

CaspACE™ FITC-VAD-FMK In Situ Marker for Apoptosis: Applications for Flow Cytometry

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Cells frequently respond to environmental changes or insults by undergoing apoptosis. Apoptosis is an essential component of the organism's repertoire of cellular responses, whether it is triggered by a genetic program for development or induced by an external factor. Researchers performing cell-based assays in a high-throughput screening (HTS) capacity are frequently interested in the apoptotic response. In cell-based assays for cancer drugs, apoptosis may be the desired response; conversely, in screens designed for therapeutic reagents for various degenerative diseases, the ability to inhibit apoptosis may be desired. Most assays for apoptosis are not readily adapted to HTS, however. Promega has recently developed a useful marker for apoptosis that can be added directly to cells in culture. The simple add, wash and analyze format makes this apoptosis marker easily adaptable for use in high-throughput screening systems.

INTRODUCTION

Apoptotic death requires the controlled degradation of the cell. Proteases play a crucial role in this programmed cell death. During apoptosis, activation of a family of cysteine aspartyl proteases, or caspases, results in proteolytic cleavage of numerous substrates. Promega has developed a fluorescein isothiocyanate (FITC) conjugate of the pan-caspase inhibitor, Z-VAD-FMK, for monitoring caspase activity in situ. Z-VAD-FMK (carbobenzoxy-valyl-alanyl-aspartyl-fluoromethylketone) is a cell-permeable, irreversible inhibitor of caspases. The replacement of the carbobenzoxy end-group with the FITC label allows in situ labeling

of activated caspases. The CaspACE™ FITC-VAD-FMK In Situ Marker (Cat.# G7461, G7462) can be added directly to living cells in culture.

In this article, we demonstrate the utility of the CaspACE™ FITC-VAD-FMK In Situ Marker in the human lymphocyte Jurkat cell line and in primary cultures of bovine pulmonary artery endothelial (BPAE) cells. Jurkat cells were treated with anti-Fas antibody to induce apoptosis via the Fas receptor-mediated pathway. BPAE cells were either treated with the apoptotic agent, staurosporine, or with lipooligosaccharide (LOS) isolated from the Gram-negative bovine pathogen, *Haemophilus somnus*.

H. somnus causes a variety of diseases in cattle, including pneumonia, abortion, thrombotic meningoencephalitis (TME), myocarditis, arthritis and others (1). The net result of systemic *H. somnus* infection is the development of septicemia, thrombosis and vasculitis. There are two phenotypes of *H. somnus*, pathogenic and asymptomatic carrier, which differ in their abilities to cause septicemia (1). LOS is a candidate virulence factor that may promote endothelial cell apoptosis in vitro, since BPAE cells treated with lipopolysaccharide (LPS) from a variety of Gram-negative enteric pathogens undergo apoptosis (2). LPS has a long carbohydrate polymer (O chain), which LOS lacks. There are differences within the structure of LOS between pathogenic and asymptomatic carrier isolates of *H. somnus*, yet they share the key core component, lipid A (3). Previous experiments in our laboratory demonstrated that purified LOS from pathogenic and asymptomatic carrier isolates

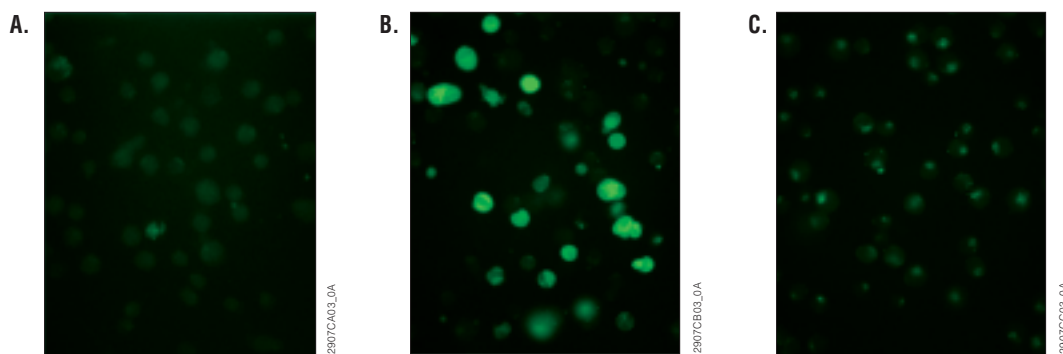


Figure 1. CaspACE™ FITC-VAD-FMK In Situ Marker is a cell-permeable, marker of activated caspases. Jurkat cells were treated with a monoclonal antibody against the Fas receptor (human anti-Fas, Clone CH-11, PanVera Corporation) to induce Fas receptor-mediated apoptosis. Jurkat cells (5×10^5 cells/ml) were treated with anti-Fas antibody (100ng/ml) for 2.5 hours. Control cells were left untreated, or were treated with the CaspACE™ FITC-VAD-FMK In Situ Marker (in DMSO, 10 μ M final concentration) for 20 minutes prior to apoptosis induction. After induction of apoptosis, the CaspACE™ FITC-VAD-FMK In Situ Marker was added directly to the cells in culture and incubated for a minimum of 20 minutes (longer

incubations can improve the signal intensity). The cells were centrifuged at 250–350 $\times g$ for 5 minutes, rinsed once in PBS, and resuspended in PBS to 1.5×10^6 cells/ml. Cells were then added to poly-L-lysine coated slides for in situ analysis. To preserve the signal for longer periods, cells can be fixed briefly (10–30 minutes) in 4% paraformaldehyde or 10% buffered formalin. Slides were mounted in Vectashield® plus DAPI (Vector Labs), and viewed with a Zeiss® fluorescence microscope. Photo images were obtained with a Spot™ II digital camera. **Panel A:** Untreated human Jurkat cells. **Panel B:** Anti-Fas treated Jurkat cells. **Panel C:** Cells pre-incubated with FITC-VAD-FMK before anti-Fas treatment.

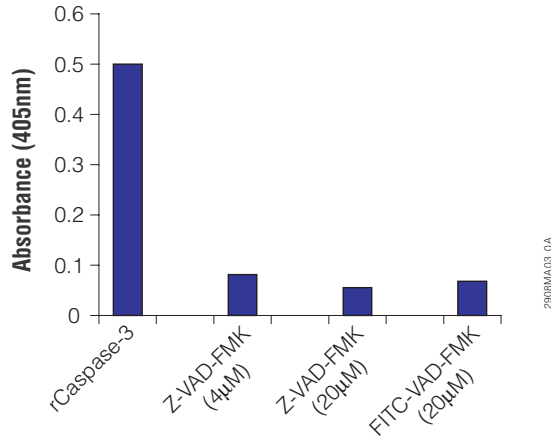


Figure 2. CaspACE™ FITC-VAD-FMK In Situ Marker irreversibly binds activated caspases and inhibits further caspase activation. The CaspACE™ Assay System, Colorimetric (Cat.# G7220), was used to measure the ability of Z-VAD-FMK (4µM and 20µM) and FITC-VAD-FMK (20µM) to block cleavage of the labeled peptide substrate, Ac-DEVD-pNA by recombinant caspase-3. Recombinant caspase-3 (Pharmingen) was used at 200ng/ml. The assay was performed as described in the *CaspACE™ Assay System, Colorimetric Technical Bulletin* (4).

induced apoptosis in BPAE cells. This experiment was designed to assess whether LOS isolated from *H. somnus* asymptomatic carrier isolate 127P induces caspase activation in BPAE cells.

The simplicity of the CaspACE™ FITC-VAD-FMK In Situ Marker labeling method makes it particularly well suited for fluorescent flow cytometry analysis of apoptotic cells. Using the CaspACE™ FITC-VAD-FMK In Situ Marker in combination with flow cytometry provides a means to rapidly monitor and quantify apoptosis.

CASPACE™ FITC-VAD-FMK IN SITU MARKER IS CELL-PERMEABLE AND INHIBITS CASPASE-3

We first demonstrated that the CaspACE™ FITC-VAD-FMK In Situ Marker is cell-permeable and can function as an inhibitor of caspases in a comparable manner to Z-VAD-FMK. FITC-VAD-FMK was tested both in situ for its ability to inhibit apoptosis in Jurkat cells (Figure 1), and in vitro for its ability to block cleavage of the caspase-3 substrate, Ac-DEVD-pNA (Figure 2). FITC-VAD-FMK was added to Jurkat cells prior to apoptosis induction with anti-Fas treatment. After anti-Fas treatment, FITC-VAD-FMK was again added to label apoptotic cells. If cells were pre-treated with FITC-VAD-FMK, apoptosis was effectively inhibited (a minimal amount of labeling was observed) and the cells were not labeled with the second incubation of FITC-VAD-FMK. Cells that were not pre-treated with the FITC-VAD-FMK became apoptotic after anti-Fas treatment and were strongly labeled with the FITC-VAD-FMK (Figure 1). Similarly, in an in vitro assay for caspase activity, FITC-VAD-FMK effectively inhibited cleavage of the Ac-DEVD-pNA (Figure 2). These in situ and in vitro results demonstrated that the CaspACE™ FITC-VAD-FMK In Situ Marker binds irreversibly to activated caspases comparably to the caspase inhibitor Z-VAD-FMK.

FLOW CYTOMETRY ANALYSIS

The CaspACE™ FITC-VAD-FMK In Situ Marker was then used to compare apoptotic cells to non-apoptotic cells by flow cytometric analysis. Untreated and anti-Fas treated Jurkat cells were labeled with FITC-VAD-FMK and counted using a Coulter EPICS® flow cytometer (Figure 3). Positive (fluorescently-labeled) cells are denoted by the M2 region of the graph; negative cells (untreated) are denoted by M1. The results indicate that 53% of anti-Fas treated cells were labeled (green line) and 5% of untreated cells were labeled (black line). The red line denotes cells that were not treated with FITC-VAD-FMK, indicating a minimal amount of autofluorescence (0.4%). The same cells that were prepared for flow cytometry analysis were also used for in situ analysis (Figure 4). After incubation with the CaspACE™ FITC-VAD-FMK In Situ Marker, samples of both anti-Fas treated and untreated cells were added to poly-L-lysine coated slides and observed with fluorescence microscopy (Figure 4). DAPI staining of the anti-Fas treated cells showed numerous cells with condensed and fragmented nuclei that were also labeled with the FITC-VAD-FMK. The percentage of anti-Fas treated cells that were FITC-positive by flow cytometric analysis corresponded well to the number of FITC-labeled cells by in situ analysis (Figure 4). For untreated cells, no labeling is seen in this image, but in a larger field of cells, a few untreated cells appeared apoptotic by DAPI staining and were also labeled with FITC-VAD-FMK.

DETECTION OF APOPTOSIS IN BPAE CELLS TREATED WITH *H. SOMNUS* LOS

The utility of the CaspACE™ FITC-VAD-FMK In Situ Marker for the analysis of apoptosis by flow cytometry was also demonstrated in cultures of bovine pulmonary artery endothelial cells. The cells were incubated with 200nM staurosporine for 6 hours to induce apoptosis, and then incubated with FITC-VAD-FMK (20µM). There was a significant increase (2.5-fold) in the number of labeled cells between untreated (5.5%) and staurosporine-treated samples (15%) (Figure 5). BPAE cells were also treated LOS (500ng/ml) isolated from *H. somnus* asymptomatic carrier isolate 127P and then incubated with the CaspACE™ FITC-VAD-FMK In Situ Marker in parallel to the untreated and staurosporine-treated cells. At a dose of 500ng/ml of LOS from strain 127P, there was a significant increase in the number of FITC-labeled cells (2-fold increase), as detected by flow cytometry (Figure 5). The results also show that treatment of BPAE cells with 500ng/ml of LOS from asymptomatic carrier isolate 127P induces significant numbers of apoptotic cells (12%). These results indicate that LOS from *H. somnus* asymptomatic carrier isolate 127P activates caspases in BPAE cells.

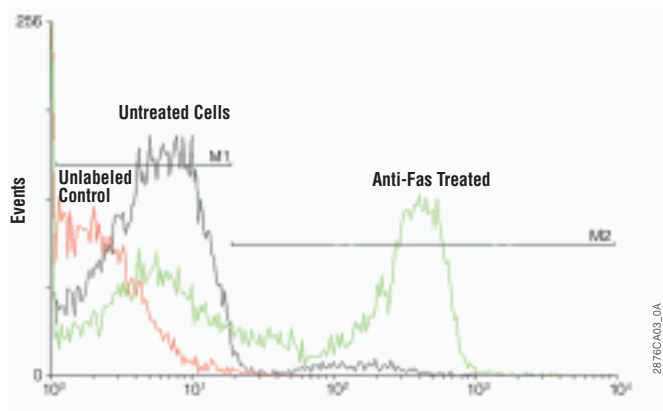


Figure 3. Fluorescence Flow Cytometry analysis of CaspACE™ FITC-VAD-FMK In Situ Marker-labeled apoptotic Jurkat cells. Human Jurkat cells were treated with 100ng/ml anti-Fas antibody for 4 hours, or left untreated, prior to incubation with 10µM CaspACE™ FITC-VAD-FMK In Situ Marker. Flow cytometry was performed using a Coulter EPICS® flow cytometer. Approximately 10,000-gated events were analyzed per sample. The results were analyzed using Windows® Multiple Document Interface (WinMDI) flow cytometry applications software. The fluorescence is plotted on a log-scale; M1 denotes unlabeled cells and M2 denotes labeled cells. There is little overlap between the untreated and anti-Fas treated cells, indicating an excellent signal-to-noise ratio.

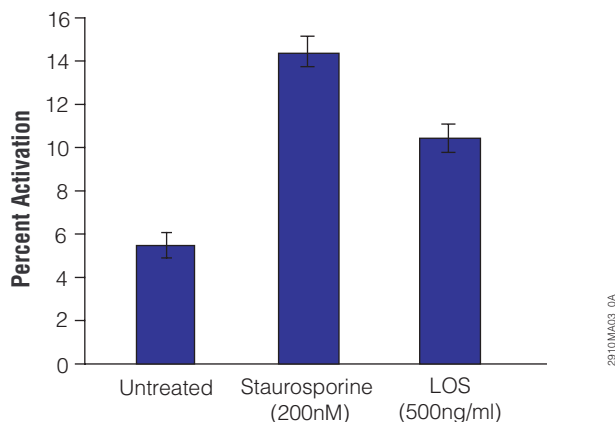


Figure 5. CaspACE™ FITC-VAD-FMK In Situ Marker labels apoptotic bovine pulmonary artery endothelial (BPAE) cells. Primary cultures of BPAE cells were treated with staurosporine (200nM) or LOS (500ng/ml) from *H. somnus* asymptomatic carrier isolate 127P for 6 hours, prior to the addition of CaspACE™ FITC-VAD-FMK In Situ Marker (20µM final concentration) directly to the culture medium. The cells were washed in PBS, fixed for 30 minutes in 4% paraformaldehyde, and washed again. Cells were then counted by flow cytometry as described in the legend to Figure 3. Data are representative of three independent experiments.

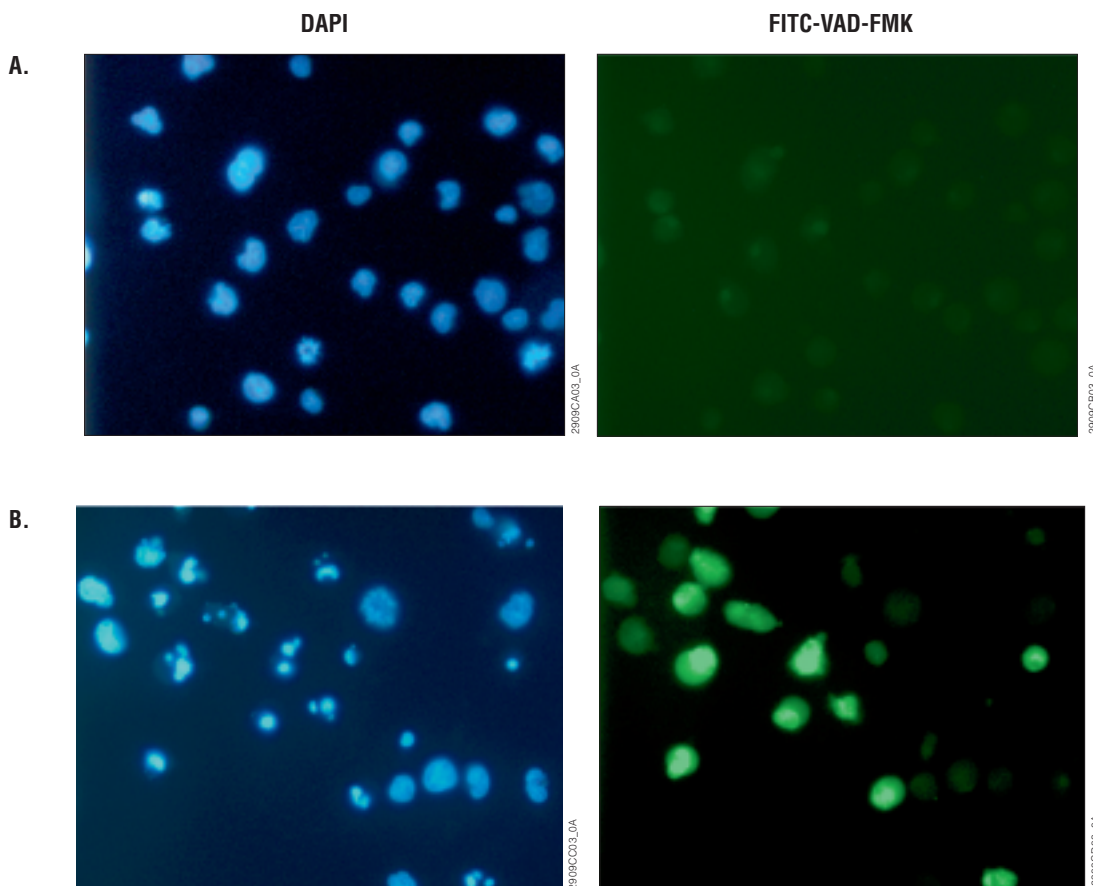


Figure 4. In situ labeling with the CaspACE™ FITC-VAD-FMK In Situ Marker corresponds to the results of flow cytometry analysis. Jurkat cells from the same batch of cells used for flow cytometry, prepared as described in the legend to Figure 3,

were analyzed by fluorescence microscopy. **Panel A:** Untreated cells. **Panel B:** Anti-Fas treated cells. The DAPI nuclear stain verifies the apoptotic morphology (condensed chromatin and fragmenting nuclei) of the FITC-labeled cells.

SUMMARY

The CaspACE™ FITC-VAD-FMK In Situ Marker is a labeled derivative of the cell-permeable irreversible pan-caspase inhibitor, Z-VAD-FMK. FITC-VAD-FMK inhibits caspases both in vitro and in situ, similar to Z-VAD-FMK. The FITC label allows in situ labeling of activated caspases, thus becoming a useful qualitative tool for assessing apoptosis, as well as a quantitative tool for flow cytometric analysis of apoptosis. Labeling of apoptotic cells was demonstrated for both a lymphocytic cell line in suspension and a primary culture of adherent endothelial cells. The simplicity of the assay and the utility for flow cytometry makes this product adaptable to high-throughput screening applications.

REFERENCES

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3. Inzana, T.J., Gogolewski, R.P. and Corbeil, L.B. (1992) *Infect. Immun.* **60**, 2943.
4. *CaspACE™ Assay System, Colorimetric, Technical Bulletin #TB270*, Promega Corporation.



MATT SYLTE



MARTHA O'BRIEN

Not pictured:

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Ordering Information

Product	Size	Cat.#
CaspACE™ FITC-VAD-FMK	50µl	G7461
In Situ Marker (5mM)	125µl	G7462
Caspase Inhibitor	50µl	G7231
Z-VAD-FMK (20mM)	125µl	G7232
CaspACE™ Assay System, Colorimetric	100 reactions 50 reactions	G7220 G7351
CaspACE™ Assay System, Fluorometric	160 assays	G3540

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