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, Colorimetric

Product Code **CASP8C**
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 found in their substrates. Caspases play a 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 assay is based on the hydrolysis of the peptide substrate Acetyl-Ile-Glu-Thr-Asp *p*-nitroaniline (Ac-IETD-pNA) by caspase 8 resulting in the release of a *p*-Nitroaniline (pNA) moiety.[7] *p*-Nitroaniline has absorbance at 405 nm (e^{mM} = 10.5). The concentration of the pNA released from the substrate is calculated from the absorbance values at 405 nm or from a calibration curve performed with defined pNA solutions.

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

The Caspase 8 Colorimetric Assay Kit provides the reagents needed for a quick and efficient detection of caspase 8 activity in crude and purified preparations of caspase 8.

The Caspase 8 Colorimetric Assay Kit is designed for the fast and sensitive detection and measurement of caspase 8 activity in crude or purified preparations. The assay can be performed in a 100 µl volume in a 96 well plate using a microplate fluorometer.

Reagents

The kit provides reagents sufficient for 100 tests of 100 µl in 96 well plates.

- **5´ Lysis Buffer** 5 ml
  Product Code L2912
  250 mM HEPES, pH 7.4, 25 mM CHAPS, 25 mM DTT

- **10´ Assay Buffer** 5 ml
  Product Code A0344
  200 mM HEPES, pH 7.4, 1% CHAPS, 50 mM DTT, 20 mM EDTA, 50% sucrose

- **Caspase 8** 5 µg
  Product Code C6849
  Lyophilized powder. Reconstitution with 50 µl of 17 MΩ·cm water results in 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 nmole of the substrate Ac-IETD-pNA per minute at pH 7.4 at 25 °C.

- **Acetyl-Ile-Glu-Thr-Asp-p-nitroaniline Substrate** 0.1 ml
  Product Code A7332
  20 mM Ac-IETD-pNA in DMSO [MW 638.6]

- **Acetyl-Ile-Glu-Thr-Asp-Aldehyde Inhibitor** 0.1 ml
  Product Code A6464
  1 mM Ac-IETD-CHO in DMSO [MW 502.7]

- ***p*-Nitroaniline**, 1 ml
  Product Code N7773
  10 mM p-NA in DMSO [MW 138.1]

- **Water** (17 MΩ·cm) 125 ml
  Product Code W3888

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

- Apoptotic cells of interest
- BSA (optional) Product Code A8022
- ELISA reader, 96 well plate
- Polypropylene test tubes and microfuge 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
Note: Use the provided 17 MΩ·cm water (Product Code W3888) in all the steps. Trace amounts of metal ions will inactivate caspase 8.

- **1´ Assay Buffer -** 20 mM HEPES, pH 7.4, 2 mM EDTA, 0.1% CHAPS, 5 mM DTT, 5% sucrose. Dilute the 10× Assay Buffer (Product Code A0344) 10-fold with the supplied 17 MΩ·cm water.

- **Caspase 8 Colorimetric Substrate** (Ac-IETD-pNA) - Dilute the caspase substrate solution (Product Code A7332) 10-fold with 1× Assay Buffer for the microwell assay to yield 2 mM solution.

- **Caspase 8 Inhibitor** (Ac-IETD-CHO) - Dilute an aliquot of the caspase inhibitor solution (Product Code A6464) 40-fold with 1× Assay Buffer to yield 25 µM.

- **Caspase 8 Positive Control**. Reconstitute the vial (5 µg) with 50 µl of the supplied 17 MΩ·cm water (~100 µg/ml). Store in aliquots at –70 °C. Just before use, dilute 10-fold with 1× Assay Buffer to 10 µg/ml in a polypropylene tube and use a sample of 10 µl in the assay.
  Note: Caspase 8 may absorb to glass surfaces, therefore all dilutions should be prepared in polypropylene tubes.

- ***p*-Nitroaniline Standard Solution -** For the 96 well plate assay, dilute the *p*-Nitroaniline solution (Product Code N7773) 50-fold with 1× Assay Buffer to 200 µM and build a calibration curve as shown in Table 2.

- **1´ Lysis Buffer** - Dilute the 5× Lysis Buffer (Product Code L2912) 5-fold with the supplied 17 MΩ·cm water.
  Note: In order to protect the cell lysate caspases from non-specific proteolysis, protease inhibitors that do not include cysteine protease inhibitors (E-64 and leupeptin) may be added.

Storage/Stability
Store the kit at –20 °C.

Procedure
When determination of caspase 8 activity in cell lysates is desired, lyse the cells with 1× Lysis Buffer with appropriate protease inhibitors.

The reaction schemes for assays of unknown samples and controls are shown in Table 1. Three controls are recommended for each caspase 8 colorimetric assay:

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

Equipment Required

- Flat bottomed 96 well plate for colorimetric assay
- ELISA plate reader

1. Place 10 µl of the diluted Caspase 8 Positive Control or x µl of the unknown sample in a well.
2. Add the appropriate amount of 1× Assay Buffer (see Table 1).
3. 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.
4. Start the reaction with the addition of 10 µl of the prepared Caspase 8 Colorimetric Substrate using a multichannel pipette.
5. Place in the ELISA reader and measure the initial absorbance at 405 nm (t = 0) and then read at 5 minute intervals for t minutes (where t can be from 20-60 minutes or even longer for very dilute samples).

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

	1´ Assay Buffer	Caspase 8 10 µg/ml	Unknown Sample	Caspase 8 Inhibitor Ac-IETD-CHO 25 µM	Caspase 8 Substrate Ac-IETD-pNA 2 mM
Reagent blank	90 µl	----	----	----	10 µl
Caspase 8 Positive Control	80 µl	10 µl	----	----	10 µl
Caspase 8 Positive Control + Inhibitor	78 µl	10 µl	----	2 µl	10 µl
Unknown Sample	90 –x µl	----	x µl	----	10 µl
Unknown Sample + Inhibitor	88 –x µl	----	x µl	2 µl	10 µl

Table 2.
p-Nitroaniline Calibration Curve

nmole pNA per well	pNA standard solution µl per well	1x Assay buffer µl per well
0	0	100
1	5	95
2	10	90
5	25	75
10	50	50
15	75	25
20	100	0

Calculation
Calculate the caspase 8 activity as nmoles of *p*-nitroaniline released per minute per ml for the unknown sample or per mg protein for the positive control.

$$\text{Activity (nmole/min/ml)} = \frac{(A_t - A_0) \times d}{(A_{1\ nmole}) \times t \times v}$$

v = volume of sample in ml
d = dilution factor
t = reaction time in minutes
$A_{1\ nmole}$ = absorbance of 1 nmole of pNA in the well
A_t = absorbance at time t minutes
A_0 = absorbance at zero time

References

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