



**Promega**

## Technical Bulletin

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# CaspACE™ Assay System, Colorimetric

INSTRUCTIONS FOR USE OF PRODUCTS G7220 AND G7351.



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# CaspACE™ Assay System, Colorimetric

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## 1. Description

The CaspACE™ Assay System, Colorimetric, provides reagents to measure the activity of caspase-3, a member of the cysteine aspartic acid-specific protease family. These proteases play key roles in apoptosis in mammalian cells (1-4). The CaspACE™ Assay System provides a colorimetric substrate and a cell-permeable inhibitor that allow highly sensitive, quantitative measurement of caspase-3 (DEVDase) activity, an early regulatory event in apoptosis. This assay system may be used with purified enzyme preparations or cell extracts and can be adapted for use in high-throughput applications.

Caspase activation occurs as a result of growth factor withdrawal, exposure to radiation or chemotherapeutic agents, or initiation of the Fas/Apo-1 receptor-mediated cell death process. Active caspases participate in a cascade of cleavage events that disable key homeostatic and repair enzymes and bring about systematic disassembly of dying cells. The biological substrates of caspases

## 1. Description (continued)

include poly-(ADP ribose) polymerase (PARP), DNA-dependent protein kinase (DNA-PK), lamins, topoisomerases, Gas2, protein kinase C  $\delta$  (PKC  $\delta$ ), sterol regulatory element binding proteins (SREBP), U1-70kDa protein and Huntingtin protein (5-8).

Several mammalian homologs of CED-3 (pro-apoptotic gene of *C. elegans*) have been described (9-12). Three subfamilies have been identified based on amino acid sequence and substrate and inhibitor specificities (13-15). Of these, caspase-3 plays a central role in the apoptotic cascade. Caspase-3 specifically cleaves at the C-terminal side of the aspartate residue of the amino acid sequence DEVD (Asp-Glu-Val-Asp).

### Assay Principle

The colorimetric substrate (Ac-DEVD-pNA) provided in the CaspACE™ Assay System, Colorimetric, is labeled with the chromophore *p*-nitroaniline (pNA). pNA is released from the substrate upon cleavage by DEVDase. Free pNA produces a yellow color that is monitored by a spectrophotometer at 405nm. The amount of yellow color produced upon cleavage is proportional to the amount of DEVDase activity present in the sample (16).

The potent, irreversible and cell-permeable pan-caspase inhibitor Z-VAD-FMK is provided in the CaspACE™ Assay System, Colorimetric. This compound inhibits activation of caspases in several models of apoptosis, in some by irreversibly binding to and blocking the cleavage site of the caspases (17-25). This inhibitor has also been used in studies of the order of events in cell death pathways (22). Z-VAD-FMK should be added directly to the cells as apoptosis is being induced. The difference between the amount of yellow color produced in the absence of inhibitor and in the presence of inhibitor is a measure of the caspase-3 activity present in the sample.

Apoptosis can be induced in experimental systems by a variety of methods that lead to caspase activation. These include:

- Treating Fas or TNF receptor-bearing cells with agonistic antibodies (26,27).
- Treating cells with DNA topoisomerase inhibitors [e.g., etoposide (28)], with the protein kinase inhibitor staurosporine (29) or with microtubule-damaging agents such as paclitaxel (30).
- Exposing cells to genotoxic damage induced by ionizing radiation (31).

## 2. Product Components and Storage Conditions

Product	Size	Cat. #
CaspACE™ Assay System, Colorimetric	1 system	G7220

For Laboratory Use. Each system contains sufficient reagents to perform 100 reactions. Includes:

- 2 × 100µl Caspase-3 Substrate Ac-DEVD-pNA (10mM)
- 125µl Caspase Inhibitor Z-VAD-FMK (20mM)
- 100µl pNA Standard (100mM)
- 5ml Caspase Assay Buffer
- 20ml Cell Lysis Buffer

Product	Size	Cat. #
CaspACE™ Assay System, Colorimetric	1 system	G7351

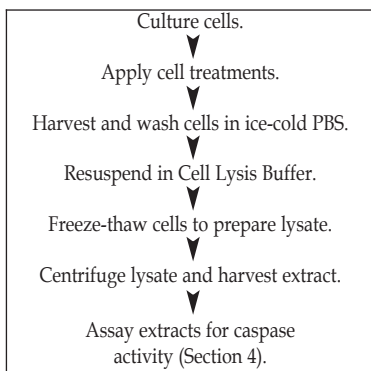
Each system contains sufficient reagents to perform 50 reactions. Includes:

- 1 × 100µl Caspase-3 Substrate Ac-DEVD-pNA (10mM)
- 50µl Caspase Inhibitor Z-VAD-FMK (20mM)
- 100µl pNA Standard (100mM)
- 5ml Caspase Assay Buffer
- 20ml Cell Lysis Buffer

**Storage Conditions:** Store the CaspACE™ Assay System, Colorimetric, at -20°C. Avoid multiple freeze-thaw cycles. Store substrates and inhibitors in aliquots at -20°C away from light and moisture.

## 3. Preparation of Cell Extracts

### 3.A. Overview



**Notes:** References 26–31 provide examples of apoptosis induction. The length of induction will vary and should be optimized by the user. Inhibited apoptosis extracts are made by adding Z-VAD-FMK inhibitor to induced cell cultures at the time of apoptosis induction.

The amount of Z-VAD-FMK inhibitor to use must be determined empirically.

If you wish to store extracts, prepare aliquots and store at -70°C. Long-term storage is not recommended.

### 3.B. Preparation of Jurkat Cell Extracts

The following procedure can be used to induce apoptosis in Jurkat cells using anti-Fas antibody (25,26). For an example of a time course for apoptosis induction with Jurkat cells, see reference 32.

#### Materials to Be Supplied by the User

(Solution compositions are provided in Section 7.)

- human Jurkat T cells (ATCC# TIB-152)
  - humidified 37°C incubator
  - RPMI 1640 medium
  - glutamine
  - anti-Fas monoclonal antibody (clone #CH-11, MBL International, Cat.# SY-001)
  - fetal bovine serum
  - PBS, ice cold
1. Grow Jurkat cells in RPMI 1640 medium containing 10% fetal bovine serum in a humidified, 5% CO<sub>2</sub> incubator at 37°C, as recommended by ATCC.
  2. Adjust the cell density to 10<sup>6</sup> cells/ml. Add 50ng/ml of anti-Fas mAb to the Jurkat cells for a positive (induced apoptosis) control. For inhibited apoptosis samples, add the Z-VAD-FMK inhibitor to the cells (final concentration of 50µM) at the same time as the anti-Fas mAb is added. Prepare negative controls using untreated cells. Incubate for 16 hours (overnight) at 37°C in a humidified, 5% CO<sub>2</sub> atmosphere.
  3. Harvest cells by centrifugation at 450 × g for 10 minutes at 4°C. Keep the cell pellet on ice. Wash 1X with ice-cold PBS, and resuspend in Cell Lysis Buffer at a concentration of 10<sup>8</sup> cells/ml.
  4. Lyse cells by freeze-thaw, then incubate on ice for 15 minutes (repeat freeze-thaw cycles as needed to ensure complete cell lysis). Centrifuge the cell lysates at 15,000 × g for 20 minutes at 4°C, and collect the supernatant fraction.
  5. Measure caspase-3 activity in cell extracts as described in Section 4. Use extract from at least 1 × 10<sup>6</sup> cells/assay. Extracts may be stored in aliquots at -70°C; avoid multiple freeze-thaw cycles. Long-term storage is not recommended.

### 4. Caspase Assay Protocol

This protocol can be used to test multiple samples. The assay is performed in a total volume of 100µl in 96-well plates; absorbance is read using a plate reader. Cell extracts are used as an enzyme source. Section 3.B describes the preparation of Jurkat cell extracts for use as controls.

For optimal results, you may need to assay several concentrations of the sample. In the 96-well plate format, add up to 20µl of cell extract (25–100µg total protein) to each reaction. If necessary, the sample may be diluted in Caspase Assay Buffer.

### Materials to Be Supplied by the User

(Solution compositions are provided in Section 7.)

- cell extract
- 37°C incubator
- 96-well plate (flat-bottom, clear polystyrene)
- plate reader
- dimethyl sulfoxide (DMSO)
- DTT, 100mM
- deionized water
- Parafilm® laboratory film or plate sealer

**!** Use a flat-bottom, clear polystyrene 96-well plate for the assay.

1. Thaw the Substrate stock solution and Caspase Assay Buffer. Warm to room temperature, and mix thoroughly before use.
2. Prepare replicate wells containing blank (no cell extract), negative control (extract from untreated cells), induced apoptosis (extract from induced cells) and inhibited apoptosis (extract from induced, inhibitor-treated cells) samples.

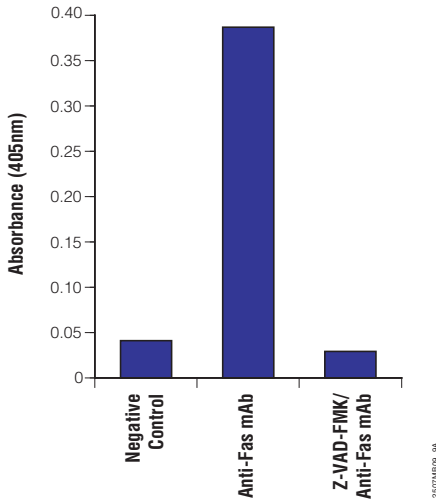
	Blank	Negative Control	Induced Apoptosis	Inhibited Apoptosis
Caspase Assay Buffer	32µl	32µl	32µl	32µl
DMSO	2µl	2µl	2µl	2µl
DTT, 100mM	10µl	10µl	10µl	10µl
untreated cell extract	–	Xµl	–	–
induced apoptosis extract	–	–	Xµl	–
inhibited apoptosis extract	–	–	–	Xµl
deionized water to final volume	98µl	98µl	98µl	98µl

**!** All reactions must have identical cell extract protein concentrations.

3. Add 2µl of DEVD-pNA Substrate (10mM stock) to all wells.
4. Cover the plate with Parafilm® laboratory film or a plate sealer, and incubate at 37°C for 4 hours. Color development may be monitored during the 4-hour incubation.

**Note:** The assay may be incubated overnight at 20–22°C. This should yield a similar absorbance to that seen after 4 hours incubation at 37°C; however, the background absorbance may increase. Color development may be monitored during the 4-hour incubation.

5. Measure the absorbance in the wells at 405nm. Calculate caspase-specific activity as described in Section 5.



**Figure 1. Measurement of caspase-3 activity in anti-Fas antibody-treated human Jurkat T cells.** Jurkat T cells ( $10^6$  cells/ml) were treated with 50ng/ml of anti-Fas antibody, anti-Fas mAb plus Z-VAD-FMK inhibitor or PBS (negative control) for 16 hours at 37°C. Cell extracts were tested for caspase-3 activity according to the assay conditions described in Section 4. Z-VAD-FMK inhibitor was added to a final concentration of 50μM.

## 5. Calculation of Results

### 5.A. Determination of Relative Absorbance

Determine the mean absorbance of each sample and the blank then calculate the relative absorbance values ( $\Delta A$ ) as:

$$A_A = (\text{mean induced apoptosis sample } A_{405}) - (\text{mean blank } A_{405})$$

$$A_N = (\text{mean negative control sample } A_{405}) - (\text{mean blank } A_{405})$$

$$A_I = (\text{mean inhibited apoptosis sample } A_{405}) - (\text{mean blank } A_{405})$$

$$\Delta A = (A_A - A_N) - (A_I - A_N)$$

**Note:** Due to natural cell death in the test population,  $A_I$  may be less than  $A_N$ . If this is the case, let  $A_I - A_N = 0$

### 5.B. Calculation of Caspase Specific Activity

1. Construct a standard curve using known amounts of pNA as described in Section 6.

- Calculate the activity (X) of caspase-3 present in each sample using the following formula:

$$X = \text{pmol pNA liberated/hour}$$

$$= \frac{\Delta A - (Y \text{ intercept of pNA std. curve})}{\text{incubation time in hours}} \times \frac{100\mu\text{l (sample volume)}}{(\text{slope of pNA std. curve } (A_{405}/\text{pmol}/\mu\text{l}))}$$

- Determine the protein concentration of each extract in  $\mu\text{g}/\mu\text{l}$  by the method of Bradford (33) using a BSA standard curve. Calculate the micrograms of protein in each  $100\mu\text{l}$  sample volume (microliters extract added  $\times$  protein concentration).
- Calculate the specific activity (SA) of caspase-3 in the cell extract as follows:

$$SA = \frac{\text{pmol pNA liberated per hour}}{\mu\text{g protein}} = \frac{X \text{ (from Step 2)}}{\mu\text{g protein (Step 3)}}$$

## 6. Construction of pNA Calibration Curves

### 6.A. Preparation of pNA Stock Solutions

- Thaw the 100mM stock solution of pNA (*p*-nitroaniline).
- Prepare a 10mM stock solution of pNA by adding 900 $\mu\text{l}$  of DMSO to the vial containing 100 $\mu\text{l}$  (100mM) pNA Standard supplied. To confirm the pNA concentration, dilute the 10mM solution 1:200 in water and read the absorbance at 405nm in a standard 1cm cuvette. The concentration can be calculated using an extinction coefficient of 10,500M<sup>-1</sup>cm<sup>-1</sup> as follows:

$$\frac{A_{405} \times 200}{10,500} = \text{concentration (Molar)}$$

**Note:** The extinction coefficient has units of M<sup>-1</sup>cm<sup>-1</sup>. A 1cm path length must be used for this measurement. A multiwell plate is not suitable. A standard spectrophotometer typically has a 1cm path length, but multiwell plate readers do not. Therefore, multiwell plate readers are not recommended for confirming the concentration of the 10mM stock solution. However, once the concentration of the 10mM stock has been confirmed, a plate reader can be used to generate a standard curve (Figure 2).

- Perform serial dilutions of the 10mM stock solution in DMSO by combining the volumes shown in Table 1 to make 1mM, 100 $\mu\text{M}$  and 10 $\mu\text{M}$  stock solutions.

**Table 1. Preparation of pNA Stock Solutions.**

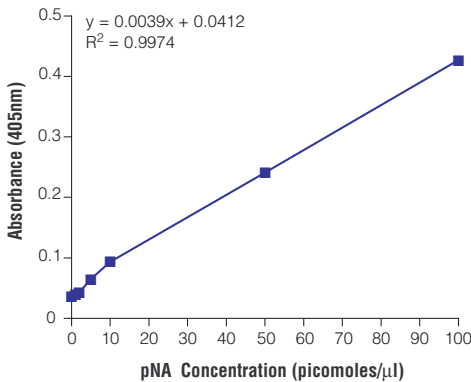
pNA Stock Solution				
10mM	1mM	100 $\mu\text{M}$	DMSO	Final pNA Solution
10 $\mu\text{l}$	–	–	90 $\mu\text{l}$	1mM
–	10 $\mu\text{l}$	–	90 $\mu\text{l}$	100 $\mu\text{M}$
–	–	10 $\mu\text{l}$	90 $\mu\text{l}$	10 $\mu\text{M}$

**6.B. pNA Calibration Curve for 96-Well Plate Assay (100µl reactions)**

1. Prepare 100µl of each pNA Standard (in duplicate) as described in Table 2.
2. Using a 96-well plate reader, measure the absorbance of each sample using a wavelength of 405nm.
3. Plot absorbance units (Y axis) versus amount of pNA in picomoles per microliter (X axis). The graph should be linear (Figure 2).
4. Calculate the slope of the calibration curve.

**Table 2. Components Required for pNA Calibration Curve (96-Well Assay).**

pNA Stock Solution					pNA Standard	
10µM	100µM	1mM	DMSO	Deionized Water	µM	pmol/µl
-	-	-	20µl	80µl	0	0
10µl	-	-	10µl	80µl	1	1
20µl	-	-	0	80µl	2	2
-	5µl	-	15µl	80µl	5	5
-	10µl	-	10µl	80µl	10	10
-	-	5µl	15µl	80µl	50	50
-	-	10µl	10µl	80µl	100	100



**Figure 2. pNA standard curve.** Absorbance values in Figure 2 were determined using a multiwell plate reader.

## 7. Composition of Buffers and Solutions

### Caspase Assay Buffer

312.5mM	HEPES (pH 7.5)
31.25% w/v	sucrose
0.3125% w/v	CHAPS (3-[(3-cholamido-propyl)-dimethylammonio]-1propane-sulfonate)

### DTT, 100mM

Prepare a 1M stock solution of DTT in deionized water. Store in aliquots at -20°C. Dilute 1:10 with deionized water to make a 100mM solution before use.

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## 9. Related Products

### Apoptosis Products

Product	Size	Cat.#
Caspase-Glo® 8 Assay*	2.5ml	G8200
	10ml	G8201
	100ml	G8202
Caspase-Glo® 9 Assay*	2.5ml	G8210
	10ml	G8211
	100ml	G8212
Caspase-Glo® 3/7 Assay*	2.5ml	G8090
	10ml	G8091
	100ml	G8092
Apo-ONE® Homogeneous Caspase-3/7 Assay	1ml	G7792
	10ml	G7790
	100ml	G7791
CaspACE™ Assay System, Fluorometric*	160 assays	G3540
CaspACE™ FITC-VAD-FMK	50µl	G7461
In Situ Marker (5mM)	125µl	G7462
Caspase Inhibitor Z-VAD-FMK (20mM)	50µl	G7231
	125µl	G7232
DeadEnd™ Colorimetric TUNEL System*	40 assays	G7130
	20 assays	G7360
DeadEnd™ Fluorometric TUNEL System	60 reactions	G3250
Anti-PARP p85 Fragment pAb	50µl	G7341
Anti-ACTIVE® Caspase-3 pAb	50µl	G7481
Anti-pS <sup>473</sup> Akt pAb	40µl	G7441
rhTNF-α	10µg	G5241
Terminal Deoxynucleotidyl	300u	M1871
Transferase, Recombinant*	1,500u	M1875

\*For Laboratory Use.

**Cell Viability Products**

<b>Product</b>	<b>Size</b>	<b>Cat.#</b>
CellTiter-Glo® Luminescent Cell Viability Assay (ATP, luminescent)	10ml	G7570
	10 × 10ml	G7571
	100ml	G7572
	10 × 100ml	G7573
CellTiter-Blue® Cell Viability Assay (resazurin, fluorometric)	20ml	G8080
	100ml	G8081
	10 × 100ml	G8082
CellTiter 96® AQueous One Solution Cell Proliferation Assay* (MTS, colorimetric)	200 assays	G3582
	1,000 assays	G3580
	5,000 assays	G3581
CellTiter 96® AQueous Non-Radioactive Cell Proliferation Assay* (MTS, colorimetric)	1,000 assays	G5421
	5,000 assays	G5430
	50,000 assays	G5440
CellTiter 96® Non-Radioactive Cell Proliferation Assay* (MTT, colorimetric)	1,000 assays	G4000
	5,000 assays	G4100
CytoTox-ONE™ Homogeneous Membrane Integrity Assay (LDH, fluorometric)	200–800 assays	G7890
	1,000–4,000 assays	G7891
CytoTox-ONE™ Homogeneous Membrane Integrity Assay, HTP (LDH, fluorometric)	1,000–4,000 assays	G7892
CytoTox 96® Non-Radioactive Cytotoxicity Assay* (LDH, colorimetric)	1,000 assays	G1780
MultiTox-Fluor Multiplex Cytotoxicity Assay* (live:dead cell ratio)	10ml	G9200
	5 × 10ml	G9201
	2 × 50ml	G9202
CytoTox-Fluor™ Cytotoxicity Assay*	10ml	G9260
	5 × 10ml	G9261
	2 × 50ml	G9262

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