



APO-DIRECT™

Flow Cytometry Kit for Apoptosis

Kit for 50 Tests

Cat. No. APT110

FOR RESEARCH USE ONLY
Not for use in diagnostic procedures

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Introduction

The *CHEMICON* APO-DIRECT™ Kit is a single-step staining method for labeling DNA breaks to detect apoptotic cells by flow cytometry (1). The kit contains all the reagents required for measuring apoptosis in cells including positive and negative control cells; washing, reaction, and rinsing buffers; and propidium iodide/RNase A solution for counter staining the total DNA.

Description of Apoptosis

Apoptosis is programmed cell death. It is believed to take place in the majority of animal cells. It is a distinct event that triggers characteristic morphological and biological changes in the cellular life cycle. It is common during embryogenesis (3), normal tissue and organ involution (4,5), cytotoxic immunological reactions (6,7) and occurs naturally at the end of the life span of differentiated cells (8,9). It can also be induced in cells by the application of a number of different agents including physiological activators, heat shock, bacterial toxins, oncogenes, chemotherapeutic drugs, ultraviolet and gamma radiation (10). When apoptosis occurs, the nucleus and cytoplasm of the cell often fragments into membrane-bound apoptotic bodies that are then phagocytized by neighboring cells. Alternatively, during necrosis, cell death occurs by direct injury to cells resulting in cellular lysing and release of cytoplasmic components into the surrounding environment often inducing an inflammatory response in the tissue. A landmark of cellular self destruction by apoptosis is the activation of nucleases that degrade the higher order chromatin structure of the DNA into fragments of 50 to 300 kilobases and subsequently into smaller DNA pieces of about 200 base pairs in length (11). Numerous reviews of the events accompanying apoptosis are available and several well-researched model systems have been described (12, 13, 14).

Measurable Features of Apoptosis

One of the most easily measured features of apoptotic cells is the break-up of the genomic DNA by cellular nucleases. These DNA fragments can be extracted from apoptotic cells and result in the appearance of “DNA laddering” when the DNA is analyzed by agarose gel electrophoresis (11). The DNA of non-apoptotic cells which remains largely intact does not display this “laddering” on agarose gels during electrophoresis. The large number of DNA fragments appearing in apoptotic cells results in a multitude of 3'-hydroxyl termini of DNA ends. This property can be used to identify apoptotic cells by labeling the DNA breaks with fluorescent-tagged deoxyuridine triphosphate nucleotides (F-dUTP). The enzyme terminal deoxynucleotidyl transferase (TdT) catalyzes a template-independent addition of deoxyribonucleoside triphosphates to the 3'-hydroxyl ends of double- or single-stranded DNA (15). A substantial number of these sites are available in apoptotic cells providing the basis for the single step fluorescent labeling and flow cytometric method utilized in the APO-DIRECT™ Kit (1,16). Non-apoptotic cells do not incorporate significant amounts of the F-dUTP owing to the lack of exposed 3'-hydroxyl DNA ends.

Kit Components

The following components are included with the APO-DIRECT™ Kit. Reagent bottles have color coded caps to aid in their identification. Sufficient reagents are provided to process 50 cell suspensions including control cells of approximately 1×10^6 cells per suspension for flow cytometry analysis. The control cells have been fixed as described (under the heading cell fixation procedure) and are in 70% (v/v) ethanol.

COMPONENT	COLOR CODE	VOLUME (mL.)	STORAGE CONDITIONS
Positive Control Cells	Brown cap	5.00	-15 to -25°C
Negative Control Cells	Natural cap	5.00	-15 to -25°C
Wash Buffer	Blue cap	100.00	2 to 8 °C
Reaction Buffer	Green cap	0.50	2 to 8 °C
TdT Enzyme	Yellow cap	0.038	-15 to -25 °C
F-dUTP	Orange cap	0.40	-15 to -25 °C
Rinsing Buffer	Red cap	100.00	2 to 8 °C
PI/RNase Staining Buffer	Amber bottle	25.00	2 to 8 °C

Precautions and Warnings

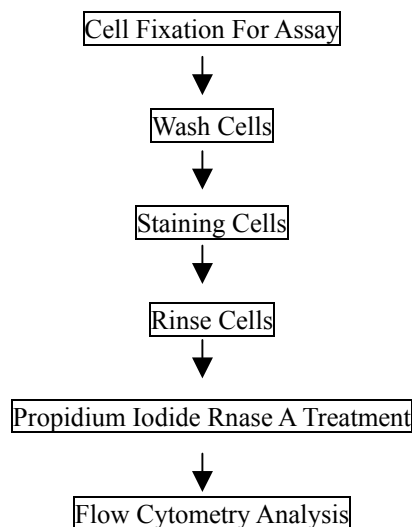
1. The components of this kit are for Research Use only and are not intended for diagnostic procedures.
2. Positive and negative control cells contain 70% (v/v) ethanol as a preservative; wash and reaction buffer contain sodium cacodylate (dimethylarsinic) as a buffer; rinsing and PI/RNase staining buffer contain 0.05% (w/v) sodium azide as a preservative. These materials are harmful if swallowed; avoid areas of contact immediately. See Material Safety Data Sheets.
3. TdT Enzyme will not freeze at -20°C , because it is in 50% (v/v) glycerol solution. Upon warming the TdT enzyme solution, centrifuge the tube for 30 seconds to force all the liquid to the bottom of the tube.

Materials Not Supplied

1. Flow Cytometer
2. Distilled Water
3. 1% (w/v) paraformaldehyde (methanol free) in PBS
4. 70% (v/v) ethanol
5. 37°C Water Bath
6. Ice Bucket
7. 12 x 75 mm flow cytometry test tubes
8. Micro-pipettor

Flow Diagram of APO-DIRECT™ APOPTOSIS ASSAY

Following is an outline of the steps employed in the APO-DIRECT™ apoptosis assay that is described in this manual.



Cell Fixation Procedure for APO-DIRECT™ Assay

Note: Cell fixation using paraformaldehyde is a required step in the APO-DIRECT™ assay. The following cell fixation procedure is a suggested method. Variables such as cell origin and growth conditions can affect the results. The fixation conditions provided below should be considered as guidelines. Additional experimentation may be required to obtain results comparable to the control cells provide with this kit. The positive and negative control cells provided in the APO-DIRECT™ Kit are already fixed.

1. Suspend the cells in 1% (w/v) paraformaldehyde in PBS, pH 7.4 at a concentration of $1-2 \times 10^6$ cells/mL.
2. Place the cell suspension on ice for 30-60 minutes.
3. Centrifuge cells for 5 minutes at 300 x g and discard the supernatant.
4. Wash the cells in 5 mL of PBS then pellet the cells by centrifugation. Repeat the wash and centrifugation.
5. Resuspend the cell pellet in the residual PBS in the tube by gently vortexing the tube.

6. Adjust the cell concentration to $1-2 \times 10^6$ cells/mL in ice-cold 70% (v/v) ethanol. Let the cells stand for a minimum of 30 minutes on ice or in the freezer. See note below.
7. Store cells in 70% (v/v) ethanol at -20°C until use. Cells can be stored at -20°C several days before use.

Note: In some biological systems storage of the cells at -20°C in 70% (v/v) ethanol for at least 12-18 hours prior to staining for apoptosis detection yields the best results in this assay.

APO-DIRECT™ PROTOCOL

The following protocol describes the method for measuring apoptosis in the positive and negative control cells that are provided in this kit. The same procedure should be employed for measuring apoptosis in the cell specimens provided by the researcher.

1. Resuspend the positive (brown cap) and negative (natural cap) control cells by swirling the vials. Remove 1 mL aliquots of the control cell suspensions (approximately 1×10^6 cell per 1 mL) and place in 12 x 75 mm centrifuge tubes. Centrifuge ($300 \times g$) the control cell suspensions for 5 minutes and remove the 70% (v/v) ethanol by aspiration being careful to not disturb the cell pellet.
2. Resuspend each tube of control cells with 1 mL of wash buffer (blue cap) for each tube. Centrifuge as before and remove the supernatant by aspiration.

Note: It is very important to remove all ethanol from the reaction tube since it will inactivate the TdT enzyme. Do not skip these washes.

3. Repeat the wash buffer treatment (step 2).
4. Resuspend each tube of the control cell pellets in 50 μL of the staining solution (prepared as described below).

STAINING SOLUTION COMPONENT	1 ASSAY	6 ASSAYS (2 CONTROLS + 4 UNKNOWN)	12 ASSAYS (2 CONTROLS + 10 UNKNOWN)
TdT Reaction Buffer (green cap)	10.00 µL	60.0 µL	120.0 µL
TdT Enzyme (yellow cap)	0.75 µL	4.5 µL	9.0 µL
Fluorescein-dUTP (orange cap)	8.00 µL	48.0 µL	96.0 µL
Distilled H ₂ O	32.25 µL	193.5 µL	387.0 µL
Total Volume	51.00 µL	306.0 µL	612.0 µL

The appropriate volume of Staining Solution to prepare for a variable number of assays is based upon multiples of the component volumes combined for 1 Assay. Mix only enough Staining Solution to complete the number of assays prepared per session. The Staining Solution is active for approximately 24 hours.

5. Incubate the cells in the Staining Solution for 60 minutes at 37°C in a temperature controlled bath. Shake cells every 15 minutes to resuspend.

***Note:** Reaction can also be carried out at 22-24°C overnight for the control cells. For unknown samples the incubation time at 37°C may need to be adjusted to longer or shorter periods depending on the characteristics of the cells supplied by the researcher.*

6. At the end of the incubation time add 1.0 mL of Rinse Buffer (red cap) to each tube and centrifuge each tube (300 x g) for five minutes. Remove the supernatant by aspiration.
7. Repeat the cell rinsing with 1.0 mL of the Rinse Buffer (red cap), centrifuge and remove the supernatant by aspiration.

FOR FLOW CYTOMETRY ANALYSIS

8. Resuspend the cells pellet in 0.5 mL of the Propidium Iodide/RNase A solution (amber bottle).
9. Incubate the cells in the dark for 30 minutes at room temperature.
10. Analyze the cells in Propidium Iodide/RNase solution by flow cytometry within 3 hours of staining.

FOR LASER SCANNING CYTOMETRY ANALYSIS

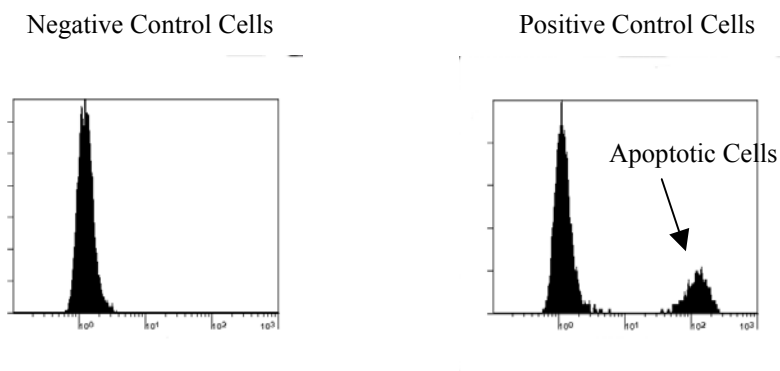
8. Dilute the Propidium Iodide/RNase A solution (amber bottle) 1 to 5 (add 1 mL of PI/RNase solution to 4 mL of Wash Buffer (blue cap)).
9. Resuspend the cells pellet in 0.5 mL of the Propidium Iodide/RNase A solution dilutant.
10. Incubate the cells in the dark for 30 minutes at room temperature.
11. Spin down cells to concentrate the suspension. Aspirate most of the supernatant and resuspend pellet in the remaining Propidium Iodide/RNase solution. Remove a small aliquot of the stained cells from the tube and place on a slide. Put cover slip over droplet of cells. Analyze the cells in Propidium Iodide/RNase solution later by laser scanning within 3 hours of staining.

Analyzing the APO-DIRECT™

Samples on the flow cytometer or laser scanning cytometer

This assay is run on a flow cytometer equipped with a 488 nm Argon Laser as the light source. Propidium Iodide (DNA) and Fluorescein (Apoptotic Cells) are the two dyes being used. Propidium Iodide (PI) fluoresces at about 623 nm and Fluorescein at 520 nm. Two dual parameter and two single parameter displays are created with the flow cytometer data acquisition software. The gating display should be the standard dual parameter DNA doublet discrimination display with DNA Area signal on the Y-axis and the DNA Width (Becton-Dickinson) or DNA Integral (Coulter) signal on the X-axis (see page 8). From this display, a gate is drawn around the non-clumped cells and the second gated dual parameter display is generated. The normal convention of this display is to put DNA (Linear Red Fluorescence) on the X-axis and the d-UTP (Log Green Fluorescence) on the Y-axis (see page 11). Two single parameter gated histograms, DNA and d-UTP, can also be added but are not necessary. By using the dual parameter display method, not only are apoptotic cells resolved but at which stage of the cell cycle they are in is also determined.

Gated Green Fluorescence Histograms (Apoptosis) of Control Cells included in the kit



Log Green Fluorescence

Figure 1: Cytometry Data of APO-DIRECT™
Negative & Positive Control Cells

Cytometer Setups

Typical Gain Settings

Becton Dickinson FACScan/Caliber Flow Cytometer

Parameter	Amplified Gain	Detector Gain
FL 1	Log	380 Volts
FL 2	1.46	414 Volts
FL 2 Width	0.87	
FL 2 Area	3.25	
	Threshold- FL2, 40	

Coulter XL Flow Cytometer

Parameter	Amplified Gain	Detector Gain
FL 1	Log	589 Volts
FL 3	2.00	698 Volts
AUX (FL3 Peak)	1.00	250 Volts
	Discriminator-AUX (FL3 Peak)	

CompuCyte Laser Scanning Cytometer

Laser Power-10mw

Contouring Parameter-PI-Sensor 3

Scan Data Display Threshold Value-600

Minimum Pixels-100

Parameter	Feature	Amplifier Gain	Offset
FL 1-Sensor 1	Log Integral	25	1950
FL 3-Sensor 3	Linear Integral	30	1950

Different Gates Used for Different Cytometers

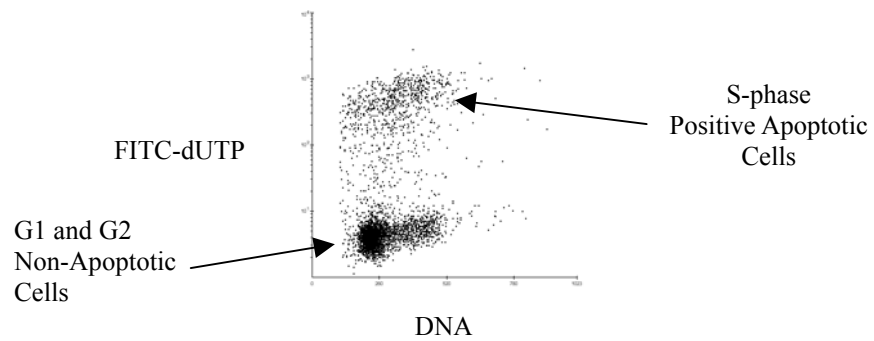
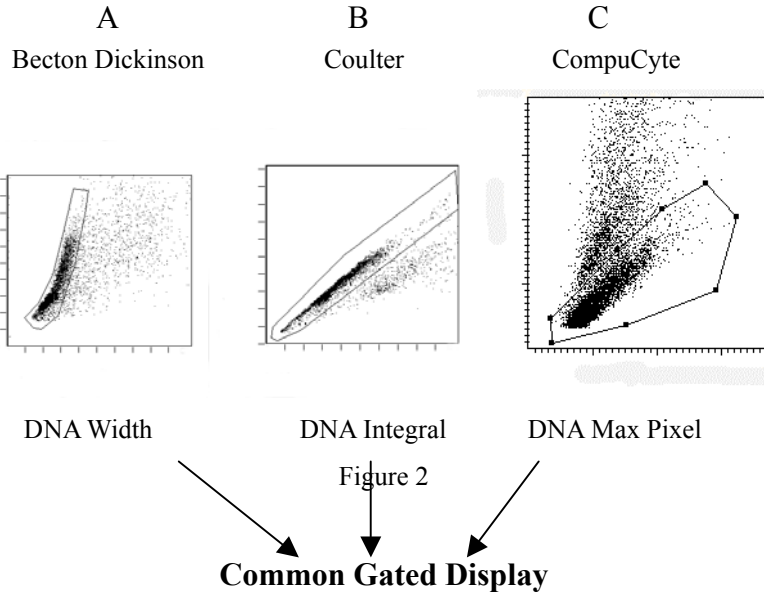


Figure 3

Figure 2 and 3 illustrate how the data should be acquired on the respective cytometer. No fluorescence compensation or gating from a scatter parameter is required.

Technical Tips and Frequently Asked Questions

1. To minimize cell loss during the assay, restrict the assay to the use of a single 12 x 75 mm test tube. If polystyrene plastic tubes are used, an electrostatic charge can build up on the sides of the tube. Cells will adhere to the side of the tube and the sequential use of multiple tubes can result in significant cell loss during the assay.
2. Occasionally a mirror image population of cells at lower intensity is observed in the PI vs FITC-dUTP dual parameter display. This population arises because during the 50 μ L DNA Labeling Reaction, some cells have become stuck to the side of the test tube and were not fully exposed to the reaction solution. This phenomenon can be overcome by washing all the cells from the side of the tube and making sure all cells are properly suspended at the beginning of the labeling reaction.
3. For those researchers using adherent cell line systems, the cells in the supernatant have a higher probability of being apoptotic than do the adherent cells. Save cells in the supernatant for assay prior to trypsinization of the adherent cell layer.
4. Cell fixation using a DNA cross-linking chemical fixative is an important step in analyzing apoptosis. Unfixed cells may lose smaller fragments of DNA that are not chemically fixed in place inside the cell during washing steps. The researcher may have to explore alternative fixation and permeabilization methods to fully exploit their systems.
5. If a low intensity of FITC staining is observed, try increasing the incubation time during the 50 μ L DNA Labeling Reaction. Some researchers have found labeling times of up to four hours at 37°C may be required for certain cell systems.
6. If the DNA cell cycle information is not required, it is not necessary to add the PI/Rnase A solution to each tube.

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Cat. No. APT110

December 2001
Revision C: 40782