

become seriously damaged. This normal physiological process is referred to as *programmed cell death*. The term *apoptosis* was originally defined to include certain morphological characteristics, including membrane blebbing, nuclear and cytoplasmic shrinkage and chromatin condensation. Since its original definition, apoptosis has found broad use in reference to all the biochemical and morphological characteristics of programmed cell death.

Cells dying by apoptosis often fragment into membrane-bound apoptotic bodies that are readily phagocytosed and digested by macrophages or by neighboring cells without generating an inflammatory response. This is in contrast to the type of cell death known as necrosis, characterized by cell swelling, chromatin flocculation, loss of membrane integrity, cell lysis and generation of a local inflammatory reaction.

Apoptosis plays important roles 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. In contrast to its beneficial effects, the inappropriate activation of apoptosis may contribute to a variety of pathogenic processes such as the extensive T cell death in AIDS as well as the loss of neuronal cells in Alzheimer's disease and following ischemic stroke (1-8).

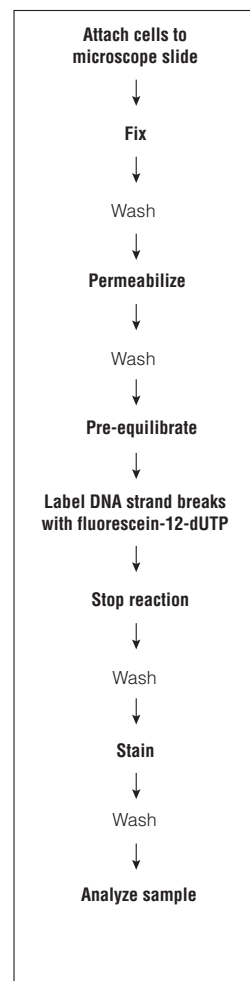
Apoptosis is a genetically controlled process, and some mechanistic aspects of apoptosis are at least partially conserved throughout evolution. The basic machinery to carry out apoptosis is (constitutively) present in all mammals; however, activation of the apoptotic process is thought to be regulated by the balance between many survival and death signals (9,10).

In many cell types, apoptosis is characterized by the generation of DNA fragments through the action of endogenous endonucleases (11-14). The DNA of apoptotic cells is cleaved into multimers of 180-200bp fragments, corresponding to the oligonucleosomal size. Therefore, the DNA of apoptotic cells typically migrates as a ladder of 180-200bp multimers on an agarose gel. The generation of single strand breaks also has been reported (15).

The Assay Principle

The DeadEnd™ Fluorometric TUNEL System measures the fragmented DNA of apoptotic cells by catalytically incorporating fluorescein-12-dUTP^(a) at 3'-OH DNA ends using the Terminal Deoxynucleotidyl Transferase, Recombinant, enzyme (rTdT). rTdT forms a polymeric tail using the principle of the TUNEL (TdT-mediated dUTP Nick-End Labeling) assay (16). The fluorescein-12-dUTP-labeled DNA can then either be visualized directly by fluorescence microscopy (Figure 1) or quantitated by flow cytometry (Figures 2 and 3).

Flow Diagram



Notes

Grow cells on slides or cytopsin cells onto slides.

Immerse slide in 4% methanol-free formaldehyde in PBS.

Permeabilize cells with Triton® X-100; permeabilize tissue sections with Proteinase K.

Add Equilibration Buffer; cover with Plastic Coverslip.

Add incubation buffer containing Equilibration Buffer, Nucleotide Mix and rTdT Enzyme; cover with Plastic Coverslip and incubate at 37°C for 1 hour. Avoid exposure to light.

Remove Plastic Coverslip; dip slides in 2X SSC.

Add propidium iodide to stain all cells. Alternatively, add DAPI nuclear stain in mounting medium and proceed to analysis.

Detect localized green fluorescence of apoptotic cells (fluorescein-12-dUTP) in a red or blue background (propidium iodide or DAPI, respectively) by fluorescence microscopy.

5746MA

Figure 1. Protocol overview for use of the DeadEnd™ Fluorometric TUNEL System in fluorescent microscopy of attached cells.

2. Product Components and Storage Conditions

Product	Size	Cat.#
DeadEnd™ Fluorometric TUNEL System	60 reactions	G3250

Includes:

- 9.6ml Equilibration Buffer
- 300µl Nucleotide Mix (6 × 50µl)
- 3 × 20µl Terminal Deoxynucleotidyl Transferase, Recombinant
- 70ml 20X SSC
- 10mg Proteinase K
- 60 Plastic Coverslips

Storage Conditions: Store the Equilibration Buffer, rTdT Enzyme and Proteinase K at -20°C. Store the Nucleotide Mix protected from light at -20°C. Avoid multiple freeze-thaw cycles of these components. Once thawed, store the 20X SSC at room temperature.

Reconstitute the Proteinase K supplied with the system in 1ml of proteinase K buffer (see Section 7) before use. The resulting Proteinase K solution will be 10mg/ml. Store aliquots of reconstituted Proteinase K at -20°C where the enzyme is stable for at least 6 months.

3. General Considerations

3.A. Light Sensitivity

The Nucleotide Mix provided in the system is light-sensitive. Protect the Nucleotide Mix as well as reaction mixtures and slides containing the Nucleotide Mix from direct exposure to light.

3.B. Safety

The Equilibration Buffer contains potassium cacodylate (dimethylarsinic acid). Avoid contact with skin and eyes. **Wear gloves and safety glasses when working with this reagent.**

4. Assay Protocol

To ensure that you are sufficiently prepared to perform the assay, please read all of the material below before attempting to use the DeadEnd™ Fluorometric TUNEL System in your application. Solution compositions are provided in Section 7.

Materials to Be Supplied by the User

- PBS
- propidium iodide (Sigma Cat.# P4170)
- optional: *SlowFade® Light* Anti-Fade Kit (Molecular Probes Cat.# S7461) or VECTASHIELD® (Vector Labs Cat.# H-1000)
- optional: VECTASHIELD® + DAPI (Vector Labs Cat.# H-1200)

Additional Materials For Cultured Cells

- 1% methanol-free formaldehyde (Polysciences Cat.# 18814) in PBS
- 4% methanol-free formaldehyde (Polysciences Cat.# 18814) in PBS
- **Note:** Paraformaldehyde can be directly substituted for methanol-free formaldehyde.
- 70% ethanol
- 0.2% Triton® X-100 solution in PBS
- 0.1% Triton® X-100 solution in PBS containing 5mg/ml BSA
- DNase I (e.g., RQ1 RNase-Free DNase, Cat.# M6101)
- 20mM EDTA (pH 8.0)
- DNase buffer
- DNase-free RNase A

Additional Materials For Paraffin-Embedded Tissue Section

- 4% methanol-free formaldehyde (Polysciences Cat.# 18814) in PBS
- **Note:** Paraformaldehyde can be directly substituted for methanol-free formaldehyde.
- xylene
- ethanol: 100%, 95%, 85%, 70% and 50% diluted in deionized water
- 0.85% NaCl solution
- proteinase K buffer
- DNase I
- DNase I buffer

Equipment to Be Supplied by the User

For Cultured Adherent Cells and Tissue Sections

- poly-L-lysine-coated or silanized microscope slides, e.g., Poly-Prep® slides (Sigma Cat.# P0425) or other appropriate pretreated slides, e.g., Superfrost® Plus glass slides (Fisher Cat.# 12-550-15) or Lab-Tek® Chamber Slides (Nunc Cat.# 177380)
- cell scraper
- Coplin jars (separate jar needed for optional DNase I positive control)
- forceps
- humidified chambers for microscope slides
- 37°C incubator
- micropipettors
- glass coverslips
- rubber cement or clear nail polish
- fluorescence microscope

4. Assay Protocol (continued)

Equipment to Be Supplied by the User (continued)

For Cell Suspensions

- tabletop centrifuge
- 37°C incubator or a 37°C covered water bath
- poly-L-lysine-coated or silanized microscope slides, e.g., Poly-Prep® slides (Sigma Cat.# P0425) or other appropriate pretreated slides, e.g., Superfrost® Plus glass slides (Fisher Cat.# 12-550-15)
- Coplin jars (separate jar needed for optional DNase I positive control)
- forceps
- glass coverslips
- humidified chambers for microscope slides
- micropipettors
- flow cytometer or fluorescence microscope

4.A. Procedure for the Analysis of Apoptosis in Adherent Cells

Preparation of Slides

Prepare sufficient poly-L-lysine-coated slides for appropriate positive and negative controls as well as all experimental samples.

Preparation of poly-L-lysine-coated slides: Pipet 50–100µl of a 0.01% w/v aqueous solution of poly-L-lysine (Sigma Cat.# P9155 or Sigma Cat.# P8920 diluted 1:10 with water) onto the surface of each precleaned glass slide. Distribute a thin layer of the poly-L-lysine solution throughout the areas to be used for fixing cells. Immediately after the slides have dried, rinse in deionized water and then allow the coated slides to air-dry for 30–60 minutes. Poly-L-lysine-coated slides may be stored at ambient temperature for several months before use.

Preparation of adherent cells on slides: Grow adherent cells on Lab-Tek® Chamber Slides. Following treatment of experimental control to induce apoptosis, wash the slides twice with PBS and process directly in the apoptosis detection assay described below.

Apoptosis Detection

1. Fix cells by immersing slides in freshly prepared 4% methanol-free formaldehyde solution in PBS (pH 7.4) in a Coplin jar for 25 minutes at 4°C.

Note: Paraformaldehyde can be directly substituted for methanol-free formaldehyde.

2. Wash the slides by immersing in fresh PBS for 5 minutes at room temperature. Repeat PBS wash.

Note: After completion of Step 2, slides may be stored for up to two weeks in 70% ethanol at –20°C or in PBS at 4°C.

3. Permeabilize cells by immersing the slides in 0.2% Triton® X-100 solution in PBS for 5 minutes.

4. Rinse slides by immersing in fresh PBS for 5 minutes at room temperature. Repeat PBS rinse.

Note: An optional **positive control** slide using DNase I may be prepared at Step 4 as described in Section 4.E.

5. Remove excess liquid by tapping the slides. Cover the cells with 100µl of Equilibration Buffer. Equilibrate at room temperature for 5–10 minutes.

6. While the cells are equilibrating, thaw the Nucleotide Mix on ice and prepare sufficient rTdT incubation buffer for all experimental and optional positive control reactions (see Section 4.E) according to Table 1. To determine the total volume of rTdT incubation buffer needed, multiply the number of experimental and positive control reactions by 50µl, the volume of a standard reaction for an area not larger than 5cm². For samples with a larger surface area, increase the volumes of reagents proportionally.

Note: Keep the Nucleotide Mix and rTdT incubation buffer solution on ice, protected from light.

Table 1. Preparation of rTdT Incubation Buffer for Experimental and Optional Positive Control Reactions.

Buffer Component	Component Volume		Number of Reactions	
	per Standard 50µl Reaction		(Experimental Reactions + Optional Positive Controls)	Component Volume
Equilibration Buffer	45µl	x	_____	= _____µl
Nucleotide Mix	5µl	x	_____	= _____µl
rTdT Enzyme	1µl	x	_____	= _____µl
Total rTdT Incubation Buffer Volume				= _____µl



For negative controls: Prepare a control incubation buffer without rTdT Enzyme by combining 45µl of Equilibration Buffer, 5µl of Nucleotide Mix and 1µl of autoclaved, deionized water. (The final volume of the negative control incubation buffer is sufficient for one standard 50µl reaction.) Process the negative control through Steps 7–16.

For positive controls: If positive controls are desired, please refer to the optional protocol in Section 4.E. Because DNase I is used in the positive control reaction, we recommend that positive control slides are processed in separate Coplin jars for subsequent steps.

4.A. Procedure for the Analysis of Apoptosis in Adherent Cells (continued)

7. Blot around the equilibrated areas with tissue paper to remove most of the 100µl of Equilibration Buffer and add 50µl of rTdT incubation buffer to the cells on a 5cm² area. Do not allow the cells to dry out.

Note: Plastic Coverslips may be cut in half before use. Fold the Coverslip edge for easy removal and handling.

-  Avoid exposing the slides to light after completion of Step 7.
8. Cover the cells with Plastic Coverslips to ensure even distribution of the reagent. Place paper towels soaked with water at the bottom of a humidified chamber. Incubate the slides at 37°C for 60 minutes inside the humidified chamber to allow the tailing reaction to occur. Cover the chamber with aluminum foil to protect from direct light.
9. Dilute the 20X SSC 1:10 with deionized water and add enough of the resulting 2X SSC to fill a standard Coplin jar (40ml). Remove the Plastic Coverslips and terminate the reactions by immersing the slides in 2X SSC in a Coplin jar for 15 minutes at room temperature.
-  Ensure that all salts of the 20X SSC are in solution before diluting (Step 9).
10. Wash the samples by immersing the slides in fresh PBS for 5 minutes at room temperature. Repeat two times for a total of three washes to remove unincorporated fluorescein-12-dUTP.
11. Stain the samples in a Coplin jar by immersing the slides in 40ml of propidium iodide solution freshly diluted to 1µg/ml in PBS for 15 minutes at room temperature in the dark.
Optional: Omit propidium iodide step and mount slides in VECTASHIELD® + DAPI (Vector Lab Cat.# H-1200) to stain nuclei. Add coverslips to the slides and proceed to Step 16.
12. Wash the samples by immersing the slides in deionized water for 5 minutes at room temperature. Repeat two times for a total of three washes.
13. Drain off excess water from the slides and wipe the area surrounding the cells with tissue paper.
14. Analyze samples immediately as described in Step 16. Alternatively, add one drop of Anti-Fade solution (Molecular Probes Cat.# S7461) to the area containing the treated cells and mount slides using glass coverslips.
15. Seal the edges with rubber cement or clear nail polish and let dry for 5–10 minutes.
16. Immediately analyze samples under a fluorescence microscope using a standard fluorescein filter set to view the green fluorescence of fluorescein at 520 ± 20nm; view red fluorescence of propidium iodide at >620nm and blue DAPI at 460nm. If necessary, slides may be stored overnight at 4°C in the dark.

Note: Propidium iodide stains both apoptotic and nonapoptotic cells **red**. Fluorescein-12-dUTP incorporation results in localized **green** fluorescence within the nucleus of apoptotic cells only.

4.B. Pretreatment of Paraffin-Embedded Tissues

Tissue sections may be formalin-fixed and paraffin-embedded for sectioning by a variety of techniques. A standard protocol is provided in reference 17.

1. Deparaffinize tissue sections (attached to microscope slides) by immersing slides in fresh xylene in a Coplin jar for 5 minutes at room temperature. Repeat one time for a total of two xylene washes.
2. Wash the samples by immersing the slides in 100% ethanol for 5 minutes at room temperature in a Coplin jar.
3. Rehydrate the samples by sequentially immersing the slides through graded ethanol washes (100%, 95%, 85%, 70%, 50%) for 3 minutes each at room temperature.
4. Wash the samples by immersing the slides in 0.85% NaCl for 5 minutes at room temperature.
5. Wash the samples by immersing the slides in PBS for 5 minutes at room temperature.
6. Fix the tissue sections by immersing the slides in 4% methanol-free formaldehyde solution in PBS for 15 minutes at room temperature.
Note: Paraformaldehyde can be directly substituted for methanol-free formaldehyde.
7. Wash the samples by immersing the slides in PBS for 5 minutes at room temperature. Repeat once for a total of two PBS washes.
8. Remove the liquid from the tissue and place the slides on a flat surface. Prepare a 20µg/ml Proteinase K solution from the reconstituted Proteinase K (10mg/ml; see Section 2) by diluting 1:500 in PBS. Add 100µl of the 20µg/ml Proteinase K to each slide to cover the tissue section. Incubate slides for 8–10 minutes at room temperature.
Note: Proteinase K helps permeabilize tissues and cells to the staining reagents in subsequent steps. For best results, optimize the length of incubation with Proteinase K. Longer incubations may be needed for tissue sections thicker than 4–6µm; however, with prolonged Proteinase K incubations, the risk of releasing the tissue sections from the slides increases in subsequent wash steps.
9. Wash the samples by immersing the slides in PBS for 5 minutes at room temperature in a Coplin jar.
10. Fix the tissue sections after washing by immersing the slides in 4% methanol-free formaldehyde solution in PBS for 5 minutes at room temperature.

4.B. Pretreatment of Paraffin-Embedded Tissues (continued)

11. Wash the samples by immersing the slides in PBS for 5 minutes at room temperature.

Note: An optional **positive control** slide may be prepared at Step 11 by treating a sample with DNase I to cause DNA fragmentation. A protocol for DNase treatment is given in Section 4.E.

12. Follow Steps 5–16 of Section 4.A to analyze apoptosis of these pretreated tissue sections.

Note: Use of a confocal microscope is strongly recommended for analyzing tissue sections.

4.C. Procedure for the Analysis of Suspension Cells by Flow Cytometry

1. Wash $3\text{--}5 \times 10^6$ cells with PBS two times by centrifugation ($300 \times g$) at 4°C and resuspend in 0.5ml of PBS.

2. Fix the cells by adding 5ml of 1% methanol-free formaldehyde for 20 minutes on ice.

Note: Paraformaldehyde can be directly substituted for methanol-free formaldehyde.

3. Centrifuge the cells at $300 \times g$ for 10 minutes at 4°C , remove the supernatant and resuspend cells in 5ml of PBS. Repeat wash once and resuspend cells in 0.5ml of PBS.

4. Permeabilize the cells by adding 5ml of 70% ice-cold ethanol. Incubate at -20°C for 4 hours. Cells can be stored in 70% ethanol at -20°C for one week.

Alternatively, cells can be permeabilized with 0.2% Triton® X-100 solution in PBS for 5 minutes at room temperature.

5. Centrifuge the cells at $300 \times g$ for 10 minutes and resuspend in 5ml of PBS. Repeat centrifugation and resuspend the cells in 1ml of PBS.

6. Transfer 2×10^6 cells into a 1.5ml microcentrifuge tube.

7. Centrifuge at $300 \times g$ for 10 minutes, remove supernatant and resuspend the pellet in 80µl of Equilibration Buffer. Incubate at room temperature for 5 minutes.

8. While the cells are equilibrating, thaw the Nucleotide Mix on ice and prepare sufficient rTdT incubation buffer for all reactions according to Table 2. To determine the total volume of rTdT incubation buffer needed, multiply the number of reactions times 50µl, the volume of a standard reaction using 2×10^6 cells.

Note: Keep the Nucleotide Mix and rTdT incubation buffer solution on ice, protected from light.

Table 2. Preparation of rTdT Incubation Buffer for Experimental Reactions.

Buffer Component	Component Volume per Standard 50µl Reaction		Number of Reactions (Experimental Reactions + Optional Positive Controls)	Component Volume
Equilibration Buffer	45µl	x	_____	= _____µl
Nucleotide Mix	5µl	x	_____	= _____µl
rTdT Enzyme	1µl	x	_____	= _____µl
Total rTdT Incubation Buffer Volume				= _____µl

For negative controls: Prepare a control incubation buffer without rTdT Enzyme by combining 45µl of Equilibration Buffer, 5µl of Nucleotide Mix and 1µl of autoclaved, deionized water. (The final volume of the negative control incubation buffer is sufficient for 1 standard 50µl reaction.) Process the negative control through Steps 9–14.

9. Centrifuge cells at $300 \times g$ for 10 minutes, remove supernatant and resuspend the pellet in 50µl of rTdT incubation buffer. Incubate in a water bath for 60 minutes at 37°C , protecting from direct light exposure. Resuspend the cells with a micropipettor at 15-minute intervals.

! Avoid exposing the slides to light after completion of Step 9.

10. Terminate the reaction by adding 1ml of 20mM EDTA. Vortex gently.

11. Centrifuge at $300 \times g$ for 10 minutes, remove supernatant and resuspend in 1ml of 0.1% Triton® X-100 solution in PBS containing 5mg/ml BSA. Repeat one time for a total of two rinses.

12. Centrifuge at $300 \times g$ for 10 minutes, remove supernatant and resuspend the cell pellet in 0.5ml of propidium iodide solution (freshly diluted to 5µg/ml in PBS) containing 250µg of DNase-free RNase A.

13. Incubate the cells at room temperature for 30 minutes in the dark.

14. Analyze cells by flow cytometry. Measure green fluorescence of fluorescein-12-dUTP at $520 \pm 20\text{nm}$ and red fluorescence of propidium iodide at $>620\text{nm}$.

Note: Propidium iodide stains both apoptotic and nonapoptotic cells **red**. Fluorescein-12-dUTP incorporation results in localized **green** fluorescence within the nucleus of apoptotic cells only.

4.D. Procedure for the Analysis of Suspension Cells by Fluorescence Microscopy

Grow suspension cells in appropriate medium. Following control or experimental treatment to induce apoptosis, centrifuge the cells at $300 \times g$ for 10 minutes at 4°C and remove the culture medium, taking care to avoid aspirating the cells. Wash the cells in PBS by centrifugation as described above and resuspend in PBS at a concentration of approximately 2×10^7 cells/ml. Pipet 50–100µl of the cell suspension onto poly-L-lysine-coated or silanized microscope slides. Gently smear the cell suspension with a clean slide. Analyze apoptotic cells as described in Section 4.A. Cytospin preparations also may be prepared from suspension cells and analyzed as described in Section 4.A.

4.E. Procedure for DNase Treatment for Positive Controls (optional)

Positive controls for detection of DNA fragmentation can be performed on adherent cells or tissue sections as described below. For adherent cells, follow Steps 1–4 as described in Section 4.A. After Step 4, prepare a positive control slide by treating the cells with DNase I as outlined below. For paraffin-embedded tissues, follow Steps 1–11 as described in Section 4.B, then prepare positive control slides after Step 11.

Note: DNase I treatment of the fixed cells results in fragmentation of the chromosomal DNA and exposure of multiple 3'-OH DNA ends for labeling. The protocol outlined below generally results in the majority of the treated cells demonstrating green fluorescence.

1. Add 100µl of DNase I buffer (Section 7) to the fixed cells and incubate at room temperature for 5 minutes.
2. Tap off the liquid and add 100µl of DNase I buffer containing 5.5–10 units/ml of DNase I (Cat.# M6101, RQ1 DNase; when using other DNases an optimization step may be required). Incubate for 10 minutes at room temperature.
3. Remove excess liquid by tapping the slide, and wash the slide extensively 3–4 times in deionized water in a Coplin jar dedicated for the positive control.
4. Process the positive control as described in Section 4.A, Steps 5–16, using separate Coplin jars.

Note: Use a **separate** Coplin jar for **positive control** slides. Residual DNase I activity from the positive control slide may introduce high background to the experimental slides.

For biological positive controls: Apoptosis may be induced in the experimental system through a variety of methods.

- Treatment of cells with the protein synthesis inhibitor, anisomycin, or the DNA topoisomerase I inhibitor, camptothecin, induces apoptosis in the human promyelocytic cell line HL-60 (18–21; see Section 5).
- Withdrawal of growth factors induces apoptosis of growth factor-dependent cell lines. For example, NGF-deprivation of PC12 cells or sympathetic neurons in culture induces apoptosis (22).
- In vitro treatment with the glucocorticoid, dexamethasone, induces apoptosis in mouse thymus lymphocytes (16,23).
- Activation of either Fas or TNF-receptor-bearing cells by the respective ligands or by cross-linking with agonist antibody induces apoptosis of those cells (24).

5. Example Protocol: Analysis of Camptothecin- or Anisomycin-Induced Apoptosis of HL-60 Cells

1. Grow HL-60 cells in RPMI 1640 medium containing 10% fetal bovine serum, 2mM glutamine, 1% penicillin and streptomycin in a humidified, 5% CO₂ incubator at 37°C.
2. Adjust the cell density to 6×10^5 cells/ml. For camptothecin treatment, use a final concentration of 0.2µg/ml (stock solution dissolved in DMSO) and incubate for 5 hours in a humidified 5% CO₂ incubator at 37°C. For anisomycin treatment, use a final concentration of 2µg/ml (stock solution dissolved in DMSO) and incubate for 2 hours in a humidified 5% CO₂ incubator at 37°C. Treat negative control cells with an equal volume of DMSO without inhibitor and incubate under the same conditions.
3. Harvest the cells and follow Steps 1–14 as described in Section 4.C for the analysis of apoptosis of cells in suspension by flow cytometry.

Figures 2 and 3 contain data generated with control and camptothecin-treated cells.

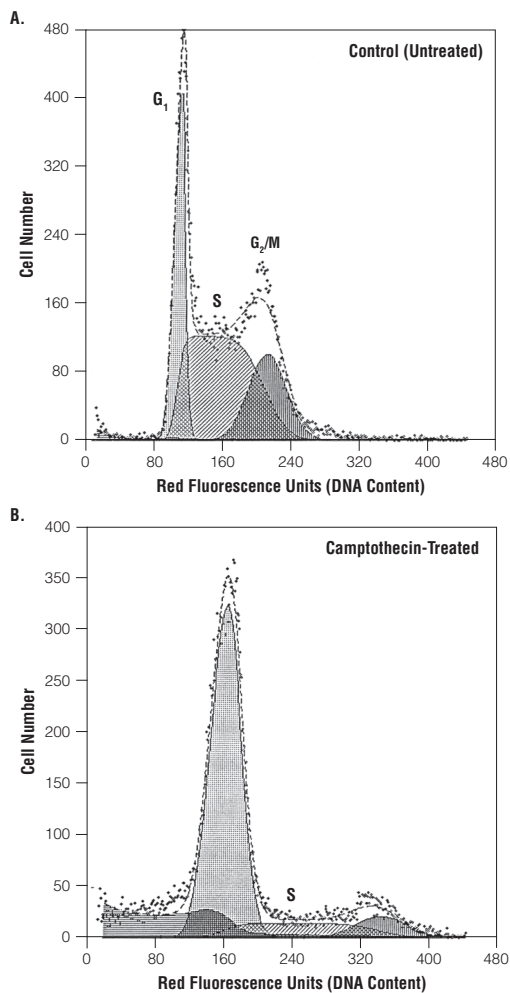


Figure 2. Flow cytometric analysis of camptothecin-induced apoptosis of HL-60 cells. HL-60 cells were incubated with and without camptothecin, and DNA breaks were labeled as described in Section 4.C for the analysis of apoptosis of cells in suspension by flow cytometry (EPICS® Profile II, Beckman Coulter, Inc.). Frequency distribution DNA histograms of control (untreated) HL-60 cells (**Panel A**) and camptothecin-treated HL-60 cells (**Panel B**) are shown. Cell cycle was analyzed using MultiCycle software (Phoenix Flow System); analysis of DNA content was performed using Elite™ software (Beckman Coulter, Inc.).

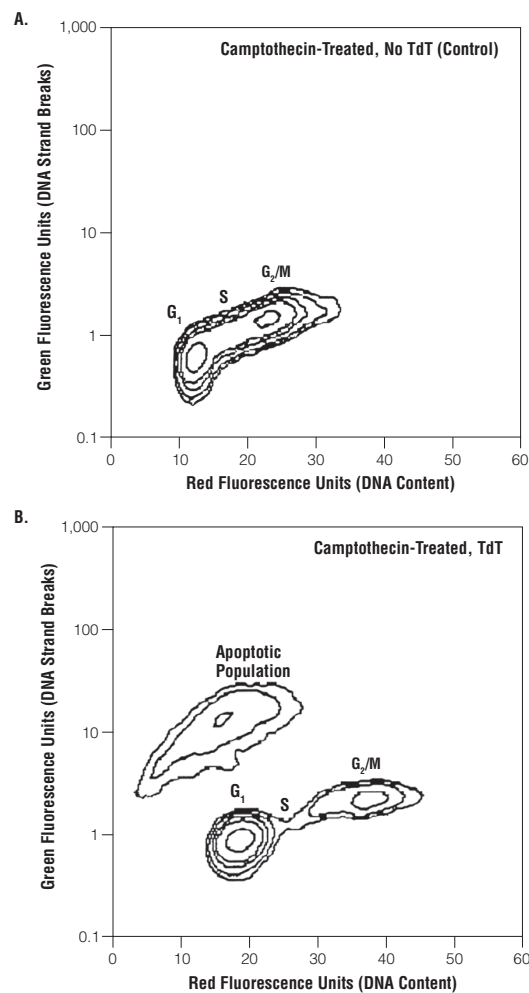


Figure 3. Detection of camptothecin-induced apoptosis of HL-60 cells in the presence (Panel B) and absence (Panel A) of TdT Enzyme. Flow cytometric analysis was performed as described in Figure 2. DNA strand breaks were analyzed using Elite™ software (Beckman Coulter, Inc.).

6. Troubleshooting

For questions not addressed here, please contact your local Promega Branch Office or Distributor. Contact information available at: www.promega.com. E-mail: techserv@promega.com

Symptoms	Causes and Comments
High background (i.e., strong fluorescent green background staining of nonapoptotic cells)	<p>Nonspecific incorporation of fluorescein-12-dUTP. Do not allow the cells to dry out at Section 4.A, Step 8, or beyond.</p> <p>At Section 4.A, Step 10, slides may be washed three times for 5 minutes with PBS containing 0.1% Triton® X-100 and 5mg/ml of BSA, followed by a single PBS wash step.</p>
Little or poor staining	<p>Insufficient permeabilization with Triton® X-100 or Proteinase K. Optimize permeabilization step by adjusting incubation time with permeabilization agent.</p>
Loss of tissue section from the slides	<p>Insufficient coating of the slide prior to attachment of tissue section. Coat microscopic slides with 3-aminopropyl triethoxysilane (TESPA; Sigma Cat.# A3648) before spreading the tissue sections according to the procedure described in reference 17. TESPA is superior to poly-L-lysine in preventing tissue detachment from the glass.</p> <p>Tissue section enzymatically digested from the slide. Optimize the Proteinase K incubation time in Section 4.B, Step 8.</p>
Few cells remaining for the final microscopic or flow cytometric analysis	<p>High number of cells lost during the procedure:</p> <ul style="list-style-type: none"> • Start with a higher number of cells. • When preparing a cell suspension for attachment to microscope slides in Section 4.D, wash cells with PBS containing 1% BSA during centrifugation. • Use a cytospin centrifuge to attach the cells to microscope slides if available. • When preparing suspension cells in Section 4.C, Step 1, wash cells with PBS containing 1% BSA during centrifugation.

7 Composition of Buffers and Solutions

Equilibration Buffer

200mM	potassium cacodylate (pH 6.6 at 25°C)
25mM	Tris-HCl (pH 6.6 at 25°C)
0.2mM	DTT
0.25mg/ml	BSA
2.5mM	cobalt chloride

proteinase K buffer

100mM	Tris-HCl (pH 8.0)
50mM	EDTA

Nucleotide Mix

50µM	fluorescein-12-dUTP
100µM	dATP
10mM	Tris-HCl (pH 7.6)
1mM	EDTA

propidium iodide solution (1mg/ml)

Weigh 10mg of propidium iodide and dissolve in 10ml of PBS. Store this solution at 0–4°C, protected from light. Dilute appropriately for use.

20X SSC

87.7g	NaCl
44.1g	sodium citrate

Dissolve in 400ml of deionized water. Adjust pH to 7.0 with HCl and bring volume to 500ml.

2X SSC

Warm 20X SSC to room temperature to ensure that all salts are in solution. Dilute 1:10 with deionized water before use to generate 2X SSC.

DNase I buffer

40mM	Tris-HCl (pH 7.9)
10mM	NaCl
6mM	MgCl ₂
10mM	CaCl ₂

1X PBS (pH 7.4)

137mM	NaCl
2.68mM	KCl
1.47mM	KH ₂ PO ₄
8.1mM	Na ₂ HPO ₄

rTdT incubation buffer

Combine the following:

90µl	Equilibration Buffer
10µl	Nucleotide Mix
2µl	rTdT Enzyme

This amount is sufficient for two reactions. Thaw ingredients on ice. Prepare the mix immediately before use and keep on ice protected from light until ready to use.

1% formaldehyde solution

Mix 90ml of PBS with 6.25ml of 16% methanol-free formaldehyde. Add a few drops of 1N NaOH, mix and adjust the pH to 7.4. Adjust the volume to 100ml with PBS. Prepare fresh for each use.

4% formaldehyde solution

Mix 70ml of PBS with 25ml of 16% methanol-free formaldehyde. Add a few drops of 1N NaOH, mix and adjust the pH to 7.4. Adjust the volume to 100ml with PBS. Prepare fresh for each use.

4% paraformaldehyde solution

Weigh 4g paraformaldehyde in a fume hood, add PBS and bring to 100ml. Dissolve by heating the closed bottle in a water bath at 65°C for 2 hours. Store the solution at 4°C, where it is stable for at least 2 weeks.

7. Composition of Buffers and Solutions (continued)

10% Triton® X-100 solution

Mix 85ml of autoclaved, deionized water and 10ml of Triton® X-100 solution in a beaker using a magnetic stir bar and a stir plate. Adjust the volume to 100ml with water.

8. Related Products

Product	Size	Cat.#
Anti-ACTIVE® Caspase-3 pAb	50µl	G7481
Apo-ONE® Homogeneous Caspase-3/7 Assay (fluorescent)	10ml 100ml	G7790 G7791
Caspase-Glo® 2 Assay*	10ml 50ml	G0940 G0941
Caspase-Glo® 6 Assay*	10ml 50ml	G0970 G0971
Caspase-Glo® 3/7 Assay* (luminescent)	2.5ml 10ml 100ml	G8090 G8091 G8092
Caspase-Glo® 8 Assay* (luminescent)	2.5ml 10ml 100ml	G8200 G8201 G8202
Caspase-Glo® 9 Assay* (luminescent)	2.5ml 10ml 100ml	G8210 G8211 G8212
DeadEnd™ Colorimetric TUNEL System*	20 reactions 40 reactions	G7360 G7130
CaspACE™ FITC-VAD-FMK In Situ Marker	50µl 125µl	G7461 G7462
Caspase Inhibitor Z-VAD-FMK	50µl 125µl	G7231 G7232
Caspase Inhibitor Ac-DEVD-CHO	100µl	G5961
Anti-PARP p85 Fragment pAb	50µl	G7341
rhTNF-α	10µg	G5241
Terminal Deoxynucleotidyl Transferase, Recombinant*	300u	M1871
RQ1 RNase-Free DNase*	1,000u	M6101

*For Laboratory Use.

Product	Size	Cat.#
MultiTox-Fluor™ Multiplex Cytotoxicity Assay* (fluorometric, nonlytic live/dead assay)	10ml 5 × 10ml†	G9200 G9201
MultiTox-Glo Multiplex Cytotoxicity Assay* (luminescent)	10ml 5 × 10ml†	G9270 G9271
CytoTox-Fluor™ Cytotoxicity Assay* (fluorometric)	10ml 5 × 10ml†	G9260 G9261
CytoTox-Glo™ Cytotoxicity Assay* (luminescent)	10ml 5 × 10ml†	G9290 G9291
CellTiter-Glo® Luminescent Cell Viability Assay (ATP, luminescent)	10ml 10 × 10ml†	G7570 G7571
CellTiter-Fluor™ Cell Viability Assay* (luminescent)	10ml 5 × 10ml†	G6080 G6081
CellTiter-Blue® Cell Viability Assay (resazurin, fluorometric)	20ml 100ml†	G8080 G8081
CytoTox-ONE™ Homogeneous Membrane Integrity Assay (LDH, fluorometric)	200-400 assays 1,000-4,000 assays	G7890 G7891
CellTiter 96® AQ _{ueous} One Solution Cell Proliferation Assay* (MTS, colorimetric)	200 assays 1,000 assays†	G3582 G3580
CellTiter 96® AQ _{ueous} Non-Radioactive Cell Proliferation Assay* (MTS, colorimetric)	1,000 assays 5,000 assays†	G5421 G5430
CellTiter 96® AQ _{ueous} MTS Reagent Powder*	250mg†	G1112
CellTiter 96® Non-Radioactive Cell Proliferation Assay* (MTT, colorimetric)	1,000 assays 5,000 assays	G4000 G4100
CytoTox 96® Non-Radioactive Cytotoxicity Assay* (LDH, colorimetric)	1,000 assays	G1780

*For Laboratory Use. †Additional sizes available.

9. References

1. Kerr, J.F.R. *et al.* (1972) Apoptosis: A basic biological phenomenon with wide-ranging implications in tissue kinetics. *Br. J. Cancer* **26**, 239-57.
2. Wyllie, A.H. *et al.* (1980) Cell death: The significance of apoptosis. *Int. Rev. Cytol.* **68**, 251-306.
3. Ellis, R.E. *et al.* (1991) Mechanisms and functions of cell death. *Annu. Rev. Cell. Biol.* **7**, 663-98.
4. Raff, M.C. (1992) Social controls on cell survival and cell death. *Nature* **356**, 397-400.
5. Martin, S.J. *et al.* (1994) Dicing with death: Dissecting the components of the apoptosis machinery. *Trends Biochem. Sci.* **19**, 26-30.
6. Cohen, J.J. *et al.* (1992) Apoptosis and programmed cell death in immunity. *Annu. Rev. Immunol.* **10**, 267-93.
7. Nagata, S. (1994) Fas and Fas ligand: A death factor and its receptor. *Adv. Immunol.* **57**, 129-44.
8. Thompson, C.B. (1995) Apoptosis in the pathogenesis and treatment of disease. *Science* **267**, 1456-62.
9. Steller, H. (1995) Mechanisms and genes of cellular suicide. *Science* **267**, 1445-49.
10. Oltvai, Z. and Korsmeyer, S.J. (1994) Checkpoints of dueling dimers foil death wishes. *Cell* **79**, 189-92.
11. Schwartzman, R.A. and Cidlowski, J.A. (1993) Apoptosis: The biochemistry and molecular biology of programmed cell death. *Endocrine Rev.* **14**, 133-51.
12. Walker, P.R. *et al.* (1991) Topoisomerase II-reactive chemotherapeutic drugs induce apoptosis in thymocytes. *Cancer Res.* **51**, 1078-85.
13. 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.
14. 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.
15. Bortner, C.D. *et al.* (1995) The role of DNA fragmentation in apoptosis. *Trends Cell Biol.* **5**, 21-6.
16. 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.
17. Ben-Sasson, S.A. *et al.* (1995) Identification of dying cells—in situ staining. *Methods Cell Biol.* **46**, 29-39.
18. Del Bino, G. *et al.* (1991) The concentration-dependent diversity of effects of DNA topoisomerase I and II inhibitors on the cell cycle of HL-60 cells. *Exp. Cell Res.* **195**, 485-91.
19. Li, X. *et al.* (1995) Single-step procedure for labeling DNA strand breaks with fluorescein- or BODIPY-conjugated deoxynucleotides: Detection of apoptosis and bromodeoxyuridine incorporation. *Cytometry* **20**, 172-80.
20. Gorczyca, W. *et al.* (1993) The cell cycle related differences in susceptibility of HL-60 cells to apoptosis induced by various antitumor agents. *Cancer Res.* **53**, 3186-92.
21. Darzynkiewicz, Z. *et al.* (1992) Features of apoptotic cells measured by flow cytometry. *Cytometry* **13**, 795-808.
22. Batistatou, A. and Greene, L.A. (1991) Aurintricarboxylic acid rescues PC12 cells and sympathetic neurons from cell death caused by nerve growth factor deprivation: Correlation with suppression of endonuclease activity. *J. Cell Biol.* **115**, 461-71.
23. Cohen, J.J. and Duke, R.C. (1984) Glucocorticoid activation of a calcium-dependent endonuclease in thymocyte nuclei leads to cell death. *J. Immunol.* **132**, 38-42.
24. Tewari, M. and Dixit, V.M. (1995) Fas- and tumor necrosis factor-induced apoptosis is inhibited by the poxvirus *crmA* gene product. *J. Biol. Chem.* **270**, 3255-60.

^(a)For Research Use Only

This product is distributed and sold under an arrangement between ENZO DIAGNOSTICS, INC., and NEN[®] LIFE SCIENCE PRODUCTS, INC., for research purposes only by the end-user in the research market and is not intended for diagnostic or therapeutic use. Purchase does not include or carry any right or license to use, develop or otherwise exploit this product commercially. Any commercial use, development or exploitation of this product without the express prior written authorization of ENZO DIAGNOSTICS, INC., and NEN[®] LIFE SCIENCE PRODUCTS, INC., is strictly prohibited.

This product or use of this product may be covered by one or more ENZO patents, including the following: U.S. Pat. Nos. 4,952,685, 5,002,885 and 5,013,831 and DK 164 407 8 and by one or more NEN[®] patents including U.S. Pat. Nos. 5,047,519, 5,151,507 and 5,608,063.

© 1996, 1999, 2001, 2003, 2006, 2007, 2008, 2009 Promega Corporation. All Rights Reserved.

Anti-ACTIVE, Apo-ONE, Caspase-Glo, CellTiter 96, CellTiter-Blue, CellTiter-Glo and CytoTox 96 registered trademarks of Promega Corporation. CaspACE, CellTiter-Fluor, CytoTox-Fluor, CytoTox-Glo, CytoTox-ONE, DeadEnd and MultiTox-Fluor are trademarks of Promega Corporation.

Elite is a trademark of and EPICS is a registered trademark of Beckman Coulter, Inc. Lab-Tek is a registered trademark of Nalge Nunc International. NEN is a registered trademark of NEN Life Science Products, Inc. Poly-Prep is a registered trademark of Bio-Rad Laboratories, Inc. *SlowFade* is a registered trademark of Molecular Probes, Inc. Superfrost is a registered trademark of Erie Scientific. Triton is a registered trademark of Union Carbide Chemicals & Plastics Technology Corporation. VECTASHIELD is a registered trademark of Vector Laboratories, Inc.

Products may be covered by pending or issued patents or may have certain limitations. Please visit our Web site for more information.

All prices and specifications are subject to change without prior notice.

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.



Promega Corporation • 2800 Woods Hollow Road
Madison, WI 53711-5399 USA • Phone 608-274-4330



Promega

www.promega.com