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ProductInformation

Caspase 6 Assay Kit, Fluorimetric

Catalog Number **CASP6F**
Storage Temperature –20 °C

TECHNICAL BULLETIN

Product Description

Caspases (**C**ysteine-requiring **Asp**artate prote**ases**) belong to a highly conserved family of cysteine proteases with specificity for aspartic acid residues of their substrates. Caspases play a central role in apoptosis. Caspase 6 (Mch2) belongs to the group of effector caspases and is localized downstream of caspase 3.[1,2] Recently, it has been suggested that caspase 6 is implicated in Alzheimer's disease.[3]

The human, recombinant caspase 6 supplied in this kit was expressed in *E. coli* as the proenzyme with a C-terminal histidine tag. It undergoes autoactivation and autoprocessing to yield 21 kDa and 19 kDa proteins (large subunit) and a 14 kDa protein containing the His tag (small subunit).[1]

The assay is based on the hydrolysis of the peptide substrate N-Acetyl-Val-Glu-Ile-Asp 7-amido-4-methyl-coumarin (Ac-VEID-AMC) by caspase 6 that results in the release of the fluorophore 7-amido-4-methyl-coumarin [AMC].[4] 7-amido-4-methylcoumarin fluorescence is observed with excitation at 360 nm and emission at 440 nm.

$$\text{Ac-VEID-AMC} \xrightarrow{\text{Caspase 6}} \text{Ac-VEID + AMC}$$

The assay is performed in a 96 well plate with a total reaction volume of 100 µl and the results are read at intervals of 5 minutes for up to 20 minutes. The final substrate concentration in this assay is 50 µM. Maximal activity is achieved at 200 µM. At 50 µM, ~50% of maximal activity is obtained.

The caspase 6 inhibitor Ac-VEID-CHO is supplied to verify the specificity of the enzyme reaction in cell extracts. A concentration of 0.5 µM will totally inhibit caspase 6 activity.

This kit provides sufficient reagents for the quick and efficient measurement of caspase 6 activity in crude and purified preparations.

Reagents

The kit is sufficient for 100 reactions in a 96 well plate.

5× Lysis Buffer — 5 ml
Catalog Number L2912
250 mM HEPES, pH 7.4, with 25 mM CHAPS and 25 mM DTT

10× Assay Buffer — 5 ml
Catalog Number A0344
200 mM HEPES, pH 7.4, with 1% CHAPS, 50 mM DTT, and 20 mM EDTA

Caspase 6, human, recombinant — 5 µg
Catalog Number C7854
Lyophilized powder. Reconstitution with 50 µl of ultrapure water will give a solution of 100 µg/ml caspase 6 in 50 mM HEPES, pH 7.5, with 0.1% CHAPS, 4 mM DTT, 500 mM NaCl, 125 mM imidazole, and 10% sucrose.
Specific Activity: >1,000 units per mg protein
Unit Definition: One unit is the amount of enzyme that will cleave 1.0 nmol of the substrate Ac-VEID-pNA per minute at pH 7.4 at 25 °C.

N-Acetyl-Val-Glu-Ile-Asp 7-amido-4-methylcoumarin (MW 673.7) — 0.25 ml
Catalog Number A0217
Caspase 6 Substrate
5 mM Ac-VEID-AMC in DMSO

N-Acetyl-Val-Glu-Ile-Asp-Aldehyde (MW 500.5) — 0.25 ml
Catalog Number A6214
Caspase 6 Inhibitor
1 mM Ac-VEID-CHO in DMSO

7-Amino-4-methylcoumarin (MW 175.2) — 1 ml
Catalog Number A7582
Standard Solution
1 mM AMC in DMSO

Reagents and Equipment Required but Not Provided
(Catalog Numbers given where appropriate)

- Cells of interest
- BSA (optional) Catalog Number A8022.
- Spectrofluorimeter with 96 well plate attachment
- 96 well plates for fluorescence assay
- Polypropylene test tubes and microcentrifuge tubes.

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

Preparation Instructions
It is recommended to use ultrapure (17 MΩ·cm or equivalent) water when preparing the reagents.

- 1× Assay Buffer (20 mM HEPES, pH 7.4, with 2 mM EDTA, 0.1% CHAPS, and 5 mM DTT) – Dilute 10× Assay Buffer (Catalog Number A0344) 10-fold with ultrapure water.

- Caspase 6 Substrate Solution (100 μM Ac-VEID-AMC) – Dilute N-Acetyl-Val-Glu-Ile-Asp 7-amido-4-methylcoumarin (Catalog Number A0217) 50-fold with 1× Assay Buffer.

- Caspase 6 Inhibitor Solution (10 μM Ac-VEID-CHO) Dilute N-Acetyl-Val-Glu-Ile-Asp-Aldehyde (Catalog Number A6214) 100-fold with 1× Assay Buffer.

- Caspase 6 Positive Control (~100 μg/ml) – Reconstitute the vial (5 μg) of Caspase 6, human, recombinant (Catalog Number C7854) with 50 μl of ultrapure water. Store in aliquots at –70 °C.
  Positive Control Working Solution (5 μg/ml) – Just prior to running assays, dilute an aliquot of the Caspase 6 Positive Control 20-fold in 1× Assay Buffer in a polypropylene tube.
  Note: Caspase 6, human, recombinant may absorb to glass surfaces; therefore, all dilutions should be prepared in polypropylene tubes.

- AMC Standard Solution (20 μM 7-Amino-4-methyl-coumarin) – Dilute the 7-Amino-4-methyl-coumarin (Catalog Number A7582) 50-fold with 1× Assay Buffer and use the AMC Standard Solution to determine a standard curve (see Table 2).

- 1× Lysis Buffer (For determination of caspase 6 activity in cell lysates) – Dilute 5× Lysis Buffer (Catalog Number L2912) 5-fold with ultrapure water and use the 1× Lysis Buffer to lyse the cells.
  Note: In order to protect caspases in cell lysates from non-specific proteolysis, protease inhibitors that do not include cysteine protease inhibitors (e.g., E-64, leupeptin) may be added.

Storage/Stability
The kit ships on dry ice and storage at –20 °C is recommended.

Procedure
The same 96 well plate may be used for reaction assays and determination of the standard curve.
96 Well Plate Assay
1. Set fluorimeter:
   Excitation: 360 nm
   Emission: 440 nm
   Slit width: 5 nm
   Note: The wavelengths of 360 nm for excitation and 440 nm for emission were found to be optimal for this system. Raising the emission wavelength to 460 nm will cause a 25% reduction in the strength of the signal. Raising the excitation wavelength to 380 nm will cause a 60% reduction in the strength of the signal.
2. Add 5 μl of the Positive Control Working Solution or x μl of the unknown sample to the appropriate well (see Table 1).
3. Add the appropriate volume of 1× Assay Buffer to each well (see Table 1).
4. Add the Caspase 6 Inhibitor Solution to the appropriate wells. Ensure that all the wells are mixed gently to avoid bubble formation and let sit for 5 minutes at room temperature.
5. Start the reaction by adding 50 μl of the Caspase 6 Substrate Solution to each well using a multichannel pipette.
6. Place the 96 well plate in the spectrofluorimeter and read at 5 minute intervals for up to t minutes (t can be from 20-60 minutes or even longer for very dilute samples).
7. Calculate the Δ fluorescence measured from zero to t minutes.

Table 1.
Reaction Scheme for 96 Well Plate Assay

Reaction	Positive Control Working Solution (5 µg/ml)	Unknown Sample	1× Assay Buffer	Caspase 6 Inhibitor Solution (10 µM Ac-VEID-CHO)	Caspase 6 Substrate Solution (100 µM Ac-VEID-AMC)
Reagent Blank	–	–	50 µl	–	50 µl
Caspase 6 Positive Control	5 µl	–	45 µl	–	50 µl
Caspase-6 Positive Control + Inhibitor Solution	5 µl	–	35 µl	10 µl	50 µl
Unknown Sample	–	x µl	50–x µl	–	50 µl
Unkonwn Sample + Inhibitor Solution	–	x µl	40–x µl	10 µl	50 µl

AMC Standard Curve for 96 Well Plate Assay

1. Add the appropriate volume of AMC Standard Solution (20 µM 7-Amino-4-methylcoumarin) to each well (see Table 2) for a standard curve from 0.1–2 nmols of AMC per well.
2. Add the indicated volume (see Table 2) of 1× Assay Buffer to the appropriate wells.

Table 2.
Scheme for AMC Standard Curve

nmol AMC per well	AMC Standard Solution (20 µM)	1× Assay Buffer
0	0 µl	100 µl
0.1	5 µl	95 µl
0.2	10 µl	90 µl
0.5	25 µl	75 µl
1.0	50 µl	50 µl
1.5	75 µl	25 µl
2.0	100 µl	0 µl

Calculation

Calculate the caspase 6 activity as nmol AMC released per minute per ml of unknown sample or mg protein of positive control.

v = volume of sample in ml
d = dilution factor
t = reaction time in minutes
FI_{1nmol} = fluorescence of 1 nmol AMC per well from the Standard Curve
ΔFI_t = difference in fluorescence between time zero and time t minutes

$$\text{Activity (nmol/min/ml)} = \frac{\Delta FI_t \times d}{FI_{1nmol} \times t \times v}$$

References

1. Fernandes-Alnemri, T., *et al*., Cancer Res., **55**, 2737-2742 (1995).
2. Bratton, S.B., *et al*., Exp. Cell Res., **256**, 27-33 (2000).
3. LeBlanc, A., J. Biol. Chem., **274**, 23426-23436 (1999).
4. Stennicke, H.R., and G. Salvesen., J. Biol. Chem., **272**, 25719-25723 (1997).
5. Alnemri, E.M., J. Cell. Biochem., **64**, 22-42 (1997).

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