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Sensitivity of the Colorimetric CaspACE[™] Assay System and Purification of Fragmented DNA from Apoptotic Cells

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ABSTRACT

In this applications note the sensitivity of the CaspACE[™] Assay System, Colorimetric, for the detection of caspase-3 activity was evaluated using both purified caspase-3 and extracts from apoptotic cells. The utility of the Wizard® Genomic DNA Purification Kit and the SV Total RNA Isolation System for the isolation of apoptotic, fragmented DNA was also demonstrated.

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Introduction

Apoptosis is an active, genetically programmed and evolutionarily conserved process by which cells self-destruct (1). The area of apoptosis research has grown substantially in the past 10–15 years, and the number of techniques utilized to detect this cellular process, and distinguish it from necrosis or accidental cell death, are numerous. Such techniques include, but are not limited to, light and electron microscopy, flow cytometry, preferential dye staining, tissue transglutaminase activity, annexin V binding, changes in ion fluxes, DNA fragmentation detection, caspase protease activity and cleavage of poly (ADP-ribose) polymerase (PARP) (2) (3) (4). Typically, two independent methods are necessary to verify that cell death is occurring via apoptosis in any particular cell or tissue system.

Many of the proteins that participate in the apoptotic pathways have been identified. At the heart of these pathways are a family of cysteine proteases, termed "caspases", which have been shown to cleave a number of intracellular targets, including PARP, DNA-dependent protein kinase (DNA-PK), lamins, topoisomerases, Gas2, protein kinase C (PKC) delta, sterol regulatory element binding proteins (SREBP), U1-70kDa protein, and Huntingtin protein (5) (6). Caspase-3-like proteases show specificity for cleavage at the C-terminal side of the aspartate residue in the sequence DEVD, while caspase-1-like proteases show specificity for cleavage at the C-terminal side of the aspartate residue in the sequence YVAD.

Promega has developed a colorimetric (CaspACE[™] Assay System, Colorimetric, Cat.# G7220) assay systems for the detection of caspase-3 (CPP32) or caspase-1 (ICE) activity. The CaspACE[™] Assay System, Colorimetric, includes a colorimetric substrate and a cell-permeable pan-caspase inhibitor (Z-VAD-FMK) that allows the highly sensitive and quantitative measurement of caspase-3 (DEVDase) activity.

A commonly used technique in apoptosis research is the detection of the characteristic, nonrandom DNA fragmentation that occurs during apoptosis. In many cell types, apoptosis is characterized by the generation of DNA fragments through the action of endogenous nucleases (7) (8) (9) (10). The DNA is cleaved internucleosomally to a population of multimers of 180–200bp fragments, which may be detected as a ladder of DNA fragments on an agarose gel. Alternately, DNA fragmentation may be detected using in vitro and in situ DNA end-labeling (TUNEL method) (11), PCR analysis (12), COMET assays (13) and ELISA systems (14). Promega offers two systems for the in situ detection of fragmented DNA using the TUNEL method: the DeadEnd[™] Fluorometric TUNEL System (Cat.# G3250), and the DeadEnd[™] Colorimetric TUNEL System (Cat.# G7130).

Studies were performed to characterize the overall sensitivity of the colorimetric CaspACE[™] Assay System. For these studies, the detection levels for caspase-3 activity were determined using purified caspase-3 enzyme, as well as cell extracts derived from Jurkat cells induced to undergo apoptosis by treatment with an anti-Fas monoclonal antibody (12). In addition, the ability of the Wizard® Genomic DNA Purification Kit (Cat.# A1120) and the SV Total RNA Isolation System (Cat.# Z3100) to purify apoptotic fragmented DNA was demonstrated.

Experimental Conditions

CASPASE ACTIVITY ASSAY

Jurkat cells (1 × 106 cells/ml) were incubated in RPMI-1640 medium containing 10% fetal bovine serum with or without 50ng/ ml anti-Fas mAb clone CH-11 (PanVera Corporation) for 16 hours at 37°C in 5% CO2. Following incubation, the cells were centrifuged, washed once in PBS, suspended to 108 cells/ml and then lysed in Cell Lysis Buffer (included with the CaspACETM Assay System, Colorimetric) by performing 2 freeze-thaw cycles. The cell lysates were subjected to centrifugation for 20 minutes at 15,000 × *g* at 4°C, and the supernatant fraction was collected and stored at -70° C. The protein concentration of each extract was determined using the Bradford method and adjusted to 5mg/ml.

Increasing amounts of either purified active caspase-3 (PharMingen; lot# M035347), untreated (control) cell extract or anti-Fas mAb-treated cell extract were combined with the appropriate amounts of Caspase Assay Buffer, DTT and water in 96 well plates (white Dynex Microfluor™ flat-bottomed plates for the fluorometric assay or clear Costar® flat-bottomed plates for the colorimetric assay) as described in the *CaspACE*[™] *Assay System, Colorimetric, Technical Bulletin,* #TB270. Samples were assayed in triplicate or quadruplicate, either in the presence of DMSO (solvent control for inhibitors) or 50µM of the caspase-3 (CPP32) inhibitor Z-VAD-FMK for the colorimetric assay. Samples were incubated for 30 minutes at 37°C in the presence or absence of the caspase-3 inhibitors, then 50µM caspase-3 substrate was added to all wells (Ac-DEVD-pNA for the colorimetric assay). The samples were incubated at 37°C for 4 hours. Both the purified caspase-3 and the control or treated cell extracts were diluted in 0.3X Caspase Assay Buffer containing 1mg/ml BSA. In addition, 1mg/ml BSA was included in the caspase assay to act as a nonspecific protein carrier. The results were then measured using a Molecular Devices SPECTRAmax® 250 plate reader (absorbance = 405nm).

DNA ISOLATION AND ANALYSIS

Jurkat cells (1 x 106 cells/ml) were incubated in RPMI-1640 medium containing 10% fetal bovine serum with or without 50ng/ml anti-Fas mAb clone CH-11 (PanVera) for 0, 3, 6 or 9 hours at 37°C in 5% CO2. Aliquots of control and treated cells (106 cells) were taken at the indicated times after antibody addition and centrifuged for 5 minutes at 500 × g in a microcentrifuge at room temperature to pellet the cells. The culture medium supernatant was removed and the cell pellets were stored at -70°C.

Genomic DNA was isolated from each cell pellet using either the Wizard® Genomic DNA Purification Kit following the protocol for cultured cells in the *Wizard*® Genomic DNA Purification Kit Technical Manual #TM050 or the SV Total RNA Isolation System, with minor protocol modifications (16; for detailed information see the *SV Total RNA Isolation System Technical Manual* #TM048). The modifications to the SV Total RNA Isolation System protocol involve a modified wash solution (70%)

ethanol instead of the supplied column wash solution) as well as digestion of the captured nucleic acid with 4mg/ml RNaseA (5µl; Cat.# A7973) in 45µl Yellow Core Buffer, instead of the DNase included in the SV Total RNA Isolation System (16). The captured DNA was eluted from the silica membrane with 100µl of room temperature nuclease-free water. Additional aliquots of control cells were taken at t = 0, and 20µl of 100bp DNA Ladder (Cat.# G2101) were added to each of these to monitor the ability of both purification systems to isolate small DNA fragments in addition to intact genomic DNA. All DNA samples were precipitated with 0.1 volumes of 3M sodium acetate (pH 5.3) and two volumes of 100% ethanol, incubated overnight at -20° C, centrifuged for 10 minutes at 17,000 × g in a room temperature microcentrifuge and resuspended in 20µl nuclease-free water. A 15µl aliquot of each DNA sample was analyzed on a 2% agarose gel and stained with ethidium bromide.

Additional aliquots of both control and treated cells were taken at the 9-hour time point, and extracts were prepared as above for the determination of caspase activity. Caspase activity was assayed to verify that the anti-Fas mAb-treated Jurkat cells had been induced to undergo apoptosis. The extracts were assayed in duplicate and values were averaged.

Results

To investigate the sensitivities of the CaspACE[™] Assay System, Colorimetric, purified caspase-3, control lysate from untreated Jurkat cells and lysate from anti-Fas mAb-treated Jurkat cells were titrated into both assay systems. The results for each amount of enzyme or extract, in the absence or presence of the appropriate caspase-3 inhibitor, are presented in Figure 1. A range of 0–1,000pg of purified caspase-3 enzyme was investigated. The detection limit is the lowest amount of analyte (caspase-3) that can be detected in a sample and may be defined by the signal that is 6 sigma (standard deviation) values above that obtained for inhibitor-treated samples. The CaspACE[™] Assay System, Colorimetric, was able to detect 100pg of purified caspase-3 enzyme (Figure 1, Panel A).



A. CaspACE[™] Assay System, Colorimetric



B. CaspACE[™] Assay System, Colorimetric

Figure 1. Caspase-3 activity detection using either the CaspACE[™] Assay System, Colorimetric, or the CaspACE[™] Assay System, Fluorometric. Caspase-3 activity was measured using either purified caspase-3 enzyme (Panel A) or lysate from untreated Jurkat cells (control) or Jurkat cells treated for 16 hours with 50ng/ml anti-FAS mAb (Panel B) using the CaspACE[™] Assay System, Colorimetric. The results are presented as either absorbance at 405nm or relative fluorescence units (RFU) for triplicate or quadruplicate samples, ± standard deviation. Note: The scale on the x-axis is not proportional.

The sensitivity of each assay system was also investigated using either control Jurkat cell extracts or extracts from Jurkat cells treated with anti-Fas mAb (Figure 1, Panels C and D). A range of 0–50µg of cell extract was added to each assay. The

CaspACE[™] Assay System, Colorimetric, was able to detect caspase-3 activity from 10µg of apoptotic cell extract (Figure 1, Panel C).

The isolation and subsequent agarose gel analysis of apoptotic, fragmented DNA is a commonly used technique to verify the apoptotic process is occurring. To demonstrate that apoptotic DNA could be successfully isolated using either the Wizard® Genomic DNA Purification Kit or with a modified protocol using the SV Total RNA Isolation System, Jurkat cells were induced to undergo apoptosis by treatment with anti-Fas mAb for up to 9 hours. This time-point has previously been shown to exhibit maximal TUNEL staining using the DeadEnd[™] Colorimetric Apoptosis Detection System (15). Aliquots of both the control and treated cells were taken at 0, 3, 6 and 9 hours and genomic DNA isolated as described above. The modified protocol for using the SV Total RNA Isolation System for DNA purification included substituting RNase A for the supplied DNase, as well as using 70% ethanol instead of the supplied column wash solution .

A. Wizard[®] Genomic DNA Purification Kit







Figure 2. Apoptotic DNA purified using either the Wizard® Genomic DNA Purification Kit (Panel A) or the SV Total RNA Isolation System (Panel B). Jurkat cells were induced to undergo apoptosis with 50ng/ml anti-Fas mAb, and aliquots of either treated or untreated cells were taken at 0, 3, 6 and 9 hours. Genomic DNA was isolated and an aliquot was separated on a 2% agarose gel. Lane 1: 5µl 100bp DNA Ladder; Iane 2: time 0 cells + 20µl 100bp DNA Ladder; Iane 3: time 0 cells; Iane 4: 3 hours –mAb; Iane 5: 6 hours –mAb; Iane 6: 9 hours –mAb; Iane 7: 3 hours +mAb; Iane 8: 6 hours +mAb; Iane 9: 9 hours +mAb.

In Figure 2, the characteristic apoptotic DNA ladder can be seen in the Jurkat cells treated for 3, 6 or 9 hours with the anti-Fas mAb, using either purification system (Figure 2, Panels A and B, Ianes 7–9). Fragmented DNA is absent or light in the untreated cells (Figure 2, Panels A and B, Ianes 3–6). DNA purification using the Wizard® Genomic DNA Purification Kit appeared to isolate more high molecular weight (intact) genomic DNA than the SV Total RNA Isolation System. This is probably due to the tight binding of high molecular weight DNA to silica membrane based systems, and thus inefficient elution

with room temperature water. The room temperature elution was performed to preferentially elute the smaller DNA fragments to increase the likelihood of obtaining apoptotic DNA. Both systems were capable of purifying small DNA fragments, as demonstrated by the cell samples that were spiked with 100bp DNA ladder (Figure 2, Panels A and B, Iane 2). The DNA isolated using the SV Total RNA Isolation System also exhibited more RNA contamination (most likely tRNAs) than that obtained using the Wizard® Genomic DNA Purification Kit. This RNA could possibly be more completely removed by a longer incubation with RNase A or by using a higher concentration of RNase A. However, the tRNA present does not interfere with the detection of the apoptotic DNA ladder.

To verify that these Jurkat cells were induced to undergo apoptosis, and as an independent indicator of apoptosis, aliquots of both the control and treated cells were taken at the 9-hour time point and assayed for caspase-3 activity using the CaspACE[™] Assay System, Fluorometric. The anti-Fas mAb treatment resulted in an approximately 6-fold increase in caspase-3 activity, verifying through an independent method that the Jurkat cells were indeed apoptotic (Table 1).

Caspase-3 Activity in Jurkat Cells Treated With Anti-Fas mAb for 9 Hours.				
Sample	Anti-Fas mAb- Treated Extract (RFU*)	Untreated Control Extract (RFU)	No Extract (RFU)	
-Caspase-3 Inhibitor	2135.0	389.0	38.0	
+Caspase-3 Inhibitor	29.5	35.0	36.0	
*Relative fluorescence un	iits			

Table 1. Caspase-3 Activity in Jurkat cells Treated with Anti-Fas mAb for 9 Hours.

Conclusions

The CaspACE[™] Assay Systems, Colorimetric is a convenient and sensitive method to quantitate caspase-3 (DEVDase) activity in cell or tissue extracts. The system is capable of detecting picogram levels of purified caspase-3, or caspase-3 in low microgram levels in cell lysates. The minimal detection limits for any particular cell or tissue extract will be highly dependent on the amount of active caspase-3 enzyme present and will thus be dependent on how efficiently and how synchronously apoptosis is induced in the target cell population. The induction of apoptosis in Jurkat cells with anti-Fas mAb is an excellent model system to study the apoptotic process.

The isolation and subsequent gel analysis of apoptotic, fragmented DNA is a powerful and relatively inexpensive method to verify that apoptosis is occurring in a given cell or tissue sample. These studies demonstrate that both the Wizard® Genomic DNA Purification Kit or a modified protocol using the SV Total RNA Isolation System can be used successfully to purify apoptotic DNA for subsequent agarose gel analysis. For these experiments, approximately one million cells were harvested per time point, and 75% of the total amount of genomic DNA isolated was analyzed on the gel. Different cell or tissue systems may require more or less sample to be analyzed to allow for visualization of the apoptotic DNA ladder.

REFERENCES

- 1. Kerr, J.F.R. and Harmon, B.V. (1991) *Apoptosis: The Molecular Basis of Cell Death*, Cold Spring Harbor Laboratory Press, New York.
- 2. Cotter, T.G. and Martin, S.J. (1996) Techniques in Apoptosis: A User's Guide, Portland Press, Ltd., London.
- 3. Schwartz, L.M. and Osborne, B.A. (1995) *Methods in Cell Biology: Cell Death*, Vol. 46, Academic Press, San Diego, CA.
- 4. Saldeen, J. and Welsh, N. (1998) Nicotinamide-induced apoptosis in insulin producing cells is associated with cleavage of poly(ADP-ribose) polymerase. *Mol. Cell. Endocrinol.* **139**, 99–107.
- 5. Nicholson, D.W. and Thornberry, N.A. (1997) Caspases: Killer proteases. TIBS 22, 299–306.
- 6. Villa, P., Kaufmann, S.H. and Earnshaw, W.C. (1997) Caspases and caspase inhibitors. TIBS 22, 388–93.
- 7. Schwartzman, R.A. and Cidlowski, J.A. (1993) Apoptosis: The biochemistry and molecular biology of programmed cell death. *Endocrine Rev.* 14, 133–51.
- 8. Walker, P.R. *et al.* (1991) Topoisomerase II-reactive chemotherapeutic drugs induce apoptosis in thymocytes. *Cancer Res.* **51**, 1078–85.
- 9. Oberhammer, F. *et al.* (1993) Apoptotic death in epithelial cells: Cleavage of DNA to 300 and/or 50 kb fragments prior to or in the absence of internucleosomal fragmentation. *EMBO J.* **12**, 3679–84.
- 10. Roy, C. *et al.* (1992) The topoisomerase II inhibitor teniposide (VM-26) induces apoptosis in unstimulated mature murine lymphocytes. *Exp. Cell Res.* **200**, 416–24.
- 11. Gavrieli, Y. *et al.* (1992) Identification of programmed cell death in situ via specific labeling of nuclear DNA fragmentation. *J. Cell. Biol.* **119**, 493–501.
- 12. Jennerwein, M.M. and Eastman, A. (1991) A polymerase chain reaction-based method to detect cisplatin adducts in specific genes. *Nucleic Acids Res.* **19**, 6209–14.
- 13. Nelms, B.E., Moravec, R. and Riss, T. (1997) Measuring apoptosis in individual cells with the comet assay. *Promega Notes* **64**, 13–6.
- 14. Salgame, P. et al. (1997) An ELISA for detection of apoptosis. Nucleic Acids Res. 25, 680-1.
- 15. Okragly, A. and O'Brien, M. (1999) Death Check[™] Assay Systems for monitoring apoptosis. *Promega* Notes **72**, 3–6.

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FIGURES

A. CaspACE[™] Assay System, Colorimetric



B. CaspACE[™] Assay System, Colorimetric



Figure 1. Caspase-3 activity detection using either the CaspACE[™] Assay System, Colorimetric, or the CaspACE[™] Assay System, Fluorometric. Caspase-3 activity was measured using either purified caspase-3 enzyme (Panel A) or lysate from untreated Jurkat cells (control) or Jurkat cells treated for 16 hours with 50ng/ml anti-FAS mAb (Panel B) using the CaspACE[™] Assay System, Colorimetric. The results are presented as either absorbance at 405nm or relative fluorescence units (RFU) for triplicate or quadruplicate samples, ± standard deviation. Note: The scale on the x-axis is not proportional.

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B. SV Total RNA Isolation System



Figure 2. Apoptotic DNA purified using either the Wizard® Genomic DNA Purification Kit (Panel A) or the SV Total RNA Isolation System (Panel B). Jurkat cells were induced to undergo apoptosis with 50ng/ml anti-Fas mAb, and aliquots of either treated or untreated cells were taken at 0, 3, 6 and 9 hours. Genomic DNA was isolated and an aliquot was separated on a 2% agarose gel. Lane 1: 5µl 100bp DNA Ladder; Iane 2: time 0 cells + 20µl 100bp DNA Ladder; Iane 3: time 0 cells; Iane 4: 3 hours –mAb; Iane 5: 6 hours –mAb; Iane 6: 9 hours –mAb; Iane 7: 3 hours +mAb; Iane 8: 6 hours +mAb; Iane 9: 9 hours +mAb.

Tables

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