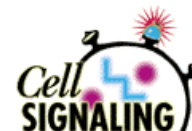


CaspACE™ Assay System, Fluorometric



By Gopa Mitra and Terry Riss*
Promega Corporation

*Corresponding author: e-mail to triss@promega.com

Promega's CaspACE™ Assay System, Fluorometric, provides reagents for measuring the activity of two cysteine aspartic acid-specific proteases caspase-1 (ICE) and caspase-3 (CPP32). Caspase is the accepted acronym for this class of enzymes, the "c" is intended to reflect a cysteine protease mechanism and the "aspase" refers to their ability to cleave after aspartic acid (1). The CaspACE™ Assay System provides fluorogenic tetrapeptide substrates and selective inhibitors, which allow highly sensitive, rapid and quantitative measurement of these enzymes. The use of the two selective substrates and inhibitors provided in the system allows discrimination between ICE and CPP32 enzymatic activities. This assay may be used to measure ICE and CPP32 activities in either purified enzyme preparations or cell extracts and can be adapted for use in high throughput assay systems.

INTRODUCTION

The caspase, or Interleukin-1beta-Converting Enzyme (ICE/CED-3), family of proteases are essential components of an evolutionarily conserved cell death pathway in multicellular eukaryotes (2). These enzymes have been shown to play key roles in inflammation and apoptosis in mammalian cells (3-5). Active caspases participate in a cascade of proteolytic cleavage events in dying cells. To date, twelve caspases have been characterized. These fall into two major subfamilies based upon sequence homology: the ICE subfamily and the CED-3 subfamily ([Table 1](#)).

Table 1. Caspases Divided into Subfamilies Based on Sequence Homology.

ICE Subfamily	CED-3 Subfamily
caspase-1 (ICE)	caspase-2
	caspase-3 (CPP32, Yama, apopain)
caspase-4 (Tx, ICH-2, ICE _{rel} II)	caspase-6 (Mch-2)
caspase-5 (ICE _{rel} III)	caspase-7 (Mch3, ICE-LAP3, CMH-1)
caspase-11 (ICH-3)	caspase-8 (FLICE, Mach1, Mch 5)
	caspase-9
	caspase-10 (Mch4)
	caspase-10b (FLICE 2)

Caspase-1, the prototype of the ICE subfamily, has a substrate specificity for the amino acid sequence YVAD (Tyr-Val-Ala-Asp), and is inhibited by the tetrapeptide inhibitor Ac-YVAD-CHO. Caspase-3 (CPP32), the prototype of the CED-3 subfamily, has a substrate specificity for the amino acid sequence DEVD (Asp-Glu-Val-Asp), and is inhibited by the tetrapeptide inhibitor Ac-DEVD-CHO (6,7). Caspase-1 (ICE) is primarily involved in procytokine activation and its role in apoptosis is uncertain; however, caspases 2, 3, 6, 7, 8 and 10 have been shown to promote apoptotic cell death pathways (4). Some of the known biological substrates of ICE-like proteases include poly(ADP-ribose) polymerase (PARP), DNA-dependent protein kinase (DNA-PK), nuclear lamins, actin, fodrin, topoisomerases, Gas2, protein kinase C (PKC)delta, sterol regulatory element binding proteins (SREBP), U1-70kDa protein, retinoblastoma tumor suppressor protein, Huntingtin protein, 45kDa subunit of DNA fragmentation factor (DFF) and PITSLRE kinase alpha2-1 (5).

ASSAY FOR CASPACE ACTIVATION USING CRUDE CELL LYSATES

Recent reports indicate that the cytotoxicity of almost all chemotherapeutic drugs is accompanied by apoptosis in susceptible cell lines (8) and that eukaryotic cells often undergo programmed cell death in response to ionizing radiation (9,10). In the present study, different mammalian cell lines were tested for activation of ICE or CPP32 enzymatic activity after treatment with either anti-Fas antibody, etoposide (a DNA topoisomerase inhibitor) and staurosporin (a protein kinase inhibitor) or after exposure to ionizing radiation. After treatment, the cells were washed with ice-cold PBS and resuspended in a hypotonic cell lysis buffer (25mM HEPES (pH 7.5), 5mM MgCl₂, 5mM DTT, 5mM EDTA, 2mM PMSF, 10µg/ml leupeptin and 10µg/ml pepstatin) to a final concentration of 10⁸ cells/ml. The cells were then lysed by exposure to four freeze-thaw cycles. The cell lysate was subjected to centrifugation at 16,000 x g for 20 minutes at 4°C and the supernatant fraction was used as the source of enzyme. ICE and CPP32 enzyme activities were measured by a fluorometric tetrapeptide substrate cleavage assay using the CaspACE™ Assay System, Fluorometric. The fluorescence values obtained may vary depending on the instrument used for detection. The assays described here were performed in a white, 96 well plate (Cliniplate, LabSystems) and fluorescence was measured using an excitation wavelength of 360nm and an emission wavelength of 460nm in a CytoFluor™ II Microwell Fluorescence Reader (PerSeptive Biosystems). For each experiment, assays were performed in triplicate. Variation between assays was less than 10%.

Figure 1, Panel A, shows the effect of anti-Fas antibody treatment on the induction of apoptosis in leukemic human Jurkat T-cells. Cells were exposed to anti-Fas antibody for 0, 2, 4, 6 and 8 hours and the percentage of apoptotic cells was monitored by flow cytometry using Promega's Apoptosis Detection System, Fluorescein (Cat.# G3250), based on the TUNEL (TdT-mediated dUTP Nick End Labeling) method. The results show an increase in apoptosis as a percentage of the cell population at 2 (16%), 4 (26%), 6 (35%) and 8 (52%) hours of exposure.

Figure 1, Panel B, shows the differential enzymatic activation of ICE and CPP32 in Jurkat T-cells after 4 hours of exposure to 100ng/ml of anti-Fas antibody. Enzyme activity was measured using the CaspACE™ Assay System, Fluorometric. The results show that anti-Fas antibody treatment specifically resulted in an almost eight-fold increase in CPP32 activity compared to that of control (untreated) cells. No change in ICE activity was observed between control cells and anti-FAS antibody-treated cells.

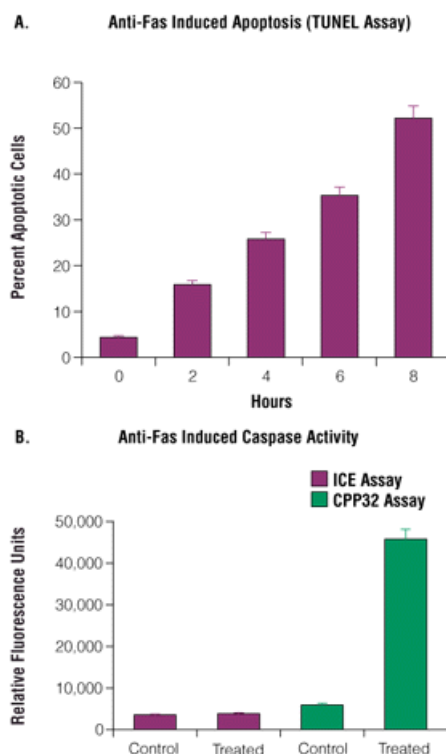


Figure 1. Jurkat T-cells treated with anti-Fas antibody. Panel A: Measurement of DNA strand breaks associated with apoptosis of Jurkat T-cells (ATCC# TIB-152) following treatment with anti-Fas antibody. Jurkat T-cells (5×10^5 cells/ml) were treated with 100ng/ml of anti-Fas antibody for 0, 2, 4, 6 and 8 hours at 37°C. Fixed cells were subjected to TUNEL procedure using Promega's Apoptosis Detection System, Fluorescein (Cat.# G3250), and were analyzed by flow cytometry as described previously (11). Values depict the mean of triplicate experiments \pm SEM. **Panel B:** ICE and CPP32 protease activities in anti-Fas antibody-treated Jurkat T-cells. Cells (5×10^5 cells/ml) were treated with 100ng/ml of anti-Fas antibody (Mab CH-11, PanVera Corporation) or PBS for 4 hours at 37°C. Cell extracts were assayed for ICE and CPP32 activities using the CaspACE™ Assay System, Fluorometric.

THE EFFECT OF STAUROSPORIN AND ETOPOSIDE ON CASPACE ACTIVITY

The results in [Table 2](#) show the effect of the broad-spectrum protein kinase inhibitor staurosporin (Sigma) and the DNA topoisomerase II inhibitor etoposide (Sigma) on the activities of ICE and CPP32 in Jurkat T-cells and HL-60 cells, respectively. Staurosporin has been shown to induce programmed cell death in many mammalian cell types (12). Similar to treatment with anti-Fas antibody, treatment of Jurkat cells with staurosporin (8 μ M for 6 hours) resulted in specific CPP32 activation. CPP32 activity was increased by eight-fold over the uninduced control culture. No activation of ICE was observed.

Table 2. Caspase Activity in Extracts of Jurkat T-cells and HL-60 Cells Treated with Staurosporin or Etoposide.

Treatment	Cell Type	ICE Activity (relative fluorescence units)		CPP32 Activity (relative fluorescence units)	
		Control	Treated	Control	Treated
Staurosporin	Jurkat T-cells	851	659	915	7,159
Etoposide	HL-60 cells	4,064	4,041	7,582	41,004

Jurkat T-cells (1 x 10⁶ cells/ml) were treated with 8 μ M staurosporin for 6 hours at 37°C. HL-60 cells (5 x 10⁵ cells/ml) were treated with 68 μ M etoposide for 5 hours at 37°C. Equivalent numbers of cells were exposed to PBS alone in control experiments. ICE and CPP32 activities in cell lysates were monitored using the CaspACE™ Assay System, Fluorometric.

Treatment of human promyelocytic leukemia HL-60 cells with 68 μ M etoposide for 5 hours resulted in a six-fold increase in CPP32 enzyme activity compared to that of untreated control cells. No change in ICE activity between control and etoposide-treated cells was observed.

THE EFFECT OF IONIZING RADIATION ON CASPACE ACTIVITY

The results shown in [Figure 2](#) demonstrate the effect of exposure to ionizing radiation on CPP32 activity in Jurkat T-cells. Exposure to 2,000 and 4,000 rads resulted in a 3.8- and 7.5-fold increase in CPP32 activity in irradiated cells, compared with untreated controls.

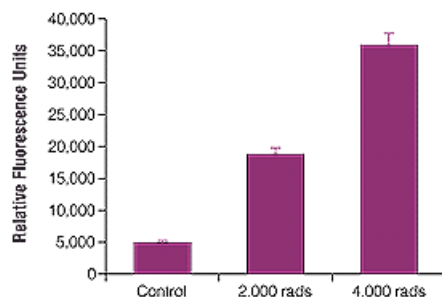


Figure 2. CPP32 activity in Jurkat T-cells after exposure to ionizing radiation (IR). Jurkat T-cells (5 x 10⁵ cells/ml) were exposed to 0, 20 or 40 gray IR for 6 hours. Irradiation was performed with a gamma-ray source (Cesium 137 gamma-irradiator, Mark-I). Cell lysates were assayed for CPP32 activity using the CaspACE™ Assay System, Fluorometric. The results shown represent the mean of 3 separate experiments \pm SEM.

The CaspACE™ Assay System, Fluorometric, provides selective reversible tetrapeptide inhibitors for both ICE and CPP32. [Figure 3, Panel A](#), shows the effects of the ICE and CPP32 inhibitors on the corresponding enzymatic activities in extracts of human monocytic THP-1 cells. The results show that 97% of the CPP32 activity in THP-1 cells was inhibited in the presence of 50 μ M of the CPP32 inhibitor, Ac-DEVD-CHO, and that 81% of the ICE activity could be inhibited in the presence of the ICE inhibitor, Ac-YVAD-CHO. [Figure 3, Panel B](#), shows the effect of Ac-DEVD-CHO on the CPP32 activity of anti-Fas antibody treated Jurkat T-cells. Treatment with 100ng/ml of anti-Fas antibody for 4 hours resulted in 8.5-fold activation of CPP32 activity over that of control cells. Pre-incubation of the anti-Fas antibody-treated cell extracts with 10 μ M of Ac-DEVD-CHO caused 98% inhibition of the Fas receptor-mediated elevation of CPP32 activity.

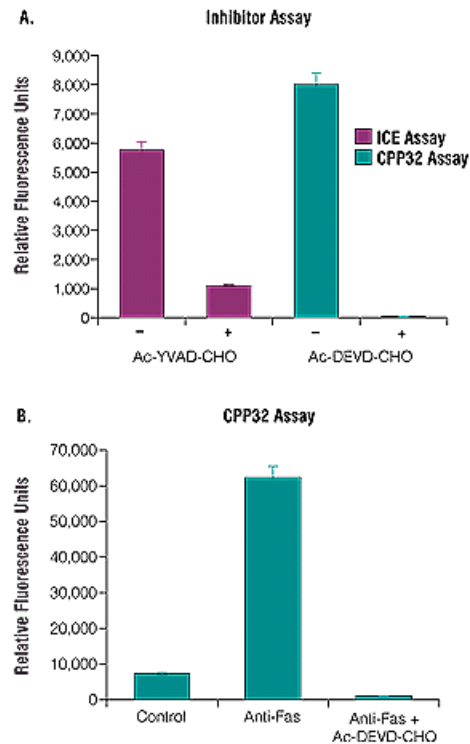


Figure 3. Enzymatic activities of ICE and CPP32 following treatment with selective tetrapeptide inhibitors. Panel A: The effects of the ICE and CPP32 inhibitors on the corresponding enzymatic activities in human monocytic THP-1 cells. THP-1 cells were grown to a concentration of 5×10^5 cells/ml in Dulbecco's modified Iscove's medium with 9% horse serum and 0.05mM beta-mercaptoethanol. Cell lysates were pre-activated by incubating at 30°C for 1 hour. Cell lysates were then incubated with and without 50 μ M ICE inhibitor (Ac-YVAD-CHO), or CPP32 inhibitor (Ac-DEVD-CHO) for 30 minutes at 30°C prior to assay. Caspase assays were performed in the presence of 50 μ M of the corresponding substrate, according to the procedure described in the *CaspACE™ Assay System, Fluorometric, Technical Bulletin #TB248*. **Panel B:** The effect of the CPP32 inhibitor on CPP32 protease activity expressed by anti-Fas antibody-treated Jurkat T-cells. Human Jurkat cells were treated with either 100ng/ml of anti-Fas antibody or PBS (control) for 4 hours at 37°C. Cell lysates were pre-incubated with either 10 μ M of CPP32 inhibitor or PBS for 30 minutes at 30°C and then assayed for CPP32 activity using the *CaspACE™ Assay System, Fluorometric*. All results represent the mean of 3 separate experiments \pm SEM.

SUMMARY

The CaspACE™ Assay System, Fluorometric, provides a highly sensitive, rapid, convenient and quantitative measurement of both ICE and CPP32, and allows discrimination between these two activities. Using this system, a marked increase in CPP32 activity was detected in cytosolic extracts of human cells that had been exposed to ionizing radiation or had been treated with anti-Fas antibody, staurosporin or etoposide. The CPP32 inhibitor, Ac-DEVD-CHO, inhibited this activity. No change in ICE activity was observed under these treatment conditions. These studies corroborate earlier observations that CPP32 is one of the major effector caspases involved in the apoptotic cell death process induced by ionizing radiation or chemotherapy (12-15). Additional experiments using cell lysates from THP-1 cells, which are known to contain ICE activity, illustrated that this assay system is capable of detecting ICE activity. The CaspACE™ Assay System has application for studies on caspase activation during animal development, homeostasis and pathology. This assay system also may be used in high throughput screening for activators or inhibitors that modulate caspase activity. For example inhibitors of ICE may prevent proinflammatory cytokine activation and have application for the treatment of inflammatory diseases, while activators of CPP32 may be used to kill malignant cells and inhibitors of CPP32 may prevent unwanted cell death in the treatment of acute degenerative diseases.

ACKNOWLEDGEMENTS

Special thanks to the State Laboratory of Hygiene at the University of Wisconsin, Madison for their assistance in performing radiation exposure experiments and in generating the flow cytometry data presented in this article.

REFERENCES

1. Alnemri, E.S. *et al.* (1996) *Cell* **87**, 171.
2. Yuan, J. *et al.* (1993) *Cell* **75**, 641.
3. Nicholson, D.W. and Thornberry, N.A. (1997) *TIBS* **22**, 299.

4. Salvesen, G.S. and Dixit, V.M. (1997) *Cell* **91**, 443.
5. Villa, P., Kaufmann, S.H. and Earnshaw, W.C. (1997) *TIBS* **22**, 388.
6. Thornberry, N.A. *et al.* (1992) *Nature* **356**, 768.
7. Nicholson, D.W. *et al.* (1995) *Nature* **376**, 37.
8. Lowe, S.W. *et al.* (1994) *Science* **266**, 8.
9. Kaufmann, S.H. *et al.* (1993) *Cancer Res.* **53**, 3976.
10. Datta, R. *et al.* (1997) *J. Biol. Chem.* **272**, 1965.
11. *Apoptosis Detection System, Fluorescein, Technical Bulletin #TB235*, Promega Corporation.
12. Jakobson, M.D. *et al.* (1996) *J. Cell. Biol.* **133**, 1041.
13. Enari, M. *et al.* (1996) *Nature* **380**, 723.
14. Schlegel, J. *et al.* (1996) *J. Biol. Chem.* **271**, 1841.
15. Martins, L.M. *et al.* (1997) *J. Biol. Chem.* **272**, 74.

Ordering Information	
Product	Cat.#
CaspACE™ Assay System, Fluorometric	G3540

Each system contains sufficient reagents to perform at least 160 reactions (standard format) or 800 reactions (96 well plate format).

Related Product	
Product	Cat.#
Apoptosis Detection System, Fluorescein	G3250

Each system contains sufficient reagents to perform 60 reactions.

© 1998 Promega Corporation. All Rights Reserved.

CaspACE is a trademark of Promega Corporation.

CytoFluor is a trademark of PerSeptive Biosystems.

Product claims are subject to change. Please contact Promega Technical Services or access the Promega online catalog for the most up-to-date information on Promega products.