

APOPTOSIS VERSUS NECROSIS

**(Adapted from the R&D Systems Apoptosis Detection Kit
Insert Cat NX50)**

BACKGROUND INFORMATION:

In addition to DNA fragmentation, apoptotic cells undergo early changes that result in the exposure of phosphatidylserine on the surface of the cell. Annexin V is a phospholipid protein that binds to the exposed phosphatidylserine and can be labeled with fluorescein to enable one to detect cells undergoing apoptosis. In addition cells can be treated with propidium iodide (PI) to determine if the cells can either take-up or exclude PI. This procedure allows for quantification of apoptotic versus necrotic cells.

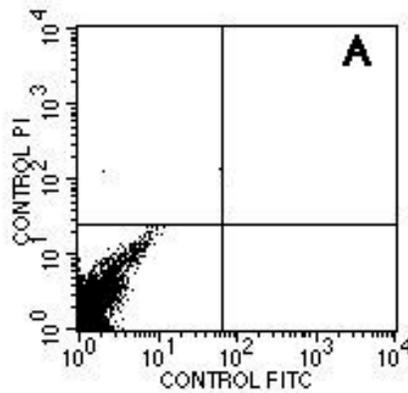
PROCEDURE:

1. Live cells are harvested and washed twice with cold PBS before resuspending in 1X binding buffer at a concentration of 1×10^6 cells/ml.
2. Transfer 100 μ l of cells (1×10^5 cells) to a 12x75 mm tubes (Becton Dickinson Cat #2058) and label tubes A, B, C, and D
3. Tube **A** is the control tube without any added reagents.
4. To tube **B**, add 10 μ l of FITC-conjugated annexin V reagent.
5. To tube **C**, add 10 μ l PI.
6. To tube **D**, add 10 μ l of FITC-conjugated annexin V and 10 μ l PI.
7. Gently mix cells, cover with aluminum foil, and incubate for 15 minutes at room temperature.
8. After incubation, DO NOT WASH CELLS, but add 400 μ l of 1X binding buffer to each tube.

NOTE: Each cell treatment and control (untreated) must have four tubes as noted above. Plan the experiment accordingly so that all tubes can be run within one hour of completing the staining. This usually is limited to a total of 40 tubes which would allow one to run one untreated control plus nine treatments (i.e. 10 treatments x 4 tubes/treatment= 40 tubes).

ANALYSIS BY FLOW CYTOMETRY

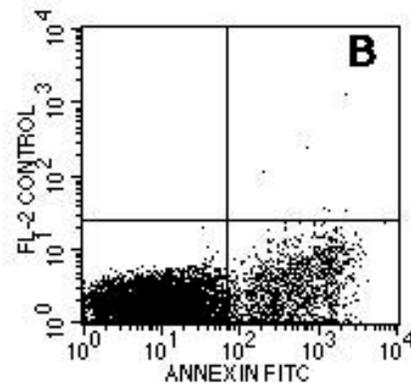
1. Run the unstained control tube and set the FSC and SSC to linear and adjust PMT's so that all the events are on-scale. Set the FL-1 (FITC) and FL-2 (PI) channels so that all the events are contained in the lower left (LL) quadrant. The data should appear as indicated in Figure 1A below. Do not save the data yet.
2. Run the FITC Control and adjust the FL-1 PMT and compensation settings so that the FITC positive cells are contained in the lower right (LR) quadrant and the negative cells remain in the LL quadrant. The data should appear as in figure 1B below. Do not save data.
3. Run the PI control tube and adjust the FL-2 PMT and compensation settings so that the PI positive cells are restricted to the upper left UL quadrant. The data should appear as in figure 1C below. Do not save data.
4. Run the control tube and collect 5000 events followed by the FITC control, PI control and the FITC+PI tubes. Note that when the tube containing both the Annexin FITC and PI is run (Figure 1D), any double positive cells detected in the upper right (UR) quadrant cannot be misrepresented as either single color FITC positive or PI positive cells since the compensation and PMT voltages were set using the single color control tubes.
5. Repeat the set of four tubes for each cell treatment.



Quadrant Statistics

Gate: No Gate
 Gated Events: 10000
 Total Events: 10000
 X Parameter: FL1-H CONTROL FITC (Log)
 Y Parameter: FL2-H CONTROL PI (Log)

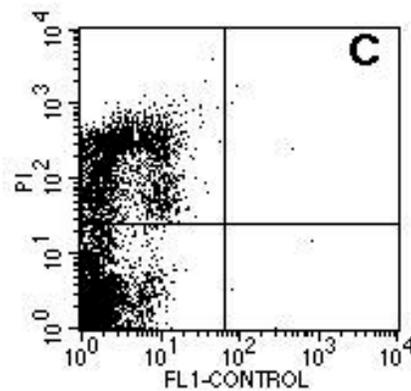
Quad	Events	% Total
UL	2	0.02
UR	0	0.00
LL	9998	99.98
LR	0	0.00



Quadrant Statistics

Gate: No Gate
 Gated Events: 10000
 Total Events: 10000
 X Parameter: FL1-H ANNEXIN FITC (Log)
 Y Parameter: FL2-H FL-2 CONTROL (Log)

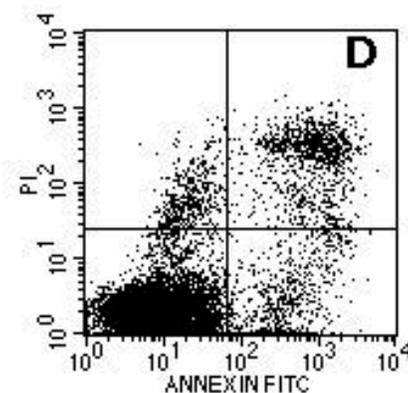
Quad	Events	% Total
UL	0	0.00
UR	7	0.07
LL	8250	82.50
LR	1743	17.43



Quadrant Statistics

Gate: No Gate
 Gated Events: 10000
 Total Events: 10000
 X Parameter: FL1-H FL1-CONTROL (Log)
 Y Parameter: FL2-H PI (Log)

Quad	Events	% Total
UL	2435	24.35
UR	3	0.03
LL	7559	75.59
LR	3	0.03



Quadrant Statistics

Gate: No Gate
 Gated Events: 10000
 Total Events: 10000
 X Parameter: FL1-H ANNEXIN FITC (Log)
 Y Parameter: FL2-H PI (Log)

Quad	Events	% Total
UL	447	4.47
UR	1032	10.32
LL	7734	77.34
LR	787	7.87

Data Interpretation: In Figure 1D, the events shown in the LL (lower left) quadrant (77%) represent viable (non-apoptotic or necrotic cells). Events in the UL (upper left) quadrant (4.5%) are indicative of necrotic cells that have taken up the PI. The percentage of early apoptotic cells that are annexin positive only, are represented in the LR (lower right) quadrant (8%) and events depicting late apoptotic cells that are both annexin and PI positive are shown in the UR (upper right) quadrant (10%).