

Caspase 8 Activity Assay Kit

C1492388

Introduction:

The caspase family of cysteine proteases has been demonstrated to play a critical role in apoptosis. Mammalian caspases can be classified into three functional groups: initiator caspases (Caspase-2, -8, -9, and -10), executioner caspases (Caspase-3, -6, and -7), and inflammatory caspases (Caspase-1, -4, -5, -11, and -12). Caspase-8 (also known as FLICE, MACH, or Mch5) is a member of the caspase family of cysteine proteases. During apoptosis, procaspase-8 is processed through autoproteolysis and/or cleavage by other caspases at aspartic acid residues. The active form of processed caspase-8 consists of large and small subunits that associate to form the active enzyme. Active caspase-8 has been shown to activate caspase-3, leading to the degradation of various cellular target proteins during apoptosis.

The Caspase 8 Activity Assay Kit is based on the hydrolysis of the peptide substrate Ac-IETD-pNA (acetyl-Ile-Glu-Thr-Asp p-nitroanilide) by caspase-8, which releases yellow p-nitroaniline (pNA). pNA exhibits strong absorbance at approximately 405 nm, allowing the detection of caspase-8 activity through absorbance measurement.

Kit Contents and Storage Conditions:

C1492388	Component	20T	50T	100T	Storage
C1492388A	Cell Lysis Buffer	5 mL	10 mL	20 mL	2-8°C
C1492388B	Reaction Buffer (2×)	10 mL	20 mL	40 mL	2-8°C
C1492388C	Ac-IETD-pNA (4 mM)	100 µL	250 µL	500 µL	-20°C. Store in the dark.
C1492388D	pNA (10 mM)	100 µL	250 µL	500 µL	-20°C. Store in the dark.
C1492388E	DTT (100×)	150 µL	400 µL	750 µL	-20°C

Materials Required but Not Provided:

- Microplate reader (capable of measuring absorbance at 405 nm).
- 96-well plate.
- Refrigerated centrifuge, ice maker.
- Adjustable pipettes and tips.
- Deionized water, phosphate-buffered saline (PBS).
- Homogenizer (for tissue samples).

Reagent Preparation:

1. Working Cell Lysis Buffer: Add DTT to Cell Lysis Buffer immediately before use (final concentration: 10 μ L DTT (100 $\times$) per 1 mL Cell Lysis Buffer). Keep on ice; store at 4°C.
2. Working Reaction Buffer (1 $\times$): Dilute Reaction Buffer (2 $\times$) with deionized water to 1 $\times$ concentration. Add DTT (final concentration: 10 μ L DTT (100 $\times$) per 1 mL 1 $\times$ Reaction Buffer). Keep on ice; store at 4°C.
3. Ac-IETD-pNA (4 mM): Ready-to-use. Thaw on ice before use; store at –20°C. Aliquot unused portions and avoid repeated freeze-thaw cycles.
4. pNA (10 mM): Ready-to-use. Thaw on ice before use; store at –20°C protected from light. Aliquot unused portions and avoid repeated freeze-thaw cycles.
5. DTT (100 $\times$): Ready-to-use. Thaw on ice before use; store at –20°C. Aliquot unused portions and avoid repeated freeze-thaw cycles.
6. Standard Curve Preparation: Prepare pNA standard solutions at 200, 100, 50, 25, 12.5, and 0 μ M using Reaction Buffer (1 $\times$, containing DTT) according to the table below. Note: Prepare a fresh standard curve for each experiment. Diluted standards are unstable and must be used within 4 hours.

Standard	Volume of Standard / Previous Standard	Volume of Reaction Buffer (1 $\times$, with DTT) (μ L)	Concentration (μ M)
Std.1	6 μ L pNA (10 mM)	294	200
Std.2	150 μ L of Std.1 (200 μ mol/mL)	150	100
Std.3	150 μ L of Std.2 (100 μ mol/mL)	150	50
Std.4	150 μ L of Std.3 (50 μ mol/mL)	150	25
Std.5	150 μ L of Std.4 (25 μ mol/mL)	150	12.5
Std.6	0	150	0

Sample Preparation:

Note: Fresh samples are recommended. Alternatively, store samples at –80°C after preparation. Thaw on ice before use, as freeze-thaw may affect stability and yield lower readings. Do not use protease inhibitors during sample preparation, as they may interfere with the assay.

1. Induce apoptosis in cells by an appropriate method. Include an uninduced control culture for each assay.
2. For adherent cells: Harvest by trypsinization. Centrifuge at 600 g, 4°C for 5 min. Carefully aspirate supernatant. Wash cell pellet twice with 1 mL PBS. Resuspend 1-5 $\times$ 10⁶ cells in 50 μ L Working Cell Lysis Buffer.
3. For suspension cells: Centrifuge at 600 g, 4°C for 5 min. Carefully aspirate supernatant. Wash cell pellet twice with 1 mL PBS. Resuspend 1-5 $\times$ 10⁶ cells in 50 μ L Working Cell

Lysis Buffer.

4. For tissues: Mince 5-20 mg tissue into small pieces. Rinse with PBS. Homogenize in 0.1 mL Working Cell Lysis Buffer on ice.
5. Incubate lysates on ice for 15-20 min.
6. Centrifuge at 16,000 g, 4°C for 15 min. Transfer supernatant to a new tube and keep on ice.
7. Measure Caspase-8 activity immediately or store samples at -80°C. Determine protein concentration using a Bradford assay.

Assay Procedure:

1. Preheat microplate reader for at least 30 min. Set wavelength to 405 nm.
2. Set up reactions in a 96-well plate as described below:

Volume (μL)	Blank Well	Test Well	Control Well (For Tissue Samples)	Standard Well
Working Reaction Buffer (1×, with DTT)	100	50	55	0
Standard	0	0	0	100
Supernatant	0	50	50	0
Ac-IETD-pNA(4 mM)	5	5	0	0

For Blank, Test, and Control Wells:

Mix thoroughly and incubate at 37°C for 1–2 hours. Measure the absorbance at 405 nm.

- Record the absorbance of the Blank Well as A_{blank} .
- Record the absorbance of the Test Well as A_{test} .
- Record the absorbance of the Control Well as A_{control} .

Calculate:

$$\Delta A_{\text{measured}} = A_{\text{test}} - A_{\text{blank}}$$

Note:

- Control Wells are required only for tissue samples.
- For tissue samples, use: $\Delta A_{\text{measured}} = A_{\text{test}} - A_{\text{control}}$.

For Standard Wells:

Measure the absorbance at 405 nm immediately after preparation.

Calculate:

$$\Delta A_{\text{standard}} = A_{\text{standard}} - A_{\text{Std.6}}$$

where $A_{\text{Std.6}}$ is the absorbance of Standard Well 6 (0 μM pNA).

Notes:

- Blank and Standard Wells require only 1-2 replicates.
- Include a Control Well for tissue samples to account for inherent color.
- Mix gently to avoid bubbles.

- If color change is weak, extend incubation time up to 4 hours.

Calculations:

Note: Provided formulas include a detailed derivation and a simplified version. They are equivalent. The simplified formula (in bold) is recommended for final calculation.

1. Standard Curve Method: Plot standard concentration (x-axis) against $\Delta A_{\text{standard}}$ (y-axis) to obtain the equation $y = kx + b$. Calculate the concentration (x, in μM) for the sample using its $\Delta A_{\text{measured}}$.
2. Percentage Increase in Activity:

$$\text{Caspase-8 Activity Increase (\%)} = [(A_{\text{test_treated}} - A_{\text{blank}}) / (A_{\text{test_control}} - A_{\text{blank}})] \times 100\%$$
3. Enzyme Activity Calculation (U/mg prot):
 One unit of enzyme activity is defined as the amount of enzyme that cleaves 1.0 nmol of the colorimetric pNA substrate per hour at 37°C under saturated substrate concentrations (per Chemicon's definition).

$$\text{Caspase-8 Activity (U/mg prot)} = 2.1 \times x \div C_{\text{pr}} \div T.$$

- x: pNA concentration from standard curve (μM).
- C_{pr} : Sample protein concentration (mg/mL).
- T: Reaction time (hours).
- 2.1: Derived from $(V_{\text{reaction_total}} \times 10^3) \div (V_{\text{sample}}) = (0.105 \text{ mL} \times 1000) \div (0.05 \text{ mL}) = 2100$, converted to 2.1 for the simplified formula.

Derivation details:

$$\text{Caspase-8 Activity (U/mg prot)} = (x \times V_{\text{reaction_total}}) \div (V_{\text{sample}} \times C_{\text{pr}}) \div T \times 10^3.$$

- $V_{\text{reaction_total}}$: Total reaction volume (0.105 mL = 1.05×10^{-4} L).
- V_{sample} : Volume of sample added (0.05 mL).
- 10^3 : Conversion factor ($1 \mu\text{mol} = 10^3 \text{ nmol}$).

Note: For samples with very high Caspase-8 activity, dilute with Working Cell Lysis Buffer before assay and multiply the result by the dilution factor (n).

Result Presentation:

Representative Standard Curve: The following data and curve are for reference only. Users must generate their own standard curve for accurate quantification.

