ApoScreen™ Annexin V Apoptosis Kit

Cat. No.	Kit Version	Quantity
10010-02	Fluorescein (FITC) Conjugated Annexin V	100 tests

INTRODUCTION

Apoptosis is a well-documented phenomenon in many cellular systems.[1] It plays a key role in tissue and organ development during embryogenesis as well as in regulating cell turnover in adult tissues. In the immune system, apoptosis provides the mechanism for deletion of autoreactive T cells in the thymus [2], low responsive B lymphocytes in germinal centers [3], and of target cells susceptible to attack by cytotoxic T lymphocytes and natural killer cells.[1, 4] Structurally, apoptosis is characterized by chromatin condensation, reduction in cell size, and endonuclease cleavage of DNA into nucleosomal size fragments.[1,4,5] In addition to these changes in cell morphology that occur during apoptosis, a major event is the loss of membrane phospholipid asymmetry, with translocation of phosphatidylserine (PS) from the inner leaflet of the phospholipid bilayer to the cell surface.[6] While the function of PS externalization is unclear, it may serve as a "signal" for recognition by phagocytic cells which respond by engulfing the apoptotic cell before loss of plasma membrane integrity. Exposure of PS on the cell surface provides a simple means for detecting cells undergoing apoptosis.

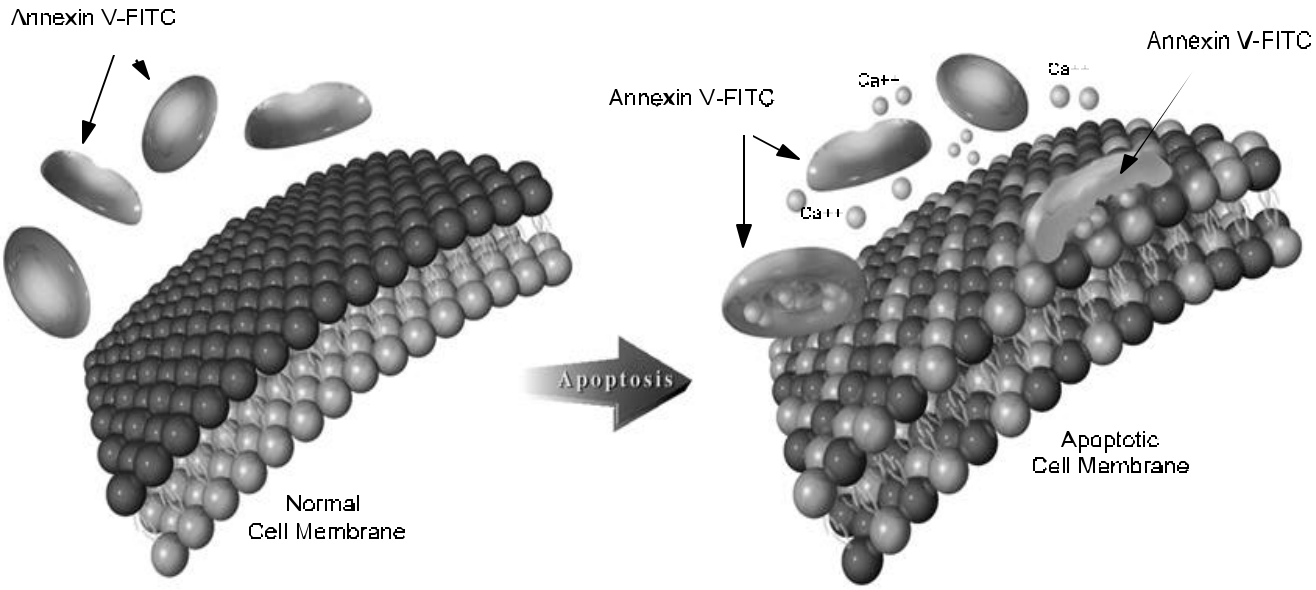

Figure 1. Illustration of the inversion of phosphatidylserine after induction of apoptosis, and the subsequent binding of Annexin V-FITC to the cell surface under well-defined calcium concentrations.

Annexin V (Mr 36-kDa), a member of the annexin family of calcium-dependent phospholipid binding proteins, has a high affinity for PS-containing phospholipid bilayers.[7,8] When PS is exposed on the extracellular face of a cell membrane, annexin V binds with an affinity of approximately 7 nM.[9] Fluorochrome conjugates of annexin V can be used to monitor changes in cell membrane phospholipid asymmetry [10-13], thereby providing a convenient tool for detection of apoptotic cells (Figure 1). Since externalization of PS occurs earlier than the nuclear changes associated with apoptosis, the ApoScreen™ Annexin V Apoptosis Kit can be used for detection of cells earlier in the apoptotic pathway than do DNA-based assays.[14] Labeled annexin V can also be used to analyze changes in membrane asymmetry in platelets [10] and erythrocytes.[13] For individual ApoScreen™ labeled annexin V reagents see product nos. 10040-02 and 10040-09.

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PRINCIPLE OF THE ASSAY

The ApoScreen™ Annexin V Apoptosis Kit employs a fluorescein-labeled annexin V (annexin V-FITC) in concert with propidium iodide (PI) to evaluate subpopulations of cells undergoing apoptosis. During the early stages of apoptosis, cells begin to display PS on the outer cell membrane where it is readily detectable by staining the cells with annexin V-FITC. As the plasma membrane becomes increasingly permeable during the later stages of apoptosis, PI can readily move across the cell membrane and bind to cellular DNA, providing a means for identifying those cells that have lost membrane integrity through mechanisms including necrosis. When cells are double stained with annexin V-FITC and PI, three different cell populations may be observed (Figure 2): (i) live cells that do not stain with either annexin V-FITC or PI; (ii) necrotic cells that stain with both reagents; and (iii) apoptotic cells that stain with annexin V-FITC only. Analysis can be performed on any flow cytometer equipped with a single laser using excitation at 488 nm. Alternatively, the cells can be examined with a fluorescence microscope equipped with filters for discrimination of FITC and rhodamine/PI.

KIT COMPONENTS

- 1 mL annexin V-FITC: Store at 2-8°C. Use as directed; no pre-dilution necessary.
- 1 mL propidium iodide (50 µg/ml): Store at 2-8°C. Use as directed; no pre-dilution necessary. Avoid skin contact and dispose of properly.
- 7 x 1.5 mL 10X binding buffer: Store at 2-8°C. Dilute 1 part 10X buffer with 9 parts dH_20 prior to use.

APOSCREEN™ ANNEXIN V STAINING PROTOCOL

The annexin V-FITC reagent is optimized to determine the frequency of cells that can be induced to enter the apoptotic pathway. Since most cell populations will contain a proportion of cells that are in the apoptotic and/or necrotic state, the number of cells undergoing induced apoptosis must be derived by subtracting the number of apoptotic cells in an untreated population from the number observed in an induced population.

NOTE: The following 4-tube protocol is designed to aid in initially setting the correct fluorescence compensation on the flow cytometer. Subsequent assays may be performed using a simpler protocol outlined in item number 1 in the Hints and Suggestions section later in this technical bulletin. Since apoptosis is a rapid and dynamic process, we recommend performing the analysis immediately after staining.

- Tube 1: unstained cells
- Tube 2: annexin V-FITC only
- Tube 3: PI only
- Tube 4: annexin V-FITC + PI

1. Wash cells twice in cold PBS; remove the PBS from the cell pellet after the second wash.
2. Resuspend cells in cold 1X binding buffer to a concentration of 1 x 10^6 to 1 x 10^7 cells/mL.
3. Add 100 uL of cells (1 x 10^5 to 1 x 10^6) to each labeled tube.
4. Add 10 uL of annexin V-FITC to tube #2 and tube #4.
5. Gently vortex each tube and incubate for 15 minutes on ice, protected from light.
6. Without washing, add 380 uL of cold 1X binding buffer to each tube. Add 10 uL of PI to tube #3 and tube #4.
7. Analyze by flow cytometry; suggested guidelines are as follows.

FLOW CYTOMETRY ANALYSIS

NOTE: The compensation instructions outlined below are for a BDIS FACScan™ flow cytometer. Compensation protocols for other instruments may differ.

1. While viewing a FSC vs SSC dot plot, run the control tube of unstained cells and set the acquisition gate on the population of interest. *Because apoptotic cells may undergo changes in light scattering properties, the gate used to identify the cells of interest should accommodate these changes.*
2. Generate a log FL1 vs log FL2 (or FL3) dot plot of the gated cells. Adjust emission PMTs so that >98% of the total events are located within the lower left quadrant and are within the first log decade on both the X and Y axis of the FL1 vs FL2 (or FL3) dot plot.
3. While viewing the FL1 vs FL2 (or FL3) dot plot of the gated cells, run tube #2 (annexin V-FITC only) to ensure that no events are recorded in the upper left and upper right quadrants of the display. If necessary, correct the compensation by increasing FL2 (or FL3) - %FL1 compensation (this may range from 15-25%).
4. While viewing the FL1 vs FL2 (or FL3) dot plot of the gated cells, run tube #3 (PI only) to ensure that no events are recorded in the upper and lower right quadrants of the display. If PI stained cells are visible in any other than the upper left quadrant, decrease the FL1 - %FL2 (or FL3) compensation (this may range from 0-1%).
5. If the flow cytometer has been properly compensated, singly stained cells should be centrally located within the upper left (FL2 or FL3/PI) or lower right (FL1/annexin V-FITC) quadrants.
6. Run samples and collect a minimum of 10,000 cells in list mode.

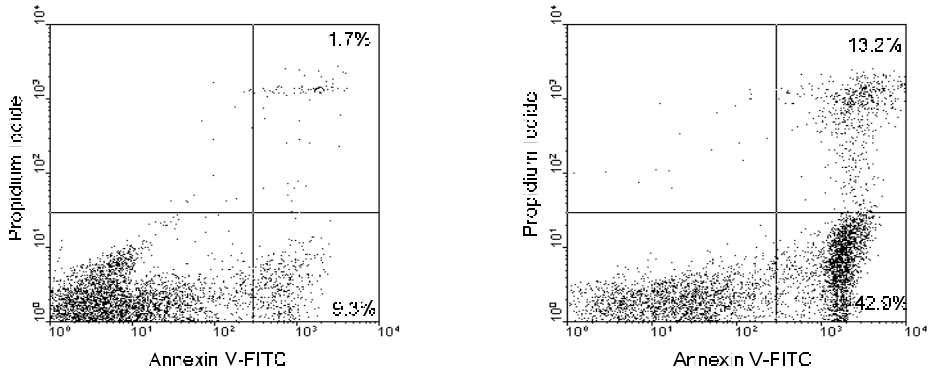

Figure 2. Two-color flow cytometry analysis of untreated (left panel) and irradiated (right panel) Balb/c thymocytes stained with PI and annexin V-FITC. Early apoptotic cells are located in the lower right quadrant of the two displays. (NOTE: Samples were analyzed on a Becton Dickinson FACScan™ flow cytometer).

7. To properly set the quadrant markers for determining the frequency of cells undergoing apoptosis, FL1 vs FL2 (or FL3) analysis should first be performed on the untreated/uninduced cell samples as follows (see Figure 2, left panel):
   a) the largest cluster of events located in the lower left region of the FL1 vs FL2 (or FL3) dot plot is the annexin V-negative population (normally this population will reside within the first two log decades of the FL1 axis). Set the vertical cursor 0.1 to 0.2 log units beyond the right edge of this annexin V-negative population.
   b) discrimination of PI^+ and PI^- subpopulations is aided if necrotic cells are present in the sample; if so it may be possible to identify an additional cluster of events in the upper right area of the dot plot (annexin V^+PI^+); if no PI^+ cells are present in the sample, distinguishing PI^+ cells is best estimated by placing the horizontal cursor 0.1 to 0.3 log units above the edge of the double-negative cluster of events (annexin V^-PI^-).
8. Cells that have been experimentally treated or are suspected of undergoing apoptosis can now be analyzed. Those events falling outside the negative staining region are considered positive staining events for either annexin V only (Figure 2, right panel, lower right quadrant) or annexin V + PI (Figure 2, right panel, upper right quadrant). The subpopulation of cells staining with annexin V-FITC only are those cells in the apoptotic pathway, while those that stain with both annexin V-FITC and PI are either necrotic or are in transition from the apoptotic to the necrotic state.

HINTS AND SUGGESTIONS

1. Once completing the initial 4-tube staining protocol and flow cytometer compensation adjustments outlined in the previous sections, subsequent assays may be performed using a simplified protocol. Such a protocol simply consists of a tube of 10^5 to 10^6 cells stained with Annexin V-FITC + PI as described above ("Tube #4" in the APOSCREEN™ ANNEXIN V STAINING PROTOCOL).
2. A negative control can consist of cells not induced to undergo apoptosis. Another type of negative control is a cell sample to which EDTA is added as a calcium chelator, just prior to staining with Annexin V-FITC + PI (annexin V binding to membrane phosphatidylserine is inhibited in the absence of free calcium); for such a control we suggest adding EDTA to give a final concentration of 5 mM; add the EDTA immediately prior to adding the annexin V-FITC reagent, then proceed with the remainder of the staining protocol and flow cytometry analysis.
3. The intensity of staining with labeled annexin V can vary from one type of cell to another, due to differences in cell size and the amount of phosphatidylserine exposed on cell surfaces. The suggested staining protocol in this kit was developed using mouse thymocytes; other cell and sample types may require differing amounts of annexin V reagent, and/or collection of differing numbers of flow cytometry events.
4. Activated platelets specifically bind annexin V (9,10), and they may also bind to nucleated blood cells such as monocytes. Therefore exercise caution when interpreting annexin V assay results in studies of nucleated blood cells that may bind to platelets. Such cells may need to be washed in PBS + 5 mM EDTA prior to the staining procedure.
5. Some methods for removing adherent cells from culture vessels can cause cell membrane damage, and may bring about spurious results in annexin V-based assays. Therefore methods for removal of adherent cells should be optimized for annexin V assays and flow cytometry analysis.
6. Adherent cells may be cultured on a suitable substrate (e.g. sterile glass coverslips) and processed using an appropriate modification of the staining protocol outlined above; however this method has not been tested in our laboratories.

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