Product Information

Caspase 3 Assay Kit, Colorimetric

CASP3C

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. Caspase 3 can process pro-Caspase 2, 6, 7, and 9 and specifically cleave most of the 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]
- 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 colorimetric assay is based on the hydrolysis by Caspase 3 of the peptide substrate acetyl-Asp-Glu-Val-Asp *p*-nitroanilide (Ac-DEVD-pNA), to give the release of a *p*-nitroaniline (pNA) moiety:

$$\text{Ac-DEVD-pNA} \xrightarrow{\text{Caspase 3}} \text{Ac-DEVD + pNA}$$

p-Nitroaniline has a high absorbance at 405 nm (E^{mM} = 10.5). The concentration of the released pNA is calculated from the absorbance values at 405 nm or from a calibration curve prepared with defined pNA solutions.

This Caspase 3 Colorimetric Assay Kit provides the reagents needed for a quick and efficient detection of Caspase 3 activity in cell lysates and in purified preparations of Caspase 3.

The assay can be performed in either of these volumes:

- 1 mL volume and measured using a spectrophotometer
- 100 µL volume in a 96-well plate using an ELISA reader.

This kit provides reagents sufficient for either:

- 100 standard 1 mL tests
- 1000 tests in 96-well plates.

Precautions and Disclaimer

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.

Reagents Provided

- 5× Lysis Buffer (Component L2912): 5 mL (250 mM HEPES, pH 7.4, 25 mM CHAPS, 25 mM DTT)
- 10× Assay Buffer (Component A0219): 20 mL (200 mM HEPES, pH 7.4, 1% CHAPS, 50 mM DTT, 20 mM EDTA)
- Caspase 3 (Component C5974, Lyophilized powder): 5 µg
  - 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, 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-pNA per minute at pH 7.4 at 25 °C.

- Ac-DEVD-pNA Substrate (Component A2559): 15 mg (Acetyl-Asp-Glu-Val-Asp *p*-nitroanilide)
- Ac-DEVD-CHO Inhibitor (Component A0835): 0.5 mg (Acetyl-Asp-Glu-Val-Asp-al)
- 4-Nitroaniline Standard (Component 185310): 1 mg
- Water (17 megohm) (Component W3888): 125 mL

Reagents and Equipment Required

(Not provided)

Example Cat. Nos. are given where appropriate.

- Cells to undergo apoptosis. The example procedure here uses Jurkat E6-1 cells.
- Apoptosis inducer. Apoptosis may be either spontaneous or induced. The example procedure here uses staurosporine (Cat. No. S4400).
- Phosphate buffered saline (PBS), such as Cat. No. D8537
- DMSO, such as Cat. No. D8418
- Bovine serum albumin (BSA), such as Cat. No. A8022
- Spectrophotometer with quartz cuvettes
- ELISA reader
- Flat-bottom, 96-well plates suitable for ELISA
- Polypropylene test tubes and microcentrifuge tubes

Storage/Stability

Store the kit at –20 °C.

Preparation Instructions

Note: Use 17 megohm water only (Component No. W3888) in all the steps.

- 1× Assay Buffer: 20 mM HEPES, pH 7.4, 2 mM EDTA, 0.1% CHAPS, 5 mM DTT
  - Dilute 10× Assay Buffer 10-fold with 17 megohm water.
- Caspase 3 substrate (Ac-DEVD-pNA): 20 mM in DMSO.
  - Dissolve the vial contents (15 mg) in 1.2 mL of DMSO to prepare a stock solution.
  - Alternatively, dissolve 1 mg of substrate in 78.5 µL of DMSO. Store at –20 °C.
  - **For assays using 96-well plates**, dilute the 20 mM stock solution to **2 mM** with 1× Assay Buffer.
- Caspase 3 inhibitor (Ac-DEVD-CHO): 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.
  - **For assays using 96-well plates**, dilute the 2 mM 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 to 5 µg/mL 20-fold either in 1× Assay Buffer containing 1 mg/mL BSA, or 1× Lysis Buffer containing 1 mg/mL BSA.
- 1× Lysis Buffer: 50 mM HEPES, pH 7.4, 5 mM CHAPS, 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 inhibitor cocktails that omit particular inhibitors of cysteine proteases (such as E-64, or leupeptin) may be added.

- *p*-Nitroaniline Standard (for developing a calibration curve for assays in 96-well plate):
  - Dissolve the vial in 0.72 of mL DMSO. Store this stock solution at –20 °C.
  - To determine the actual concentration, dilute a sample of the stock solution 100-fold in 1× Assay Buffer.
  - Determine absorbance at 405 nm using a quartz cuvette.
  - Calculate the actual concentration of the stock solution, using the molar absorptivity of E^{mM} = 10.5 at 405 nm.
- Staurosporine: 1 mg/mL in DMSO

Procedure

The following procedure is an example using Jurkat cells induced to apoptosis using staurosporine and lysed prior to the determination of Caspase 3 activity.

Three controls are recommended for each Caspase 3 Colorimetric Assay:

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

Preparation of Cell Lysates from Apoptotic Cells

1.1.1. Induce apoptosis in a cell suspension of Jurkat cell (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.

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

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

1.1.4. Remove the supernatant by gentle aspiration.

1.1.5. Wash the cell pellets once with 1 mL of PBS. Centrifuge the cells and remove the supernatant completely by gentle aspiration.

1.1.6. Suspend the cell pellets in 1× lysis buffer at a concentration of 100 µL per 10^7 cells.

1.1.7. Incubate the cells on ice for 15-20 minutes.

1.1.8. Centrifuge the lysed cells at 16,000 to 20,000 × *g* for 10-15 minutes at 4 °C.

1.1.9. Transfer the supernatants to new tubes.

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

1 mL Volume Assay Method

- Equipment required:
  - Test tubes
  - Spectrophotometer
  - 1 mL quartz cuvettes
- The positive control volume recommended in the reaction scheme is compatible with the expected activity found in $0.5 \times 10^6 – 1.5 \times 10^6$ apoptotic Jurkat cells. The positive control and sample volumes can be increased if required.
- Use quartz cuvettes **only**, since plastic cuvettes attenuate the absorption at 405 nm.
- Yellowish color is visualized by the naked eye at approximately 0.2 Optical Density (OD) at 405 nm.

1. Place 10 µL of cell lysate or Caspase 3 Positive Control in the appropriate tubes as indicated in Table 1.
2. Add 1× Assay Buffer to each of the tubes as indicated in Table 1.
3. Add 10 µL of Caspase 3 inhibitor to the appropriate tubes.
4. Start the reaction by adding 10 µL of Caspase 3 substrate to each tube.
   4.1. Mix gently.
   4.2. Cover the tubes.
   4.3. Incubate at 37 °C for 1.5 to 2 hours.
      4.3.1. If the signal is too low, continue the incubation overnight.
   4.4. Read the Absorbance at 405 nm.

5. Calculate the Caspase 3 activity in µmol of pNA released per min per mL of cell lysate or positive control based on the formula:
   - Activity, in µmol pNA/min/mL = $(OD \times D) / E^{mM} \times T \times V)$
   - Where:
     - $E^{mM} = 10.5$
     - V = volume of sample in mL
     - D = dilution factor
     - T = reaction time in minutes

96-Well Plate Microassay Method

- Equipment required:
  - Flat bottom 96-well plate
  - ELISA reader
- The positive control volume recommended in the reaction scheme is compatible with the expected activity found in 0.25×10^6 to 1×10^6 apoptotic Jurkat cells. The positive control and sample volumes can be increased if required.
- Yellowish color is visualized by the naked eye at approximately 0.2 OD at 405 nm (OD405).

1. Place 5 µL of cell lysate or Caspase 3 Positive Control in the appropriate wells as indicated in Table 2.
2. Add 1× Assay Buffer to each of the wells as indicated in Table 2.
3. Add the Caspase 3 Inhibitor to the appropriate wells.
4. Start the reaction by adding 10 µL of Caspase 3 substrate to each well.
   4.1. Mix gently by shaking.
   4.2. Try to avoid forming bubbles in the wells.
   4.3. Cover the plate.
   4.4. Incubate at 37 °C for 70-90 minutes.
      4.4.1. If signal is too low, continue the incubation overnight.
5. Read the Absorbance at 405 nm.
6. Calculate the results using a *p*-nitroaniline calibration curve. This method is recommended for accurate results to avoid miscalculations that stem from incompatibility of the ELISA reader and the plastic plates.

p-Nitroaniline (pNA) Calibration Curve

1. Prepare a series of *p*-nitroaniline solutions at the concentration range of 10 µM–200 µM by diluting the *p*-nitroaniline stock solution in 1× Assay Buffer.
2. Add 100 µL of each dilution to a well.
   2.1. Include 100 µL of assay buffer as a blank.
3. Read the Absorbance at 405 nm.
4. Prepare a calibration curve of the absorbance values versus the concentrations of the *p*-nitroaniline solutions.
   4.1. Alternatively, plot the OD405 values versus the amount of *p*-nitroaniline per well, in µmol, using the following values as a guideline.

***p*-Nitroaniline Calibration Curve**

µM *p*-Nitroaniline	µmol *p*-Nitroaniline per 100 µL
10	0.001
20	0.002
50	0.005
100	0.01
200	0.02

      4.1.1. Calculate the Caspase 3 activity in µmol pNA released per min per mL of cell lysate or positive control, based on the formula:
   - Activity, µmol pNA/min/mL = $(\mu mol\ pNA \times D) / (T \times V)$
   - Where:
     - V = volume of sample in mL
     - D = dilution factor
     - T = reaction time in minutes

References

1. Nicholson, D.W. *et al*., *Nature*, **376(6535)**, 37-43 (1995).
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3. Kamada, S. *et al*., *Proc. Natl. Acad. Sci. USA*, **95(15)**, 8532-8537 (1998).
4. Cohen, G.M., *Biochem. J*., **326(Pt 1)**, 1-16 (1997).
5. Porter, A.G., and Jänicke, R.U., *Cell Death Differ*., **6(2)**, 99-104 (1999).

Table 1. Reaction scheme for 1 mL Volume Assay Method

	Cell lysate	Caspase 3 (5 µg/mL)	Assay buffer	Caspase 3 inhibitor Ac-DEVD-CHO (2 mM)	Caspase 3 substrate Ac-DEVD-pNA (20 mM)
Reagent blank	----	----	990 µL	----	10 µL
Non-induced cells	10 µL	----	980 µL	----	10 µL
Non-induced cells + inhibitor	10 µL	----	970 µL	10 µL	10 µL
Induced cells	10 µL	----	980 µL	----	10 µL
Induced cells + inhibitor	10 µL	----	970 µL	10 µL	10 µL
Caspase 3 positive control	----	10 µL	980 µL	----	10 µL
Caspase 3 positive control + inhibitor	----	10 µL	970 µL	10 µL	10 µL

Table 2. Reaction scheme for 96-Well Plate Microassay Method

	Cell lysate	Caspase 3 (5 µg/mL)	1× Assay buffer	Caspase 3 inhibitor Ac-DEVD-CHO (200 µM)	Caspase 3 substrate Ac-DEVD-pNA (2 mM)
Reagent blank	----	----	90 µL	----	10 µL
Non-induced cells	5 µL	----	85 µL	----	10 µL
Non-induced cells + inhibitor	5 µL	----	75 µL	10 µL	10 µL
Induced cells	5 µL	----	85 µL	----	10 µL
Induced cells + inhibitor	5 µL	----	75 µL	10 µL	10 µL
Caspase 3 positive control	----	5 µL	85 µL	----	10 µL
Caspase 3 positive control + inhibitor	----	5 µL	75 µL	10 µL	10 µL

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