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

# ProductInformation

**Caspase-8 Assay Kit, Fluorimetric**

Product Code **CASP-8-F**
Store at –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 central role in apoptosis. Caspase-8, known also as Mch5,[1] MACH,[2] and FLICE[3] is localized at the top of the hierarchy of the caspase cascade and is a member of the "upstream" or initiator family of caspases. Caspase-8 exists in the cell as an inactive proenzyme of 55 kDa. It is converted to the active form, consisting of 18 and 12 kDa subunits, upon its recruitment to the cytoplasmic domain of activated death receptors such as Fas, via the adaptor protein FADD.[4] The activation of the proenzyme is triggered by the protein's aggregation, which leads to auto- or transprocessing. Caspase-8 activates dowstream caspases (3, 6 and 7) that cleave key cellular substrates and lead to apoptotic death of the cells.[5,6]

The Caspase-8 fluorimetric assay is based on the hydrolysis of the peptide substrate Acetyl-Ile-Glu-Thr-Asp-7-amino-4-methyl coumarin (Ac-IETD-AMC) by Caspase-8 resulting in the release of a 7-amino-4-methyl coumarin (AMC) moiety.[7] The excitation and emission wavelengths of AMC are 360 nm and 440 nm, respectively. The concentration of the AMC released can be calculated from a calibration curve performed with defined AMC solutions.

$$\text{Ac-IETD-AMC} \xrightarrow{\text{Caspase-8}} \text{Ac-IETD + AMC}$$

The Caspase-8 Fluorimetric Assay Kit is designed for the fast and sensitive fluorimetric detection and measurement of Caspase-8 activity in crude or purified preparations of Caspase-8. The assay can be performed in a 2 ml volume and measured using a fluorimeter or in a 100 µl volume in a 96 well plate using a microplate fluorimeter.

### Components

Sufficient for 40 standard tests of 2 ml or 800 tests of 100 µl in 96 well microplates

- **5x Lysis Buffer**, Product Code L 2912 5 ml
  250 mM HEPES, pH 7.4, 25 mM CHAPS, 25 mM DTT
- **10x Assay Buffer**, Product Code A 0344 20 ml
  200 mM HEPES, pH 7.4, 1% CHAPS, 50 mM DTT, 20 mM EDTA, 50% sucrose
- **Caspase-8**, Product Code C 6849 5 µg
  Lyophilized powder. Reconstitution with 50 µl of 17 megohm deionized water will give a solution of 100 µg/ml caspase-8 in 20 mM Tris-HCl, pH 8.0, 0.1% CHAPS, 20 mM 2-mercaptoethanol, 500 mM NaCl, 2.5 mM EDTA, 150 mM imidazole, and 10% sucrose.
  **Specific Activity:** >500 units per mg protein.
  **Unit Definition:** One unit is the amount of enzyme that will cleave 1.0 nmol of the substrate Ac-IETD-pNA per minute at pH 7.4 at 25 °C.
- **Acetyl-Ile-Glu-Thr-Asp-7-amido-4-methyl-coumarin Substrate**, Product Code A 7457 1 ml
  1.5 mM Ac-IETD-AMC in DMSO, [MW 675.69]
- **Acetyl-Ile-Glu-Thr-Asp-Aldehyde Inhibitor**, Product Code A 6464 0.5 ml
  1 mM Ac-IETD-CHO in DMSO, [MW 502.7]
- **7-Amino-4-methylcoumarin Standard**, Product Code A 7582 1 ml
  1 mM AMC in DMSO [MW 139]

Reagents and Equipment Required but Not Provided
(Product Codes are given where appropriate)

- Cells of interest
- BSA (optional) Product Code A 8022.
- Spectrofluorimeter with 96 well plate attachment
- 96 well plate for fluorimetry
- 3 ml cuvette (Polymethacrylate: Product Code C0793 or Quartz: Product Code C 9167)
- Polypropylene test tubes and microfuge tubes.

**Precautions and Disclaimer**
For laboratory use only. Not for drug, household or other uses.

**Storage**
Store the kit at –20°C.

**Preparation Instructions**
Note: **Use 17 megohm deionized water in all the steps.** Trace amounts of metal ions will cause inactivation of caspase-8.

- **1x Assay Buffer:** 20 mM HEPES, pH 7.4, 2 mM EDTA, 0.1% CHAPS, 5 mM DTT, 5% sucrose. Dilute 10x assay buffer 10-fold with 17 megohm deionized water.
- **Caspase-8 Fluorimetric Substrate** (Ac-IETD-AMC), 1.5 mM in DMSO. Use as-is for cuvette assay or dilute 10-fold with 1x Assay Buffer for the microwell assay.
- **Caspase-8 Inhibitor** (Ac-IETD-CHO), 1 mM in DMSO. Dilute with 1x Assay Buffer 40-fold to yield 25 µM and aliquot.
- **Caspase-8 Positive Control**. Reconstitute the vial (5 µg) with 50 µl of 17 megohm deionized water (~100 µg/ml) Store in aliquots at –70 °C. For the microwell assay, dilute just before use to 10 µg/ml (10-fold dilution) in 1x Assay Buffer in a polypropylene tube and use a sample of 5 µl in the assay.
  For the 2 ml assay, dilute just before use to 10 µg/ml (10-fold) in 1x Assay Buffer containing 0.5 mg/ml bovine serum albumin (BSA) in a polypropylene tube and use a sample of 5 µl in the assay.
  Note: Caspase-8 may absorb to glass surfaces, therefore all dilutions should be prepared in polypropylene tubes.
- **AMC Standard Solution** – 1 mM in DMSO
  **For microwell assay**: dilute the stock solution 100-fold with 1x Assay Buffer to 10 µM and build a calibration curve from 0.1-1 nmols of AMC (10-100 µl per well) as shown below.
  **For cuvette assay**: dilute the stock solution 100-fold with 1x Assay Buffer to 10 µM and build a calibration curve from 0.05-2 nmols of AMC (5-200 µl per cuvette) as shown below.

  Note: This range may have to be adjusted to suit the specific instrument used for fluorimetric analysis of the sample.
  When determination of Caspase-8 activity in cell lysates is desired, dilute 5x Lysis Buffer 5-fold with 17 megohm deionized water and lyse the cells with this solution.

  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, leupeptin) may be added.

**Procedure**
The following procedure is an example using Jurkat cells induced to apoptosis using staurosporine and lysed prior to the determination of caspase 8 activity.

Three controls are recommended for each Caspase-8 fluorimetric assay:

- Inhibitor-treated Caspase-8 (for measuring the nonspecific hydrolysis of the substrate)
- Caspase-8 positive control.
- Reagent blank (negative control)

**Example of Preparation of Cell Lysates from Apoptotic Cells**

1. Induce apoptosis in a cell suspension of Jurkat cells (at least $10^7$ 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 x *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

**Table 1. Reaction Scheme for 96 Well Plate Microassay Method (Total Volume = 100 µl)**

| | 1x Assay Buffer | Caspase-8 10 µg/ml | Unknown Sample | Caspase-8 Inhibitor Ac-IETD-CHO 25 µM | Caspase-8 Substrate Ac-IETD-AMC 150 µM |
|---|---|---|---|---|---|
| Reagent blank | 90 µl | ---- | ---- | ---- | 10 µl |
| Caspase-8 Positive Control | 85 µl | 5 µl | ---- | ---- | 10 µl |
| Caspase-8 Positive Control + Inhibitor | 83 µl | 5 µl | ---- | 2 µl | 10 µl |
| Unknown Sample | 90 –X µl | ---- | X µl | ---- | 10 µl |
| Unkonwn Sample + Inhibitor | 88 –X µl | ---- | X µl | 2 µl | 10 µl |

**96 Well Plate Microassay Method**

Equipment Required

- 96 well microplate for fluorimeter
- Microplate fluorimeter

1. Set fluorimeter:
   Excitation: 360 nm,
   Emission: 440 nm,
   Slit width: 5 nm.
2. Place 5 µl of the diluted Caspase-8 Positive Control or X µl of the unknown sample in a well.
3. Add the appropriate amount of 1x Assay Buffer (see Table 1).
4. Add the Caspase-8 Inhibitor to the appropriate wells. Ensure that all the cells are mixed gently to avoid bubble formation and let sit for 5 minutes at room temperature.
5. Start the reaction with the addition of 10 µl of the Caspase-8 Substrate Solution with a multichannel pipette.
6. Place in the plate reader of the spectofluorimeter 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 fluoresecnce formed from zero to t minutes.
8. Calculate the results using an AMC calibration curve (see Table 2).

**Table 2. AMC Calibration Curve**

| nmol AMC per well | AMC std. 10 µM µl per well | 1x Assay Buffer |
|---|---|---|
| 0 | 0 | 100 µl |
| 0.1 | 10 | 90 µl |
| 0.25 | 25 | 75 µl |
| 0.5 | 50 | 50 µl |
| 0.75 | 75 | 25 µl |
| 1.0 | 100 | 0 µl |

Calculation

Calculate the caspase-8 activity as nmol AMC released per min 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
$Fl_{1nmol}$ = fluorescence of 1 nmol in the microwell from the calibration curve
$\Delta Fl_t$ = difference in fluorescence between time zero and time t minutes

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

**Table 3. Reaction Scheme for 2 ml Cuvet Assay Method**

| | 1x Assay Buffer | Caspase-8 10 µg/ml | Unknown Sample | Caspase-8 Inhibitor Ac-IETD-CHO 25 µM | Caspase-8 Substrate Ac-IETD-AMC 1.5 mM |
|---|---|---|---|---|---|
| Blank | 1980 µl | | | | 20 µl |
| Caspase-8 Positive Control | 1975 µl | 5 µl | | | 20 µl |
| Caspase-8 Positive Control + Inhibitor | 1935 µl | 5 µl | | 40 µl | 20 µl |
| Unknown Sample | 1980 –X µl | | X µl | | 20 µl |
| Unknown Sample + Inhibitor | 1940 –X µl | | X µl | 40 µl | 20 µl |

**2 ml Cuvet Assay Method**

Equipment Required

- Polypropylene Test tubes or Eppendorf tubes (the enzyme is absorbed to glass tubes)
- 3 ml fluorimeter cuvet
- Spectrofluorimeter

1. Set the fluorimeter:
   Excitation: 360 nm,
   Emission: 440 nm,
   Slit width: 2.5 nm.
   Note: Conditions for fluorimetric measurement of sample: The wavelengths of 360 nm excitation and 440 nm 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 Caspase-8 Positive Control (diluted 10 fold) or X µl of unknown sample (see Table 3).
3. Add Caspase-8 Inhibitor to the appropriate tubes and incubate for 5 minutes at room temperature.
4. Start the reaction with 20 µl of 1.5 mM Caspase-8 Fluorimetric Substrate Solution.
5. Use a kinetic program with readings every minute for 10 minutes; (if necessary this period can be lengthened and the interval changed i.e. every 5 minutes for 60 min).
6. Run a reagent blank as shown in Table 3.
7. Calculate the activity using the value for the fluorescence of 1 nmol per reaction mixture from the standard curve for AMC (see Table 4)

**Table 4. AMC Calibration Curve**

| nmol AMC per cuvette | AMC std 10 µM µl per cuvette | 1x Assay Buffer µl per cuvette |
|---|---|---|
| 0 | 0 | 2000 |
| 0.05 | 5 | 1995 |
| 0.1 | 10 | 1990 |
| 0.2 | 20 | 1980 |
| 0.5 | 50 | 1950 |
| 1.0 | 100 | 1900 |
| 1.5 | 150 | 1850 |
| 2.0 | 200 | 1800 |

Calculation

Calculate the Caspase-8 activity as nmol AMC released per min per ml of cell lysate or per mg protein of positive control.

v = volume of sample in ml

d = dilution factor

t = reaction time in minutes

$Fl_{1nmol}$ = fluorescence of 1 nmol AMC in the cuvette

$\Delta Fl$ = difference of fluorescence between time t and time zero

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

**References**


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