

4. Gently vortex the cells and incubate for 15 min at RT (25°C) in the dark.
5. Add 400 µl of 1X binding buffer to each tube. Analyze by flow cytometry within one hour.

NOTE: Methods for utilizing Annexin V binding on adherent cells (i.e., monolayer) have been described (see refs 2). *However, these methods are not performed as a routine quality control for the Annexin V-FITC Apoptosis Detection Kit.*

STANDARD CONTROLS FOR SETTING UP FLOW CYTOMETRY

The following controls are used to set up compensation and quadrants:

1. Unstained cells.
2. Cells stained with Annexin V-FITC alone (no PI).
3. Cells stained with PI alone (no Annexin V-FITC).

Other Staining Controls

A cell line that can be easily induced to undergo apoptosis should be used to obtain positive control staining with Annexin V-FITC and with both Annexin V-FITC and PI. It is important to note that the basal level of apoptosis and necrosis varies considerably within a population. Thus, even in the absence of induced apoptosis, most cell populations will contain at least a minor percentage of cells that are positive for apoptosis (Annexin V-FITC positive, PI negative or Annexin V-FITC and PI positive). The untreated population is used to define the basal level of apoptotic and dead cells. The percentage of cells that have been induced to undergo apoptosis is then determined by subtracting the percentage of apoptotic cells in the untreated population from percentage of apoptotic cells in the treated population.

INDUCTION OF APOPTOSIS by CAMPTOTHECIN

The following protocol is routinely used in house to test the Annexin V-FITC.

Materials

1. Prepare camptothecin stock solution (SIGMA # C-9911): 1 mM in DMSO.
2. Jurkat T cells (ATCC #TIB-152).

Procedure

1. Add camptothecin (final conc. 4-6 µM) to 1 x 10⁶ Jurkat cells.
2. Incubate the cells for 4-6 hr at 37°C.
3. Proceed with the Annexin V-FITC Staining Protocol to measure apoptosis.

REFERENCES

1. Schmid, I. (1992) Cytometry 13:204-208; O'Brien, M.C. (1995) Cytometry 19:243-255; Vermes, IC (1995) J. Immunol. Meth. 184:39-51;
2. Casciola-Rosen, L. (1996) Proc. Natl. Acad. Sci. USA 93:1624-1629; van Engeland, M. (1996) Cytometry 24:131-139.

ANNEXIN V-FITC APOPTOSIS DETECTION KIT

For the detection and apoptotic cells

Human Annexin V-FITC Kit # ANV-F-100

Kit Components, 96 tests	Cat #
Annexin V-FITC, 100 tests (0.5 ml); supplied in a buffer containing BSA and 0.05% Azide.	ANV-F-101
Propidium Iodide Staining Solution 2.0 ml in PBS (pH 7.4) (50 ug/ml)	ANV-F-102
Annexin V Binding Buffer, 10X Concentrate 50 ml solution	ANV-F-103
Instruction Manual	

Introduction

Apoptosis is a form of cell death that permits the removal of damaged, senescent or unwanted cells in multicellular organisms, without damage to the cellular microenvironment. Apoptosis is a normal physiologic process which occurs during embryonic development as well as in maintenance of tissue homeostasis. The apoptotic program is characterized by certain morphologic features, including loss of plasma membrane asymmetry and attachment, condensation of the cytoplasm and nucleus, and internucleosomal cleavage of DNA. Loss of plasma membrane is one of the earliest features. In apoptotic cells, the membrane phospholipid phosphatidylserine (PS) is translocated from the inner to the outer leaflet of the plasma membrane, thereby exposing PS to the external cellular environment. Annexin V is a 35-36 kDa Ca^{2+} dependent phospholipid-binding protein that has a high affinity for PS, and binds to cells with exposed PS. It is estimated that approximately 50 exposed phospholipid monomers bind per Annexin V molecule. Therefore, Annexin V-FITC (fluorochromes) serves as a sensitive probe for flow cytometric analysis of cells that are undergoing apoptosis.

Externalization of PS occurs in the earlier stages of apoptosis. Therefore, Annexin V-FITC staining can identify apoptosis at an earlier stage than assays based on nuclear changes such as DNA fragmentation. **Annexin V-FITC** staining precedes the loss of membrane integrity which accompanies the latest stages of cell death resulting from either apoptotic or necrotic processes. Therefore, staining with Annexin V-FITC is typically used in conjunction with a vital dye such as Propidium Iodide (**PI**) to allow the investigator to identify early apoptotic cells (Annexin V-FITC positive, PI negative). For example, cells that are viable are Annexin V-FITC and PI negative; cells that are in early apoptosis are Annexin V-FITC positive and PI negative; and cells that are in late apoptosis or already dead are both Annexin V-FITC and PI positive. However, when apoptosis is measured over time, cells can be often tracked from Annexin V-FITC and PI negative (viable, or no measurable apoptosis), to Annexin V-FITC positive and PI negative (early apoptosis, membrane integrity is present) and finally to Annexin V-FITC and PI positive (end stage apoptosis and death). The movement of cells through these three stages suggests apoptosis. In contrast, a single observation indicating that cells are both Annexin V-FITC and PI positive, in of itself, reveals less information about the process by which the cells underwent their demise.

ADI human Annexin V-FITC kit is a highly sensitive assay for the detection of apoptotic cells.

SPECIFICITY AND PREPARATION

Annexin V-FITC is a sensitive probe for identifying apoptotic cells. It binds to negatively charged phospholipid surfaces (K_d of $\sim 5 \times 10^{-2} \mu M$) with a higher specificity for phosphatidylserine (PS) than most other phospholipids. Defined calcium and salt concentrations are required for Annexin V-FITC binding as described in the Annexin V-FITC Staining Protocol. **Purified recombinant Annexin V** was conjugated to FITC under optimum conditions. Annexin V-FITC is routinely tested using primary cells or cell lines induced to undergo an apoptotic death.

USAGE AND STORAGE

For flow cytometry (5 μl /test). Store Annexin V-FITC at 4°C.

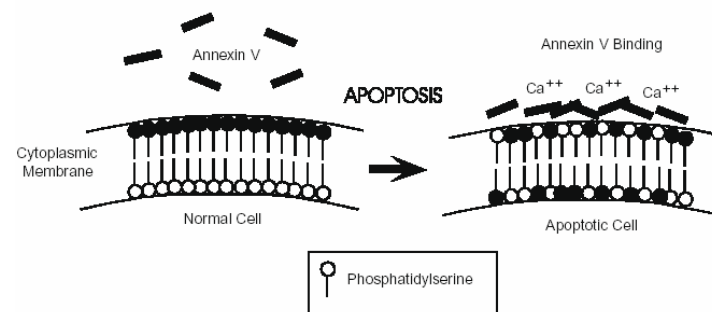


Figure 1. Schematic representation of Annexin V binding to apoptotic cell.

Annexin V-FITC Staining Procedure

Supplied Reagents

- Annexin V-FITC** (Cat. No. ANV-F101). Use 5 μl per test. Store at 4°C.
- Propidium Iodide** (Cat. No. ANV-F103). Use 5 μl per test. PI (Propidium Iodide) is a convenient, ready-to-use solution of the nucleic acid dye that can be used for the exclusion of nonviable cells in flow cytometric assays. PI fluorescence is detected in the far red range of the spectrum (650 nm long-pass filter). Store at 4°C.
- 10X Annexin V Binding Buffer**. (Cat. No. ANV-F103). 0.1 M HEPES/NaOH (pH 7.4) 1.4 M NaCl, 25 mM $CaCl_2$ (sterile filtered). Dilute 1:10 with water to prepare 1X solution. Store the 10X concentrate and working solution at 2.8°C.

Staining Procedure

- Wash cells twice with cold PBS and then resuspend cells in 1X binding buffer at a concentration of 1×10^6 cells/ml.
- Transfer 100 μl of the solution (1×10^5 cells) to a 5 ml culture tube.
- Add 5 μl of Annexin V-FITC and 5 μl of PI.