APO-DIRECT™

A complete kit for measuring Apoptosis
by flow or laser scanning cytometry using the TUNEL assay
Catalog # AD1001

Description of Kit

The Phoenix Flow Systems, Inc. **APO-DIRECT™** reagent kit is a two color TUNEL(Terminal deoxynucleotide transferase dUTP Nick End Labeling) assay for labeling DNA breaks and total cellular DNA to detect apoptotic cells by flow cytometry or laser scanning cytometry(1). The kit contains the instructions and reagents required for measuring apoptosis in cells including; positive and negative control cells for assessing reagent performance; washing, reaction and rinsing buffers for processing individual steps in the assay; terminal deoxynucleotidyl tranferase enzyme (TdT), fluorescein isothiocyanate(FITC) deoxyuridine triphosphate (FITC-dUTP) for labeling DNA breaks and propidium iodide/RNase A solution for counter staining the total DNA.

Contents of the APO-DIRECT™ Kit

The **APO-DIRECT™** Kit is shipped in one container and consists of two packages. One package is shipped at ambient temperature and should be stored at 2-8C upon arrival. The other package is styrofoam containing frozen ice packs. The contents of this styrofoam container should be stored at -20C upon arrival. Phoenix Flow Systems, Inc. has determined this shipping method is adequate to maintain the integrity of the kit components. **Upon arrival store the reagents at the appropriate temperatures**.

Reagent bottles have color coded caps to aid in their identification. Sufficient reagents are provided to process 50 cell suspensions including 5 ml positive and 5 ml negative control cell suspensions of approximately 1 x 10^6 cells per ml in 70% (v/v) ethanol. The control cells are derived from a human lymphoma cell line and have been fixed as described on page 5.

APO-DIRECT™ Kit Components:

COMPONENT	COLOR CODE	PART NUMBER	VOLUME (ml)	STORAGE CONDITIONS
Positive Control Cells	brown cap	CC1002	5.000	-15 to -25C
Negative Control Cells	white cap	CC1001	5.000	-15 to -25C
Wash Buffer	blue cap	ADWB13	100.000	2 to 8C
Reaction Buffer	green cap	ADRXB14	0.500	2 to 8C
TdT Enzyme	yellow cap	ADTD15	0.038	-15 to -25C
Rinsing Buffer	red cap	ADRB17	100.000	2 to 8C
FITC dUTP	orange cap	ADFU16	0.400	-15 to -25C
PI/RNase Staining Buffer	amber bottle	ADPR18	25.000	2 to 8C

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For Research Use Only

For information or to place an order, please call 1-800-886-3569
Phoenix Flow Systems, Inc.
6790 Top Gun St., Suite 1
San Diego, CA 92121 USA
Tel: (858) 453-5095 FAX: (858) 453-2117
www.phoenixflow.com

Precautions and Warnings

1. The components of this kit are for **Research Use Only** and are not intended for diagnostic procedures.

2. Components part numbers CC1002 and CC1001 contain 70% (v/v) ethanol as a preservative; ADWB13 and ADRXB14 contain sodium cacodylate (dimethylarsinic) as a buffer; ADRB17, and ADPR18 contain 0.05% (w/v) sodium azide as a preservative. These materials are harmful if swallowed; avoid skin contact, wash immediately with water. See Material Safety Data Sheets.

3. TdT Enzyme (ADTD15) 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.

Reagents and Materials Required, but not supplied:

1. Flow Cytometer or Laser Scanning Cytometer
2. Distilled water
3. 1% (w/v) paraformaldehyde (methanol free) in Phosphate Buffered Saline (PBS)
4. 70% (v/v) ethanol
5. 37C Water Bath
6. Ice Bucket
7. 12 x 75 mm flow cytometry test tubes
8. Pipets and Pipetting Aids
9. **LSC users-** clean glass slides and glass coverslips

Definition of Apoptosis

Apoptosis is programmed cell death. It is believed to take place in the majority of eukaryotic cells. It is a distinct event that triggers characteristic morphological and biological changes in the cellular life cycle. It is common during embryogenisis(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 fragment 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 kilobase pairs 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/laser scanning 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.

APO-DIRECT™ TUNEL ASSAY

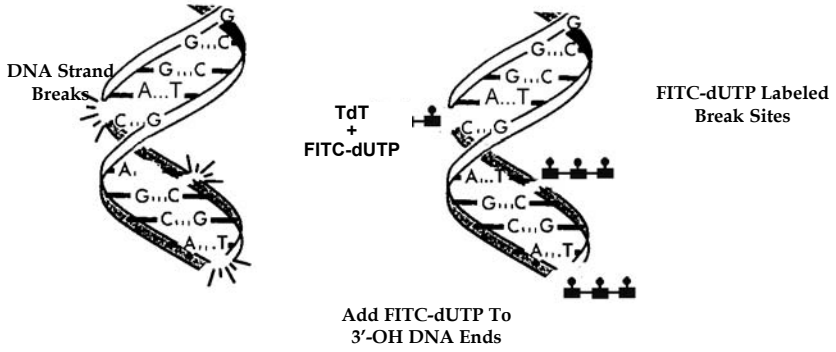

Figure 1: Diagrammatic representation of the APO-Direct TUNEL assay. Addition of fluoroscein deoxyuridine triphosphate nucleotides at the 3'-OH sites of DNA strand breaks of the apoptotic cells is catalyzed by terminal deoxynucleotidyl transferase (TdT).

Flow Diagram of APO-DIRECT™ Apoptosis Assay

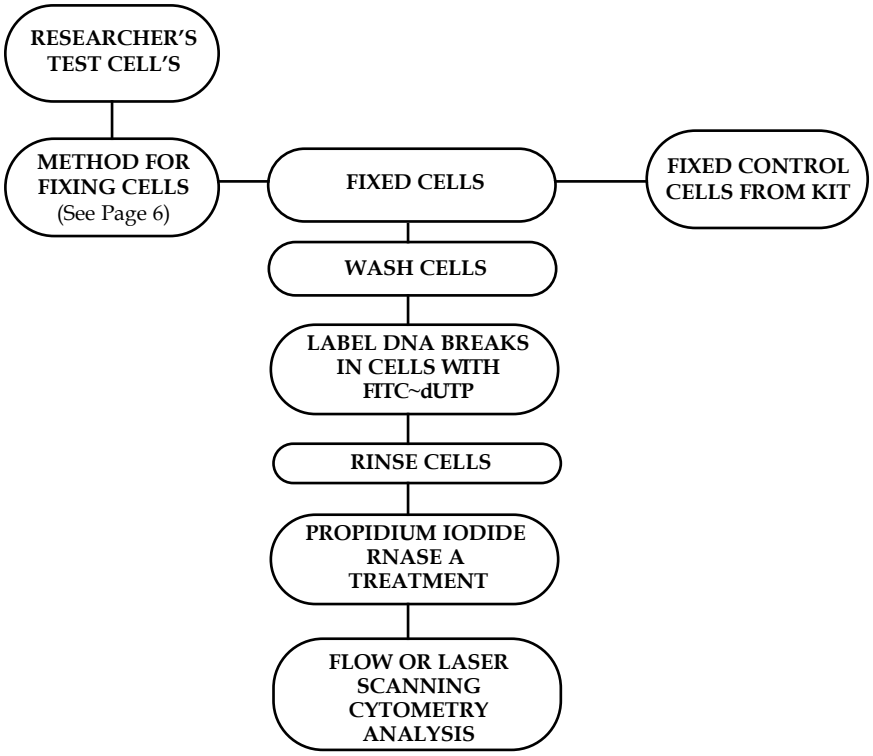

Figure 2: *Flow diagram used in the APO-DIRECT™ Apoptosis Assay. The positive and negative control cells are supplied in the kit and are already fixed. The cells supplied by the researcher should be fixed by the researcher according to the protocol suggested on page 6.*

Cell Fixation Procedure for APO-DIRECT™ Assay

NOTE: Cell fixation using paraformaldehyde is a **required** step in the **APO-DIRECT**™ assay to cross link the DNA in the cells. Ethanol treatment is required to permeabilize the cells. 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 provided with this kit. The **positive** and **negative control cells** provided in the **APO-DIRECT**™ **KIT** are already fixed as outlined below.

1. Suspend the cells in 1%(w/v) paraformaldehyde in PBS, pH 7.4 at a concentration of 1-2 x 10^6 cells/ml.

2 Place the cell suspension on ice for 30-60 minutes.

3. Centrifuge the 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 x 10^6 cells/ml in 70% (v/v) ice cold ethanol. Let 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 -20C until use. Cells can be stored at -20°C several days before use.

Note: In some biological systems storage of the cells at -20C 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™ STAINING 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 x 10^6 cell per 1 ml) and place in 12 x 75 mm centrifuge tubes. Centrifuge (300 x g) the control cell suspensions for 5 min utes 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 **DNA Labeling Solution** (prepared as described below).

STAINING SOLUTION COMPONENT	1 ASSAY	6 ASSAYS (2 controls+4 unknown)	12 ASSAYS (2 controls+10 unknown)
Reaction Buffer (green cap)	10.00µl	60.0µl	120.0µl
TdT Enzyme (yellow cap)	0.75µl	4.5µl	9.0µl
FITC-dUTP (orange cap)	8.00µl	48.0µl	96.0µl
distilled H2O	32.25µl	193.5µl	387.0µl
Total Volume	51.00µl	306.0µl	612.0µl

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

5. Incubate the cells in the **DNA Labeling Solution** for 60 minutes at 37C in a temperature con trolled bath. Shake cells every 15 min. to resuspend.

   NOTE: The DNA Labeling Reaction can also be carried out at 22-24C overnight for the control cells. For samples other than the control cells provided in the kit, incubation times at 37C 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 super natant by aspiration.

APO-DIRECT™ STAINING PROTOCOL (cont.)

FOR FLOW CYTOMETRY ANALYSIS

8. Resuspend the cell 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 cell pellet in 0.5 ml of the **Propidium Iodide/RNase A** dilutant.

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

11. Spin down cells to concentrate suspension. Aspirate most of supernatent and resuspend pellet in remaining **Propidium Iodide/RNase A 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 A solution** by laser scanning within 3 hours of staining.

Analyzing the APO-DIRECT™ Samples on the flow or laser scanning cytometer

This assay is run on a flow/laser scanning cytometer equipped with a 488 nm Argon Laser as the light source. Propidium Iodide (DNA) and FITC (Apoptotic Cells) are the two dyes being used. Propidium Iodide (PI) fluoresces at about 623 nm and FITC at 520 nm. Two dual parameter and two single parameters displays are created with the cytometer data acquisition software. The instrument settings and acquisition protocols for the different cytometers are illustrated on pages 10 & 11. For the flow cytometer the gate should be the standard dual parameter DNA doublet discrimination display with the DNA Area signal on the Y-axis and the DNA Width (Becton-Dickinson), (figure 5A) or DNA Integral (Coulter) signal on the X-axis, (figure 5B). For the CompuCyte laser scanning cytometer, the gate should be DNA Area on the Y-axis and DNA (PI) Max Pixel on the X-axis, (figure 5C). From the appropriate gating display, a region 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, (figure 6). 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.

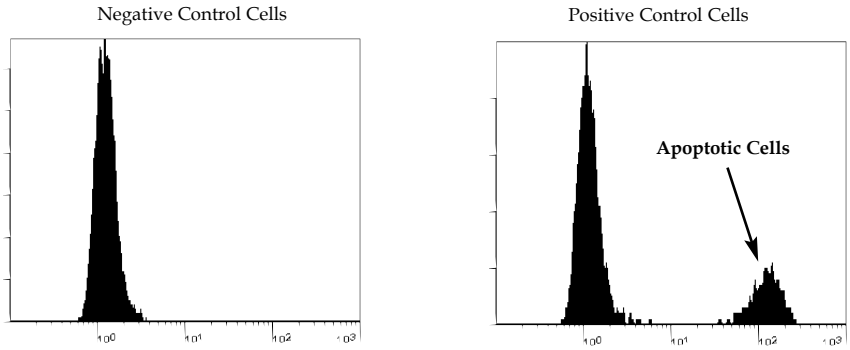

Figure 3: Cytometry Data of APO-DIRECT™ Negative & Positive Control Cells

Cytometer Setups

Typical Gain Settings

Becton Dickinson FACScan/Caliber Flow Cytometer

Parameter	Amplifier Gain	Detector Gain
FL 1	Log	380 Volts
FL 2	1.46	414 Volts
FL 2 Width	.87	
FL 2 Area	3.25	
	Threshold- FL2, 40	

Coulter XL Flow Cytometer

Parameter	Amplifier 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

Figure 4

Different Gates Used for Different Cytometers

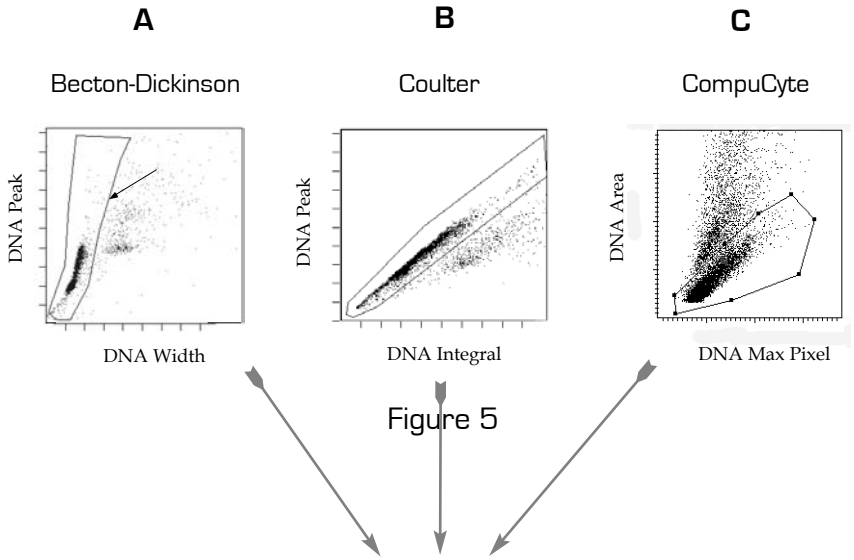

Figure 5

Common Gated Display

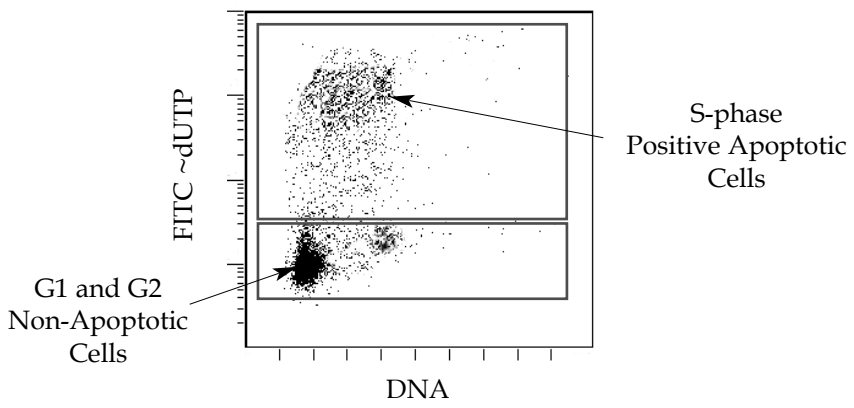

Figure 6

Figure 5 and 6 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 test tubes are used, an electro-static 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 µl **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 side of the tube and mak ing 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 crossing linking chemical fixative is an important step in analyzing apopto sis. 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 permeabiliza tion methods to fully exploit their systems.

5. If a low intensity of FITC staining is observed, try increasing the incubation time during the 50 µl **DNA Labeling Reaction**. Some researchers have found labeling times of up to four hours at 37C 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** solu tion to each tube.

References

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