3050 Spruce Street
Saint Louis, Missouri 63103 USA
Telephone 800-325-5832 • (314) 771-5765
Fax (314) 286-7828
email: techserv@sial.com
sigma-aldrich.com

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ProductInformation

Caspase 3 Assay Kit, Fluorimetric

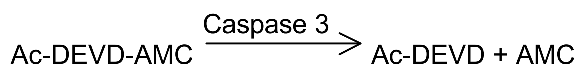
Catalog Number **CASP3F**
Storage Temperature –20 °C

TECHNICAL BULLETIN

Product Description

Caspases (Cysteine-requiring Aspartate protease) are a family of proteases that mediate cell death and are important to the process of apoptosis. Caspase 3 (also referred to as CPP32, Yama, and apopain) is a member of the CED-3 subfamily of caspases and is one of the critical enzymes of apoptosis. Caspase 3, which is an effector caspase, is the most studied of mammalian caspases. It can process caspase 2, 6, 7, and 9 proenzymes and specifically cleave most caspase related substrates known to date, including many key proteins such as the nuclear enzyme poly(ADP-ribose) polymerase (PARP),[1] the inhibitor of caspase-activated deoxyribonuclease (ICAD),[2] and gelsolin and fodrin, which are proteins involved in apoptosis regulation.[3] This cleavage is part of the mechanism leading to cell death. In addition, caspase 3 plays a central role in mediating nuclear apoptosis including chromatin condensation and DNA fragmentation as well as cell blebbing.[5] Caspase 3 activity is tissue, cell type, or death stimulus specific.[5]

The caspase 3 fluorimetric assay is based on the hydrolysis of the peptide substrate acetyl-Asp-Glu-Val-Asp-7-amido-4-methylcoumarin (Ac-DEVD-AMC) by caspase 3, resulting in the release of the fluorescent 7-amino-4-methylcoumarin (AMC) moiety.



The excitation and emission wavelengths of AMC are 360 nm and 460 nm, respectively. The concentration of the AMC released can be calculated from a standard curve determined with defined AMC solutions.

The Caspase 3 Fluorimetric Assay Kit is designed for the fast and sensitive fluorimetric detection and measurement of caspase 3 activity in cell extracts or purified preparations of caspase 3. The assay can be performed in a 2 ml volume and measured using a fluorimeter or in a 200 μl volume in a 96 well plate using a microplate fluorimeter.

Components

Sufficient for 100 standard tests of 2 ml or 1,000 tests in 96 well plates

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

10× Assay Buffer — 20 ml
200 mM HEPES, pH 7.4, with 1% CHAPS, 50 mM DTT, and 20 mM EDTA
Catalog Number A0219

Caspase 3 — 5 μg
Lyophilized powder. Reconstitution with 50 μl of 17 megohm water will give a solution of 100 μg/ml caspase 3 in 50 mM HEPES, pH 7.4, with 0.1% CHAPS, 10 mm DTT, 100 mm NaCl, 1 mM EDTA, and 10% sucrose.
Specific Activity: >1 unit per mg protein.
Unit Definition: One unit is the amount of enzyme that will cleave 1.0 μmol of the substrate Ac-DEVD-AMC per minute at pH 7.4 at 25 °C.
Catalog Number C5974

Ac-DEVD-AMC Substrate — 2.5 mg
Acetyl-Asp-Glu-Val-Asp-7-amido-4-methylcoumarin
Catalog Number A1086

Ac-DEVD-CHO Inhibitor — 0.5 mg
Acetyl-Asp-Glu-Val-Asp-al
Catalog Number A0835

7-Amino-4-methylcoumarin Standard — 1 mg
Catalog Number A9891

Water (17 megohm) — 125 ml
(sufficient volume for 70 standard tests of 2 ml or 700 tests in 96 well plates)
Catalog Number W3888

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

- Cells to undergo apoptosis.
- Apoptosis inducer. Apoptosis may be either spontaneous or induced. The procedures in this bulletin use staurosporine, Catalog Number S4400.
- Phosphate buffered saline (PBS), Catalog Number D8537
- DMSO, Catalog Number D8418
- BSA (optional), Catalog Number A8022
- Cuvette fluorimeter with 3 ml cuvettes
- Microplate fluorimeter and 96 well microplate for fluorimeter
- Spectrophotometer with quartz cuvettes
- 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
Use 17 megohm water (Catalog Number W3888) to prepare reagents.

1× Assay Buffer (20 mM HEPES, pH 7.4, with 2 mM EDTA, 0.1% CHAPS, and 5 mM DTT) - Dilute the 10× assay buffer 10-fold with 17 megohm water.

Caspase 3 Substrate (Ac-DEVD-AMC) Solution (10 mM in DMSO) - Dissolve the vial contents (2.5 mg) in 370 μl of DMSO. Store at –20 °C.

Caspase 3 Inhibitor (Ac-DEVD-CHO) Solution (2 mM in DMSO) - Dissolve the vial contents (0.5 mg) in 500 μl of DMSO to prepare a stock solution. Store at –20 °C.
Note: For assays using 96 well plates dilute the stock solution to **200 μM** with 1× Assay Buffer.

Caspase 3 Positive Control - Reconstitute the vial (5 μg) with 50 μl of 17 megohm water (100 μg/ml). Store in aliquots at –70 °C.
Just before use, dilute an aliquot 200-fold (0.5 μg/ml) in 1× Assay Buffer or 1× Lysis Buffer in a polypropylene tube. Alternatively, dilute the caspase 3 Positive Control in 1× Assay Buffer containing 1 mg/ml BSA or in 1× Lysis Buffer containing 1 mg/ml BSA.

Reaction Mixture for 96 well plate assay - Add 5 μl of 10 mM Ac-DEVD-AMC to 3 ml of 1× Assay Buffer. Prepare 200 μl per reaction. Store in the dark until use.

Reaction mixture for 2 ml cuvette assay - Add 3.3 μl of 10 mM Ac-DEVD-AMC to 2 ml of 1× Assay Buffer. Prepare 2 ml per reaction. Store in the dark until use.

7-Amino-4-methylcoumarin (AMC) Standard Solution (~10 mM), used to prepare solutions for standard curve. Dissolve the vial contents (1 mg) in 0.57 ml of DMSO. Store the Standard Solution at –20 °C. Determine the concentration of the 10 mM 7-Amino-4-methylcoumarin Standard Solution spectrophotometrically. Dilute a sample of the Standard Solution 200-fold with 1× Assay Buffer and measure the A_{354} using quartz cuvettes. Calculate the actual concentration using the millimolar extinction coefficient of $\varepsilon^{mM} = 16$ (354 nm).

1× Lysis Buffer (50 mM HEPES, pH 7.4, with 5 mM CHAPS and 5 mM DTT) - Dilute 5× Lysis Buffer 5-fold with 17 megohm water.
Note: In order to protect the cell lysate caspases from non-specific proteolysis, protease inhibitors that do not include cysteine protease inhibitors (e.g., E-64 and leupeptin) may be added.

Staurosporine Solution - Prepare a solution containing 1 mg/ml in DMSO.
Note: The CHX-ACT Apoptosis Made Easy Kit (Catalog Number CHXACT) may be used in conjuction with the assay procedures in this kit. The CHXACT kit contains solutions of two enhancers of death receptor induced apoptosis (cycloheximide and actinomycin D) and the broad spectrum apoptosis inducer (staurosporine).

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

Procedure
Three controls are recommended for each caspase 3 fluorimetric assay:

1. Inhibitor-treated cell lysate control (for measuring the nonspecific hydrolysis of the substrate)
2. Caspase 3 positive control.
3. Reagent blank (negative control)

Separate procedures (1 and 2) are presented as guidelines for the fluorimetric assay of caspase 3 activity for cell lines grown in suspension (Jurkat cells) and adherent cell lines (HeLa and HepG2 cells).

1. Procedure for Fluorimetric Assay of Caspase 3 Activity from Cell Lines Grown in Suspension

A. Preparation of Cell Lysates from Apoptotic Cells (Jurkat Cells) Grown in Suspension

The following procedure uses Jurkat cells induced to apoptosis using staurosporine and lysed prior to the determination of caspase 3 activity.

1. Induce apoptosis in a cell suspension of Jurkat cells ($\geq 10^7$ cells) by addition of staurosporine to a final concentration of 1 μg/ml. Reserve a sample of non-induced cells for a zero time control.

2. Incubate for 2.5–3 hours at 37 °C in a 5% CO_2 atmosphere.

3. Pellet the induced cells and the control cells by centrifugation at 600 × *g* for 5 minutes at 4 °C.

4. Remove the supernatant by gentle aspiration.

5. Wash the cells once with PBS. Centrifuge the cells and remove the supernatant by gentle aspiration.

6. Suspend the cell pellets in 1× Lysis Buffer at a concentration of 500 μl per 10^7 cells.

7. Incubate the cells on ice for 15–20 minutes.

8. Centrifuge the lysed cells at 14,000 × *g* for 10–15 minutes at 4 °C.

9. Transfer the supernatants to new tubes.

10. Analyze the lysates immediately or freeze in liquid nitrogen and store in aliquots at –70 °C

B. 96 Well Plate Assay for Jurkat Cell Lysate

Equipment Required:
96 well microplate for fluorimeter
Microplate fluorimeter

Table 1.
Reaction Scheme for 96 Well Plate Assay

	1× Assay Buffer	**Caspase 3 (0.5 μg/ml)**	**Cell lysate**	**Caspase 3 inhibitor Ac-DEVD-CHO (200 μM)**	**Reaction Mixture**
Reagent Blank	5 μl	–	–	–	200 μl
Caspase 3 positive control	–	5 μl	–	–	200 μl
Caspase 3 positive control + inhibitor	–	5 μl	–	2 μl	200 μl
Non-induced cells	–	–	5 μl	–	200 μl
Non-induced cells + inhibitor	–	–	5 μl	2 μl	200 μl
Induced cells	–	–	5 μl	–	200 μl
Induced cells + inhibitor	–	–	5 μl	2 μl	200 μl

1. Set fluorimeter:
   excitation – 360 nm
   emission – 460 nm
   slit width – 5 nm
2. Add 5 μl of cell lysate or caspase 3 positive control to the appropriate microplate well as indicated in Table 1.
3. Add 5 μl of 1× assay buffer to the reagent blank well.
4. Add the caspase 3 inhibitor to the appropriate wells as indicated in Table 1.
5. Add 200 μl of reaction mix to each of the wells and mix gently. Try to avoid forming bubbles in the wells.
6. Cover the plate and incubate at room temperature in the dark for 1–1.5 hours.
7. Read the fluorescence of the samples. Zero the fluorimeter using the Reagent Blank well. If the values are too low for accurate measurement, continue the incubation for another 30–60 minutes and then read the plate. To measure the kinetics of caspase 3 activity in cell lysates or in the positive control, measure the fluorescence of the plate every 10–15 minutes over 1–1.5 hours.
8. Calculate the results using an AMC standard curve (Section 1C).

C. AMC Standard Curve for the 96 Well Plate Assay
A series of 5–6 diluted AMC solutions in the concentration range of 100 nM to 5 μM are used to determine the standard curve. This range is recommended for 200 μl assays. In the event that a smaller volume is used, adjust the AMC solutions concentration range accordingly.

1. AMC Working Solution - Dilute the 10 mM AMC Standard Solution to 10 μM (1,000-fold) with 1× Assay Buffer.

2. Use Table 2 as a guideline for preparation of AMC solutions from the 10 μM AMC Working Solution.

Table 2.
AMC Standard Curve Solutions for 96 Well Plate Assay

AMC Solution	nmol AMC in 200 μl	AMC Working Solution (10 μM)	1× Assay Buffer
6 μM	1.2	180 μl	120 μl
4 μM	0.8	120 μl	180 μl
2 μM	0.4	60 μl	240 μl
1 μM	0.2	30 μl	270 μl
500 nM	0.1	15 μl	285 μl
100 nM	0.02	3 μl	297 μl

3. Set fluorimeter: excitation – 360 nm
emission – 460 nm
slit width – 5 nm

4. Place 200 μl of each solution into the appropriate microplate well.

5. Place 200 μl of 1× Assay Buffer into the blank well.

6. Zero the fluorimeter on the blank well.

7. Determine the fluorescence values of the solutions.

8. Draw the curve of fluorescence intensity values versus the concentration of the AMC solutions (standard curve). Alternatively, plot the OD values versus the amount of AMC per well in nmols, taking into account the actual concentration of 10 mM AMC solution as determined spectrophotometrically.

9. Calculate the caspase 3 activity in nmole of AMC released per min per ml of cell lysate or positive control based on the formula:

$$\text{Activity, nmol AMC/min/ml} = \frac{\text{nmol AMC} \times d}{t \times v}$$

Where: v - volume of sample in ml
d - dilution factor
t - reaction time in minutes

D. 2 ml Cuvette Assay Method
Equipment Required:
Polypropylene test tubes
3 ml quartz fluorimeter cuvette
Fluorimeter

Table 3.
Reaction Scheme for 2 ml Cuvette Assay

	1× Assay buffer	**Caspase 3 (0.5 μg/ml)**	**Cell lysate**	Caspase 3 inhibitor Ac-DEVD-CHO (2 mM)	**Reaction Mixture**
Blank	5 μl	–	–	–	2 ml
Caspase 3 positive control	–	5 μl	–	–	2 ml
Caspase 3 positive control + inhibitor	–	5 μl	–	2 μl	2 ml
Non-induced cells	–	–	5 μl	–	2 ml
Non-induced cells + inhibitor	–	–	5 μl	2 μl	2 ml
Induced cells	–	–	5 μl	–	2 ml
Induced cells + inhibitor	–	–	5 μl	2 μl	2 ml

1. Set fluorimeter:
   excitation – 360 nm
   emission – 460 nm
   slit width – 5 nm

2. Add 5 µl of cell lysate or caspase 3 positive control to the appropriate polypropylene tubes.

3. Add 5 µl of 1× Assay Buffer to the blank tube.

4. Add the Caspase 3 Inhibitor to the appropriate tubes as indicated in Table 3.

5. Start the reaction by adding 2 ml of Reaction Mix to each of the tubes. Mix gently.

6. Incubate the tubes in room temperature in the dark for 25–30 minutes.

7. Read the fluorescence of the samples in a cuvette. Zero the fluorimeter on the blank. If the values are too low for accurate measurement, continue the incubation for another 15–20 minutes and then read the solutions again. To measure the kinetics of caspase 3 activity in cell lysates or in the positive control, measure the fluorescence of the test solution in the cuvette every 10 minutes over 30–40 minutes.

8. Calculate the results using an AMC standard curve (Section 1E).

E. <u>AMC Standard Curve for 2 ml Cuvette Assay</u>
A series of 5–6 diluted AMC solutions in the concentration range of 25–250 nM are used to determine the standard curve. In the event cuvettes other than the size indicated are used, adjust the AMC solutions concentration range accordingly.

1. AMC Working Solutions - Dilute the 10 mM AMC Standard Solution to 10 µM (1,000-fold) and to 1 µM (10,000-fold) with 1× Assay Buffer.

2. Use Table 4 as a guideline for preparation of AMC solutions from the 10 µM and 1 µM AMC Working Solutions.

Table 4.
AMC Standard Curve Solutions for 2 ml Cuvette Assay

AMC Solution	nmole AMC in 2 ml	AMC Working Solution		1× Assay Buffer
		10 µM	1 µM	
250 nM	0.5	50 µl	–	1.95 ml
200 nM	0.4	40 µl	–	1.96 ml
150 nM	0.3	30 µl	–	1.97 ml
100 nM	0.2	–	200 µl	1.8 ml
75 nM	0.15	–	150 µl	1.85 ml
50 nM	0.1	–	100 µl	1.9 ml
25 nM	0.05	–	50 µl	1.95 ml

3. Set the fluorimeter:
   excitation – 360 nm
   emission – 460 nm
   slit width – 5 nm

4. Zero the fluorimeter using 2.0 ml of 1× Assay Buffer.

5. Determine the fluorescence values of the solutions.

6. Draw the curve of the intensity values versus the concentration of the AMC solutions (standard curve). Alternatively, plot the OD values versus the amount of AMC per cuvette in nmols, taking into account the actual concentration of the AMC solution as determined spectrophotometrically.

7. Calculate the caspase 3 activity in nmole of AMC released per min per ml of cell lysate or positive control based on the formula:

$$\text{Activity, nmol AMC/min/ml} = \frac{\text{nmole AMC} \times d}{t \times v}$$

Where: v - volume of sample in ml
d - dilution factor
t - reaction time in minutes

2. Procedure for Fluorimetric Assay of Caspase 3 Activity in Adherent Cell Lines

A. Procedure for Preparation of HeLa cells

1. Preparation Instructions

   Culture vessel - 96-well, flat bottom plate (Catalog Number M9780)

   Growth Medium - DMEM (Catalog Number D5671) supplemented with 10% fetal calf serum (FCS), 4 mM glutamine (Catalog Number G7513), and Pen/Strep (Catalog Number P0781).

   DMSO Medium (0.5% in growth medium) - Add 5 μl of DMSO to 1 ml of growth medium.

   Staurosporine Solution (200 μg/ml in DMSO) – Dilute 1 mg/ml Staurosporine Solution 5-fold in DMSO.

   Staurosporine Medium (1 μg/ml in growth medium) Add 5 μl of 200 μg/ml Staurosporine Solution to 1 ml of growth medium.

2. Preparation of Apoptotic HeLa Cells
   Note: Perform each test in triplicate.

   a. Plate 2×10^4 cells per well.
   b. When cells are confluent (after 40–48 hours), prepare the following samples by replacing the medium with:
      - 200 μl of Growth Medium – blank
      - 200 μl of DMSO Medium – negative control
      - 200 μl of Staurosporine Medium – positive control

      Incubate for 4–6 hours.

   c. Aspirate the medium carefully by holding the plate almost vertical and touching the well wall rather than well bottom.
   d. Determine caspase 3 activity (Section 2C or 2D).

B. Procedure for Preparation of HepG2 cells

1. Preparation Instructions

   Culture vessels - gelatin coated 75 cm^2 tissue culture flask (Catalog Number C7296) and 96-well, flat bottom plate (Catalog Number M9780) coated with 0.1% gelatin solution.

   Growth Medium - DMEM (Catalog Number D5671) supplemented with 10% FCS, 4 mM glutamine Catalog Number G7513), and Pen/Strep (Catalog Number P0781).

   DMSO Medium (0.5% in growth medium) - Add 5 μl of DMSO to 1 ml of growth medium.

   Staurosporine Solution (200 μg/ml in DMSO) – Dilute 1 mg/ml Staurosporine Solution 5-fold in DMSO.

   Staurosporine Medium (1 μg/ml in growth medium) Add 5 μl of 200 μg/ml Staurosporine Solution to 1 ml of growth medium.

   HepG2 cells grow in clumps and do not spread over the tissue culture vessel. To allow the culture to reach confluency, the cells are grown on vessels coated with 0.1% gelatin solution. When sub-culturing the cells, the cell suspension is transferred to a conical tube and the large clumps are allowed to precipitate for 1–2 minutes.

2. Preparation of Apoptotic HepG2 Cells
   Note: Perform each test in triplicate.

   a. Grow HepG2 cells to 70–80% confluency on a gelatin coated 75 cm^2 tissue culture flask.
   b. Trypsinize the cells with 1.5 ml of Trypsin-EDTA solution.
   c. As soon as the cells detach, add 10 ml of Growth Medium and suspend the cells by pipetting.
   d. Transfer the cells to a 15 ml conical tube and let large clumps precipitate for 1–2 minutes.
   e. Transfer the cell suspension to a cell reservoir (Catalog Number R9384) and plate 100–200 μl per well of a gelatin coated 96-well plate.
   f. When cells are confluent (after 40–48 hours), prepare the following samples by replacing the medium with:
      - 200 μl of Growth Medium – blank
      - 200 μl of DMSO Medium – negative control
      - 200 μl of Staurosporine Medium – positive control

      Incubate for 4–6 hours.

   g. Aspirate the medium carefully by holding the plate almost vertical and touching the well wall rather than well bottom.
   h. Determine caspase 3 activity (Section 2C or 2D).

C. <u>96 Well Assays for Adherent Cells - Immediate Caspase 3 Assay</u>

1. Set fluorimeter:
    a. excitation – 360 nm
    b. emission – 460 nm
    c. slit width – 5 nm

2. Place the plate containing the adherent cells on ice. Verify it is level.
3. Add 25 μl of 1× Lysis Buffer.
4. Incubate on ice for 15–20 minutes.
5. Add 200 μl of 1× Assay Buffer containing substrate and mix well by pipetting.
6. Transfer 200 μl to a fluorimeter multiwell plate.
7. Prepare two wells of substrate blanks and two wells of Caspase 3 positive controls.
8. Read fluorescence in a kinetic mode every 10 minutes for 40–60 minutes at room temperature.
9. If the fluorescence values are low, continue reading or incubate the plate at 37 °C to enhance caspase 3 activity rate (this is needed for HeLa cells, where the number of cells per well/test is low).
10. Calculate the results using an AMC standard curve (Section 1C).

D. <u>96 Well Assays for Adherent Cells – Delayed Caspase 3 Assay</u>

1. Place the plate containing the adherent cells on ice.
2. Add 120 μl of 1× Assay Buffer.
3. Cover the plate and store at –70 °C.

    2 to 3 days later:

4. Set fluorimeter:
    a. excitation – 360 nm
    b. emission – 460 nm
    c. slit width – 5 nm
5. Prepare 1× Assay Buffer with double the concentration of substrate (add 10 μl of 10 mM Ac-DEVD-AMC to 3 ml of 1× Assay Buffer).
6. Thaw the plate at room temperature.
7. As soon as the solutions are thawed, put the plate on ice.
8. Add 120 μl of the substrate containing Assay Buffer (prepared in step 5) to the plate and mix by pipetting.
9. Transfer 200 μl to a fluorimeter multiwell plate.
10. Prepare two wells of substrate blanks and two wells of caspase 3 positive controls.
11. Read fluorescence in a kinetic mode every 10 minutes for 40–60 minutes at room temperature.
12. If the fluorescence values are low, continue reading or incubate the plate at 37 °C to enhance caspase 3 activity rate (this is needed for HeLa cells, where the number of cells per well/test is low).
13. Calculate the results using an AMC standard curve (Section 1C).

References

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