solution in 10% sucrose containing 50 mM HEPES, pH 7.5, 5 mM DTT, 0.1% CHAPS, 1 mM EDTA, and 50 mM NaCl.

Caspase 9 is expressed in *E. coil* as a C-terminal histidine-tagged protein and appears as a two-subunit protein by SDS-PAGE. The 36 kDa subunit consists of the N-terminal prodomain plus the large subunit. The 13 kDa small subunit contains the histidine tag. It exhibits basal activity. It can be further activated via interaction of its N-terminal prodomain with the activator protein Apaf-1 in the presence of cytochrome c and dATP.[1]

Purity: minimum 90% (SDS PAGE)

Activity: minimum 2000 units per mg protein

Unit Definition: One unit will cleave 1.0 nmole of the substrate Ac-LEHD-AFC per hour at pH 6.5 at 25 °C.

Precautions and Disclaimer

This product is for R&D use only, not for drug, household, or other uses. Please consult the Safety Data Sheet for information regarding hazards and safe handling practices.

Storage/Stability

The product ships on dry ice and it is recommended to store the product at –70 °C. Aliquot to avoid repeated freeze-thaw cycles. Storage in 'frost-free' freezers is not recommended.

Assay Procedure
The assay is based on the hydrolysis of the peptide substrate Acetyl-Leu-Glu-His-Asp-7-amino-4-trifluoromethyl coumarin (Ac-LHED-AFC, Catalog Number A5845) by caspase 9, resulting in the release of a 7-amino-4-trifluromethyl coumarin (AFC) moiety.[7] The AFC product is detected using an excitation wavelength of 400 nm and an emission wavelengh of 505 nm.

Ac-LEHD-AFC ⟶ fluorescent AFC

Prepare the following solutions for the assay of Caspase 9 activity. Use ultrapure water for buffer preparation.

Assay Buffer: 50 mM MES, pH 6.5, 10% PEG 8000, 0.1% CHAPS, 5 mM DTT, and 1 mM EDTA.

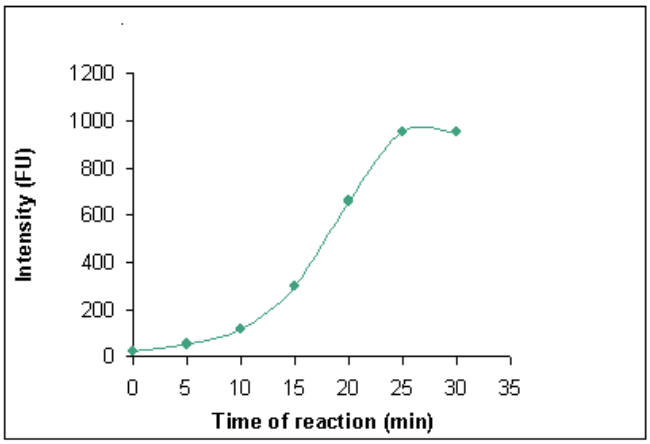
Substrate Stock Solution: Prepare a 10 mM solution by adding 650 μL of dry DMSO to the vial containing 5 mg of the substrate (Ac-LEHD-AFC, Catalog Number A5845, MW = 765.7).

Substrate Working Solution: Dilute the 10 mM Ac-LEHD-AFC stock solution 50-fold to a concentration of 200 μM with Assay Buffer. Prepare the solution in a polypropylene disposable tube.
Note: Prepare a fresh Substrate Working Solution of 200 μM Ac-LEHD-AFC before each assay.

AFC Stock Solution: Prepare a 10 mM solution by adding 1 ml of dry DMSO to 2.3 mg of AFC (MW = 229.2, E^{mM} = 17 at 382 nm). Store the solution protected from light. Dilute an aliquot of the AFC Stock Solution 200-fold with Assay Buffer. Measure the absorbance at 382 nm, and calculate the actual concentration of the prepared AFC Stock Solution using the extinction coefficient (E^{mM}). This AFC Stock Solution will be used to determine a calibration curve.

The assay is performed in a volume of 100 μL and fluorescence is measured using a 96 well plate.
Note: There is a lag time of 10–20 minutes until the enzyme activity is detectable. This delay reflects the need for caspase 9 to aggregate for activation, which is aided by the 10% PEG 8000 included in the reaction mixture. The presence of PEG 8000 in the reaction mixture is necessary for the caspase 9 activity. The lag time of the enzyme reaction is shown in Figure 1.

Figure 1.
Caspase 9 Activity



1. Fluorimeter settings for the assay:
   Excitation wavelength: 400 nm
   Emission wavelength: 505 nm
   Slit width: 5 nm

Table 1.
Reaction Scheme

	Caspase 9 Solution 0.25-0.6 mg/mL	Ac-LEHD-AFC Working Solution
Blank	–	100 μL
Test	0.5 μg (0.85 to 2 μL)	100 μL

Note: Perform the assay in duplicates.

2. Add 0.5 μg of caspase 9 (0.85 to 2 μL depending on the caspase 9 concentration) to the appropriate wells (see Table 1).
3. Add 100 μL of Substrate Working Solution to the wells, mix gently. Try to avoid bubble formation.
4. Obtain the zero reading on the blank well.
5. Incubate the plate in the fluorimeter at room temperature. Measure fluorescence intensity every 5–10 minutes for 40–60 minutes.
6. Determine the reaction rate [Δ Fluorescence intensity (FI)/minute] in the linear phase of the enzymatic reaction (15-25 minutes).
   Note: The enzymatic activity has a lag time of 10-20 minutes.
7. Calculate the activity using the AFC calibration curve.

Preparation of the AFC Calibration Curve

Table 2.
Solutions for the AFC Calibration Curve

AFC Concentration	nmole AFC per 100 µL
0 (Assay Buffer)	0
500 nM	0.05
1 µM	0.1
2 µM	0.2
4 µM	0.4
6 µM	0.6
8 µM	0.8
10 µM	1

1. Prepare a series of dilutions of the AFC Stock Solution with Assay Buffer in the concentration range of 500 nM to 10 µM. Use Table 2 as a guideline. Keep the solutions protected from light.
2. Determine the fluorescence intensity (Fl) of 100 µL of each solution. Obtain zero reading on the blank well (Assay Buffer only).
3. Plot the Fluorescence intensity (Fl) values versus nmole of AFC per well.
4. Calculate the slope of the calibration curve (Fl/nmole).

Results
Calculations:

$$\text{Units/mL} = \frac{\Delta\text{Fl/min}}{\text{Slope}} \times \frac{d}{V} \times 60$$

where:
d = enzyme sample dilution factor
V = Volume of the caspase 9 in the reaction in mL (0.001-0.003 mL)
Slope = Slope of the Calibration curve (Fl/nmol)
ΔFl/min = Reaction rate of the sample in the linear phase of the reaction

Activity = Units/mL
Unit definition: One unit will cleave 1.0 nmole of the substrate Ac-LEHD-AFC per hour at pH 6.5 at 25 °C.

References

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