Annexin V-CFS

Catalog Number: NX50
Lot Number: LKJ04
100 Tests

Reagents Provided

Fluorescein-conjugated human Annexin V: 1 mL of Annexin V-fluorescein-labeled at a concentration of 10 μg/mL.

Reagents Not Provided

Annexin V Binding buffer: 10 mM HEPES/NaOH (pH 7.4) + 140 mM NaCl + 2.5 mM $CaCl_2$

Reagent Preparation

Fluorescein-conjugated Annexin V: Use as is; no preparation necessary. Open vial under a containment hood and keep reagent sterile. No preservatives added. Reagents are stable for **twelve months** from date of receipt when stored in the dark at 2° - 8° C.

Sample Staining

1. Cells to be used for staining should be washed with cold PBS twice and then resuspended in an appropriate binding buffer at a concentration 1 x 10^6 cells/mL.
2. Transfer 100 μL of the cells (1 x 10^5 cells) to a 5 mL culture tube.
3. Add 10 μL of fluorescein-conjugated Annexin V reagent.
4. Gently vortex the cell mixture and then incubate for 15 minutes at 20° - 25° C (room temperature) in the dark.
5. Following the incubation and without washing the cells of excess reagents, add 400 μL of an appropriate binding buffer to each tube. Analyze on a flow cytometer as soon as possible (within 1 hour of completion of cell staining).

We recommend also setting up three additional control tubes that will aid in setting up electronic compensation and quadrant statistics: a) unstained cells; b) cells stained with Annexin V-fluorescein only; c) cells stained with PI only. Positive staining with both the Annexin V and PI can only be demonstrated in populations of cells that contain a fraction of cells that are dying. We also recommend setting up the above three control tubes using a cell line that can serve as a positive staining control for both Annexin V and PI. Example of such cells are the U-937 human cell line cultured in RPMI + 10% FCS + 2 - 4 ng/mL of TNF-α for 2 - 3 hours or murine thymocytes left in culture media for 1 - 2 hours.

Please refer to the technical notes below for suggestions on how to set up proper instrument compensation. This procedure may need to be modified, depending upon the type of cells used.

Analyzing the Stained Cells by Flow Cytometry

Proper flow cytometric analysis of Annexin V-FITC and propidium iodide stained cells requires that electronic compensation of the instrument be set to exclude any overlap of the two emission spectra. Since this is directly related to the voltage settings on the PMTs, electronic compensation settings will vary between instruments. We recommend analyzing single stained cells at the beginning of the experiment and adjusting for any spectral overlap by electronic compensation.

1. Run the control tube of unstained cells and while looking at a FS vs SS linear dot plot of the cells, gate on the population of interest. *Note that cells can undergo changes in their light scattering properties following tissue culture. Make sure that the scatter gate used to identify cells of interest will accommodate these changes in the light scattering properties of the cells.*
2. Generate a log FL1 vs log FL2 dot plot of the above light scatter gated cells and ensure that > 98% of events are centrally distributed within the lower left quadrant bordered by the first log decade scale on both the X and Y axis of the fluorescence dot plot.
3. Run the Annexin V-fluorescein only stained cells and while looking at the log FL1 vs log FL2 dot plot ensure that no events accumulate in the upper left and upper right quadrants. Events in the two upper quadrants are indicative of spectral overlap; in this case fluorescein emissions are being detected by the FL2 PMT. To correct this, increase the % compensation of FL1 signals being detected by the FL2 PMT (this may range between 1 - 5%). If this adjustment does not fully eliminate FL2 positive signals, reduce the voltage being applied to the FL2 PMT.
4. Run the PI only stained cells and while looking at the log FL1 vs log FL2 dot plot ensure that no events accumulate in the upper right and lower right quadrants. Events in these quadrants are indicative of spectral overlap; in this case PI emissions are being detected by the FL1 detector. To correct this problem, adjust the electronic compensation of the instrument by increasing the % compensation of FL2 signals being detected by the FL1 PMT (this may range between 15 - 25%). If this adjustment fails to completely eliminate FL1 positive events, reduce the voltage being applied to the FL1 PMT.
5. If PMT voltages were adjusted during the above compensation exercise, we recommend repeating steps 3 and 4 in order to ensure that the instrument has not been over compensated. This is evidenced by the appearance of singly positive events very close to either axis. A properly compensated instrument should result in fluorescence displays of singly stained cells centrally located within the first log decade of each axis.
6. Since most untreated cell populations will contain a resident percentage of cells that stain with Annexin V and PI, the frequencies of positive and negative staining cells determined following usage of the kit must be derived by subtracting the resident frequency of positive staining cells in the resting or untreated population from the frequencies determined in the treated cell populations.
7. Setting quadrant cursor positions for determination of the frequency of cells that stain positive for Annexin V is best achieved by first performing flow cytometric analysis of the resting or untreated cells of interest that have been stained with both Annexin V and PI and then identifying the following clusters of events on the log FL1 vs log FL2 dot plot:

a. *Setting the FL1 Cursor Position:* the largest cluster of events located in the lower left region of the histogram is the Annexin V negative staining population (normally this population will reside within the first two log decades of the FL1 axis). Position the vertical cursor 0.1 to 0.2 log units beyond the edge of this “Annexin V negative” population.

b. *Setting the FL2 Cursor Position:* distinction of the PI^+ and PI^- staining population is aided if sufficient number of double positive are present in the population being analyzed. Under these conditions it may be possible to identify two cluster of events, one being located towards the lower right hand side of the histogram (ANN^+/PI^-) and other at the upper right hand side of the histogram (ANN^+/PI^+). The horizontal cursor may be placed at the gap between these two cluster of events. In the event that no PI^+ cells are present in the population being analyzed, the horizontal cursor position setting used to distinguish PI^+ cells is best estimated by using the double negative staining population as a reference point and then placing the cursor 0.1 to 0.3 log units above the edge of the double negative cluster of events. Ideally, a combination of the above described two techniques should allow accurate cursor placement to identify the various staining populations.

8. Cells that have been experimentally treated can be now be analyzed by flow cytometry after having been stained with both the Annexin V and PI reagent in the kit. Those events falling outside of the “negative staining” regions identified in steps 6 & 7 are considered positive staining events for either Annexin V only or Annexin V and PI.
See page 3 for typical fluorescence dot plots.

Technical Notes

It is advisable that the staining and subsequent analysis of cells be performed as rapidly as possible. Although fixation of cells with 1.0% formaldehyde is not recommended in this procedure, it may be possible with certain cell types to treat Annexin V and propidium iodide stained cells with such a fixative following a single washing step of the cells. Cells are then resupended in 1X binding buffer and fixative may be added. This latter procedure however normally results in decreased staining intensity for both Annexin V and propidium iodide.

Figure 1

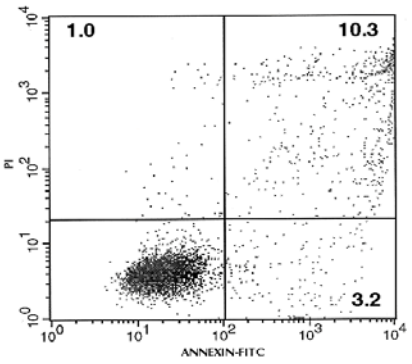

Figure 2

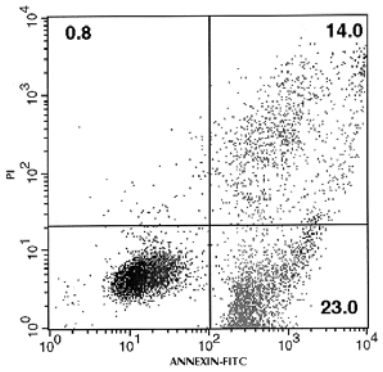

Figure 3

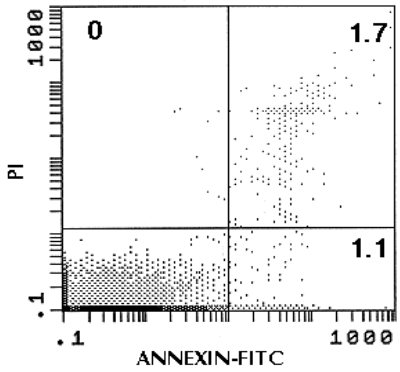

Figure 4

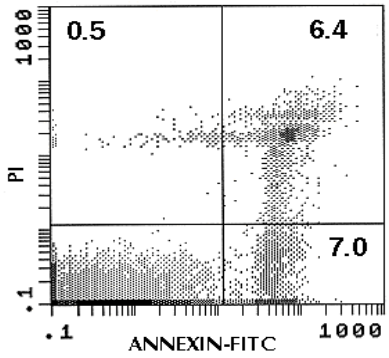

Figure 1 - Log fluorescence dot plot of Annexin V-fluorescein and PI stained U-937 cells. Untreated U-937 cells. Cell analysis was performed on a FACSCalibur (Becton Dickinson).

Figure 2 - Log fluorescence dot plot of Annexin V-fluorescein and PI stained U-937 cells. U-937 cells were cultured RPMI medium + 10% FCS + 3 ng/mL of rhTNF-α (R&D Systems Inc.) for 3 hours at 37° C prior to being stained. Cell analysis was performed on a FACSCalibur (Becton Dickinson).

Figure 3 - Log fluorescence dot plot of Annexin V-fluorescein and PI stained murine thymocytes. Thymocytes were isolated from Balb/c mice and immediately stained and FACS analysed. Cell analysis was performed on an EPICS-XL (Coulter).

Figure 4 - Log fluorescence dot plot of Annexin V-fluorescein and PI stained murine thymocytes. Thymocytes were isolated from Balb/c mice, cultured in RPMI medium + 10% FCS at 37° C for 3 hours and then stained for subsequent FACS analysis. Cell analysis was performed on an EPICS-XL (Coulter).