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Detection of Apoptotic Cells using the Apoptosis Detection System, Fluorescein

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Promega's Apoptosis Detection System, Fluorescein, is designed for the specific detection and quantitation of apoptotic cells within a cell population consisting of both apoptotic and nonapoptotic cells. The system, which is based on the TUNEL assay, uses no radioisotopes or antibodies and provides for simple, rapid and accurate detection of apoptotic cells.

Introduction

Most cells from higher eukaryotes have the ability to self-destruct by activation of an intrinsic cellular suicide program when they are no longer needed or have become seriously damaged. This normal physiological process is referred to as *programmed cell death* (PCD). The term apoptosis, which is often equated with PCD, refers to morphological alterations exhibited by dying cells that include cell shrinkage, membrane blebbing, chromatin condensation and fragmentation (reviewed in reference 1). Cells undergoing apoptosis often fragment into membrane-bound apoptotic bodies that are readily phagocytosed and digested by macrophages or neighboring cells without generating an inflammatory response (1). These changes distinguish apoptosis from cell death by necrosis. Necrosis refers to the morphology most often seen when cells die from severe and sudden injury, such as ischemia, sustained hyperthermia or physical or chemical trauma. In necrosis, there are early changes in mitochondrial shape and function, the cell loses its ability to regulate osmotic pressure, swells, and ruptures. The contents of the cell are spilled into the surrounding tissue, resulting in generation of a local inflammatory response (2-4).

Apoptosis plays an indispensable role in the development and maintenance of homeostasis and in the maturation of nervous and immune systems. It is also a major defense mechanism of the body, removing unwanted and potentially dangerous cells such as self-reactive lymphocytes, virus-infected cells and tumor cells (5-8).

In most cell types, the biochemical characteristics of apoptotic response include activation of endogenous calcium- and magnesium-dependent endonucleases, leading to fragmentation of the chromosomal DNA. Initially, the DNA fragments are large (50-300kb) but are later digested to oligonucleosomal size (multimers of 180-200bp). The formation of this distinct DNA ladder is considered to be a biochemical hallmark of apoptosis (9-12).

Promega's Apoptosis Detection System, Fluorescein, is designed for the specific detection and quantitation of apoptotic cells within a cell population consisting of both apoptotic and nonapoptotic cells. The system uses no radioisotopes and provides for simple, rapid and accurate detection of apoptotic cells *in situ* at the single cell level or in cell suspensions. The fragmented DNA of apoptotic cells is labeled by catalytically incorporating fluorescein-12-dUTP* at the 3´-hydroxyl ends of the fragmented DNA using the enzyme Terminal Deoxynucleotidyl Transferase (TdT), which forms a polymeric tail using the principle of the TUNEL (TdT-mediated dUTP Nick-End Labeling) assay (13,14). The fluorescein-dUTP-labeled DNA then can be visualized directly by fluorescence microscopy or quantitated by flow cytometry. The system can be used to assay apoptotic cell death in a wide variety of applications, including cultured cells, frozen and formalin-fixed paraffin-embedded tissue sections.

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Performance Characteristics of Apoptosis Detection System Fluorescein

The performance characteristics of Promega's Apoptosis Detection System were determined in several applications, including *in situ* detection of cell death in cultured cells and paraffin-embedded tissue sections, and flow cytometric analysis of apoptotic cells.

Visualization of apoptotic cultured cells by fluorescence microscopy

Treatment of the human promyelocytic cell line HL-60 with the DNA topoisomerase I inhibitor camptothecin induces apoptosis (15-16). The protocol for use of the Apoptosis Detection System with fluorescence microscopy (Figure 1) was used to analyze a population of camptothecin-treated HL-60 cells. The treated cells were incubated with fluorescein-12-dUTP in the presence or absence of TdT Enzyme. Fluorescein-12-dUTP is incorporated at the 3´-hydroxyl ends of fragmented DNA, resulting in green fluorescence within apoptotic cells. After the enzymatic labeling procedure was completed, the cells were counter-stained with propidium iodide which, in contrast to fluorescein-12-dUTP, stains both apoptotic and nonapoptotic cells red.

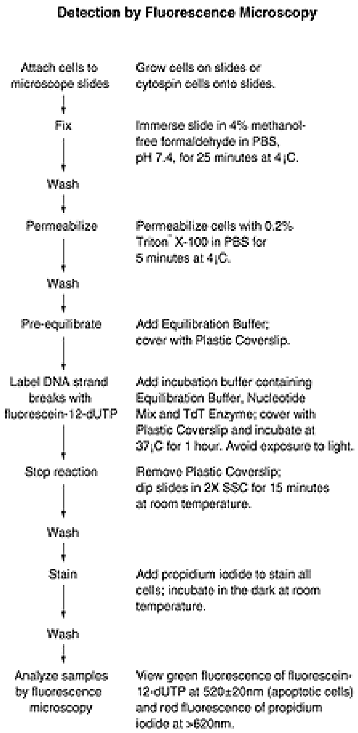

Figure 1. Protocol overview for use of the Apoptosis Detection System, Fluorescein, with fluorescence microscopy.

In Figure 2A, the apoptotic cells are labeled green and the nonapoptotic population is stained red. No detectable labeling occurred when cells were incubated with fluorescein-12-dUTP in the absence of TdT Enzyme (Figure 2B).

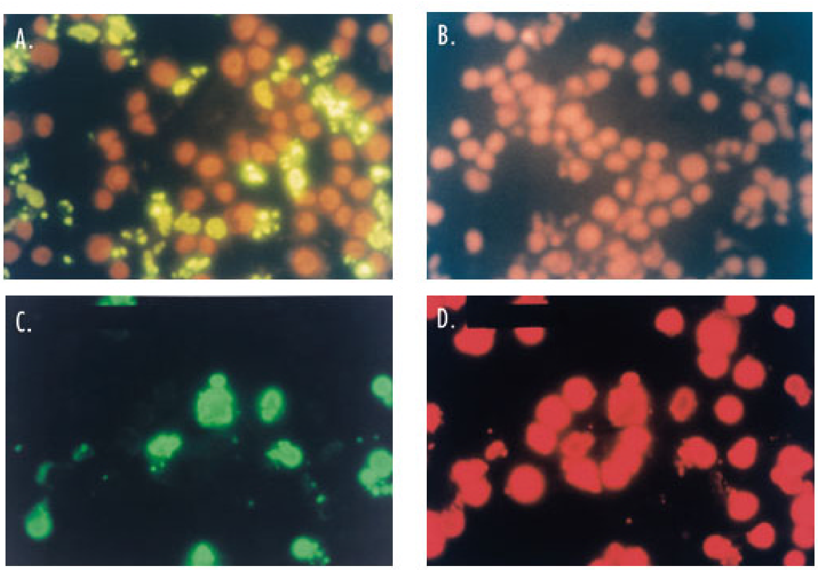

Figure 2. Labeling of DNA strand breaks associated with apoptosis using the Apoptosis Detection System, Fluorescein. HL-60 cells (6 x 10^5 cells/ml) cultured in RPMI 1640 medium containing 10% fetal bovine serum were incubated in the presence of camptothecin (0.5µg/ml) for 4 hours at 37°C. The cells were analyzed as indicated below and then counter-stained with propidium iodide (PI). **Panel A:** The cells were analyzed (17) for apoptosis by TUNEL labeling in the presence of TdT Enzyme. **Panel B:** The cells were analyzed (17) for apoptosis by TUNEL labeling in the absence of TdT Enzyme. Cells in Panels A and B were viewed by epifluorescence using standard fluorescein excitation and emission filters with a Leitz EPIVERT fluorescence microscope (magnification = 320x). **Panel C:** The cells were analyzed (17) for apoptosis by TUNEL labeling in the presence of TdT Enzyme. Green fluorescence (fluorescein) was viewed using fluorescein excitation and emission filters. **Panel D:** Red fluorescence (PI) of the same field shown in Panel C was viewed using rhodamine excitation and emission filters. Cells in Panels C and D were viewed by epifluorescence using a Zeiss Axioskope fluorescence microscope (magnification = 630x).

Figures 2C and 2D show representative photomicrographs of the same field containing camptothecin-treated HL-60 cells analyzed using the Apoptosis Detection System in the presence of TdT Enzyme. In Figure 2C, specific incorporation of fluorescein-12-dUTP in apoptotic cells (green) was viewed using fluorescein excitation and emission filters. Figure 2D shows the red fluorescent propidium iodide staining of both apoptotic and nonapoptotic cells viewed using rhodamine excitation and emission filters.

Visualization of apoptotic cells in paraffin-embedded tissue sections

Paraffin sections of normal human neonatal foreskin were deparaffinized, treated with Proteinase K and subjected to limited DNase I digestion. Proteinase K treatment permeabilizes the tissue and cells to the staining reagents, while the limited DNase I treatment fragments the chromosomal DNA in many of the cells resulting in exposure of multiple 3´-hydroxyl ends at which fluorescein-12-dUTP can be incorporated. The sections were labeled using the Apoptosis Detection System, Fluorescein, and analyzed by fluorescence microscopy (Figure 3). The cells exhibiting green fluorescence have incorporated fluorescein-12-dUTP into the nicked DNA strands. In contrast, the cells exhibiting red fluorescence have sustained very little nicking from the DNase I treatment and, therefore, display the red background staining of the propidium iodide rather than the green of the fluorescein label.

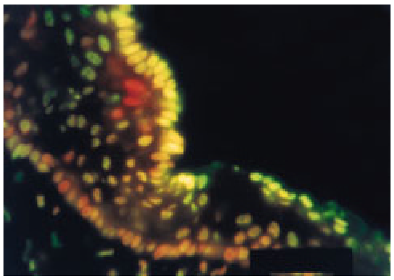

Figure 3. Fluorescence microscopy analysis of paraffin sections of normal human neonatal foreskin. Sections were deparaffinized, treated with Proteinase K (20µg/ml) and then exposed to DNase I (100ng/ml) for 10 minutes (14) prior to analysis using the Apoptosis Detection System, Fluorescein (17). Tissue sections were stained with propidium iodide following fluorescein-labeling and viewed under a fluorescence microscope using standard fluorescein excitation and emission (long pass) filters.

Flow cytometric analysis of apoptotic HL-60 cells

An overview of the protocol for use of the Apoptosis Detection System, Fluorescein, for the analysis of cells by flow cytometry is shown in Figure 4.

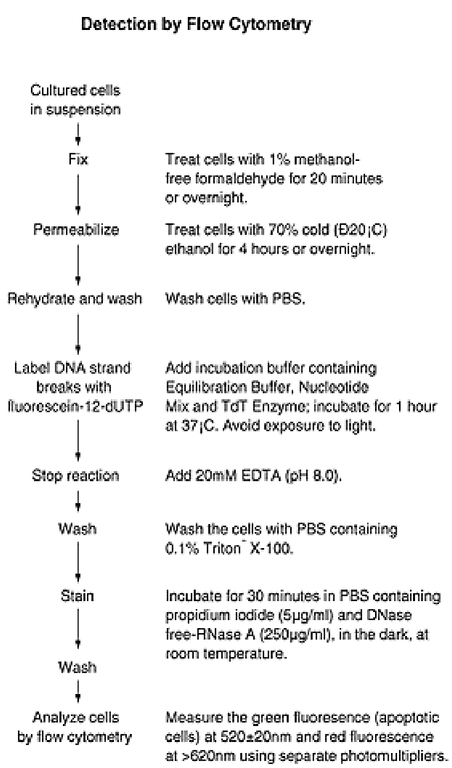

Figure 4. Protocol overview for use of the Apoptosis Detection System, Fluorescein, for analysis of cells by flow cytometry.

This protocol was used for the flow cytometric analysis of camptothecin-induced apoptosis of HL-60 cells. The cells were incubated with or without camptothecin and DNA breaks were labeled with fluorescein-12-dUTP. Flow cytometric measurement of the DNA content of the camptothecin-treated cells showed the presence of cells with a fractional DNA content typical of apoptosis (marked as "Apoptotic Population" in Figure 5). The cell cycle analysis of the unaffected cell population in the treated and untreated cultures showed marked differences in the proportion of cells in the various phases of the cell cycle. A pronounced loss of S phase cells, from 49% (control) to 14%, was observed in the camptothecin-treated culture suggesting that camptothecin treatment preferentially induced apoptosis of cells progressing through S phase as previously reported in the literature (15,16).

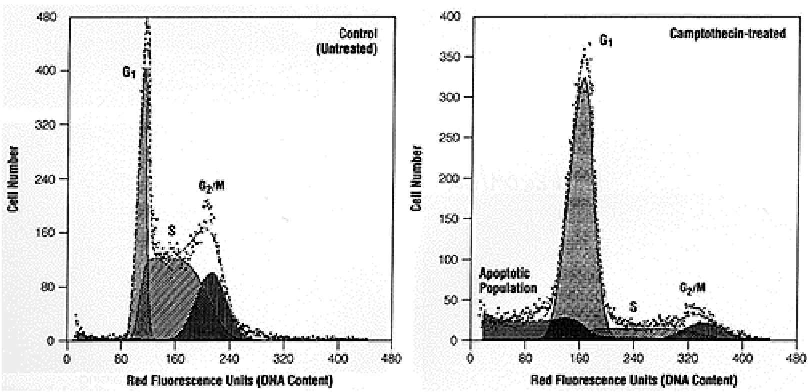

Figure 5. Flow cytometric analysis of camptothecin-induced apoptosis of HL-60 cells. HL-60 cells (6 x 10^5 cells/ml) were incubated in the presence or absence of camptothecin (0.2µg/ml) at 37°C for 5 hours. DNA strand breaks were labeled using the Apoptosis Detection System, Fluorescein (17). The cells were then counter-stained with propidium iodide as described (17) and analyzed by flow cytometry using an Epics Profile ll flow cytometer (Coulter Corporation, Miami, Florida). Analysis of cell cycle distributions was performed using Multicycle software (Phoenix Flow Systems, San Diego, CA); analysis of DNA content was performed using Elite Software (Coulter Corporation). Control (untreated) cells are shown in the left panel and camptothecin-treated cells are shown in the right panel.

Figure 6 shows the direct and specific detection of apoptotic cells by TdT-mediated labeling of DNA strand breaks with fluorescein-12-dUTP. Dual parameter analysis showed that approximately 25% of the total cell population was apoptotic as measured by incorporation of fluorescent label into DNA strand breaks, and the majority of the apoptotic cells were in the S phase.

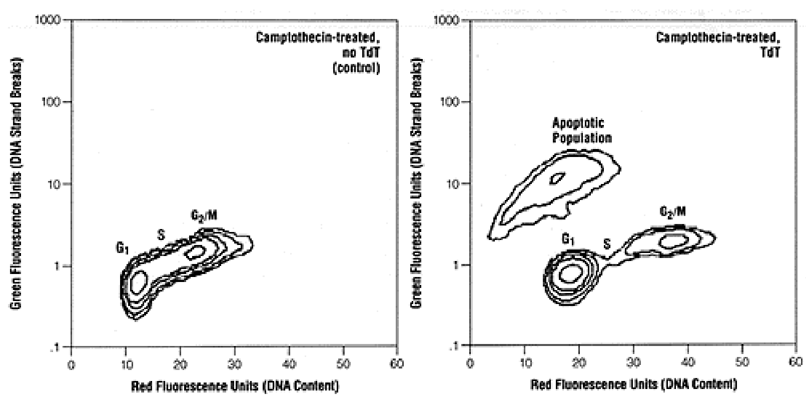

Figure 6. Detection of camptothecin-induced apoptosis of HL-60 cells in the presence and absence of TdT Enzyme. HL-60 cells (6 x 10^5 cells/ml) were incubated in the presence of camptothecin (0.2µg/ml) at 37°C for 5 hours. The cells were analyzed for apoptosis using the Apoptosis Detection System, Fluorescein (17) in the absence (left panel) or presence (right panel) of TdT Enzyme. Flow cytometric analysis was performed as described in Figure 5. The red DNA fluorescence (propidium iodide) associated with all the cellular DNA and the green fluorescence (fluorescein) associated with the DNA strand breaks were quantitated using Elite software (Coulter Corporation).

Summary

Promega's Apoptosis Detection System, Fluorescein, allows for specific, rapid and direct *in situ* labeling of DNA strand breaks associated with apoptosis. The system can be used to detect and quantitate apoptotic cells at the single cell level for cultured cells, paraffin-embedded tissues and by flow cytometry. This system will find its application for researchers studying apoptosis in diverse areas of cell biology.

References

1. McConkey, F.J. and Orrenius, S. (1994) *Trends Cell Biol.* **2,** 370.
2. Cohen, J.J. and Duke, R.C. (1992) *Ann. Rev. Immunol.* **10,** 267.
3. Nagata, S. (1994) *Adv. Immunol.* **57,**129.
4. Thompson, C. (1995) *Science,* **267,** 1456.
5. Wyllie, A.H. *et al.* (1980) *Int. Rev. Cytol.* **68,** 251.
6. Raff, M.C. (1992) *Nature* **356,** 397.
7. Martin, S.J. *et al.* (1994) *Trends Biochem. Sci.* **19,** 26.
8. Steller, H. (1995) *Science* **267,** 1445.
9. Schwartzman, R.A. and Cidlowski, J.A. (1993) *Endocrine Rev.* **14,** 133.
10. Oberhammer, F. *et al.* (1993) *EMBO J.* **12,** 3679.
11. Roy, C. *et al.* (1992) *Exp. Cell. Res.* **200,** 416.
12. Bortner, C.D. *et al.* (1995) *Trends Cell Biol.* **5,** 21.
13. Gavrieli, Y. *et al.* (1992) *J. Cell. Biol.* **119,** 493.
14. Ben-Sasson, S. *et al.* (1995) *Meth. Cell Biol.* **46,** 29.
15. Li, X. *et al.* (1995) *Cytometry* **20,** 172.
16. Gorczyca, W. *et al.* (1993) *Cancer Res.* **53,** 3186.
17. *Apoptosis Detection System, Fluorescein Technical Bulletin* #TB235, Promega Corporation.

Ordering Information

Product	Cat. #
Apoptosis Detection System, Fluorescein	G3250