User Manual

ApoAlert®
Annexin V
User Manual

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I. Introduction

Apoptosis, or programmed cell death, plays a fundamental role in many normal biological processes as well as several disease states (Wyllie *et al.*, 1980; Ellis *et al.*, 1991; Cohen *et al.*, 1992;Thompson, 1995; Nicholson, 1996). Apoptosis can be induced by various stimuli that all produce the same end result: systematic and deliberate cell death.

One method for studying apoptosis detects changes in the position of phosphatidylserine (PS) in the cell membrane. In non-apoptotic cells, most PS molecules are localized at the inner layer of the plasma membrane, but soon after inducing apoptosis, PS redistributes to the outer layer of the membrane, and becomes exposed to the extracellular environment (Martin *et al.*, 1995; Fadok *et al.*, 1992a; 1992b; 1993). PS translocation precedes other apoptotic events, thus allowing early detection of apoptosis.

Exposed PS can be easily detected with annexin V, a 35.8-kDa protein that has a strong affinity for PS. The specificity of the annexin V-PS interaction is reflected by competitive binding studies in which annexin V binding to apoptotic cells was specifically inhibited by PS liposomes, but was unaffected by liposomes containing other phospholipids (Martin *et al.*, 1995). The assay is nonenzymatic, and can be used with flow cytometry or fluorescence microscopy.

PS externalization occurs independently of the stimulus used. For example, treatment with antibodies to the Fas cell surface receptor induces apoptosis in certain cell types like cells of the immune system (Yonehara *et al.*, 1989; Rouvier *et al.*, 1993; Grifith, 1995). Fas-induced apoptosis in Jurkat T cells is profiled with the Annexin V Assay in Figure 1.

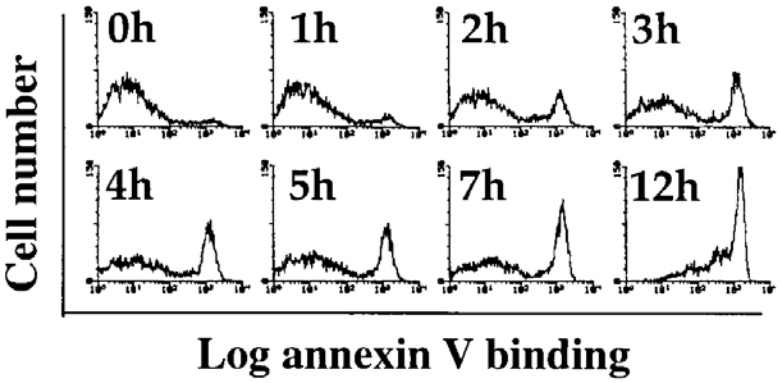

Figure 1. Annexin V-FITC staining of apoptosis in Jurkat T cells. Apoptosis was induced with 200 ng/ml anti-Fas (IgM, CH11). At the indicated times, cells were incubated for 5 min with 1.0 µg/ml annexin V-FITC in binding buffer, and 5,000 cells were examined by flow cytometry analysis. For details, see Martin *et al.*, 1995. Data reproduced with author's permission.

II. List of Components

Store all components at 4°C.

Cat. No. 630109 (50 assays)	Cat. No. 630110 (200 assays)	
• 250 µl	1 ml	**Annexin V-FITC** (20 µg/ml in Tris-NaCl)
• 2 x 25 ml	2 x 100 ml	**1X Binding Buffer**
• 500 µl	2 ml	**Propidium Iodide** (50 µg/ml in 1X binding buffer)

Cat. No. 630201

- 500 µl **Annexin V-FITC** (100 µg/ml)

Cat. No. 630202

- 100 ml **10X Annexin V Binding Buffer**

Notes:

- Under acid conditions, sodium azide yields hydrazoic acid, which is extremely toxic. Azide compounds should be discarded by flushing with running water. We recommend these precautions to avoid deposits in metal piping, in which explosive condition can develop. If the event of skin or eye contact, wash excessively with water. Discard all samples and material coming in contact with sodium azide with due precaution.
- Do not expose reagent to strong light during storage and incubation.
- Microbial contamination of reagents may cause incorrect results.
- Propidium iodide is toxic. Handle with extreme caution.

III. Additional Materials Required

Annexin V Protocol:

- **PBS**

	Final conc.	To prepare 2 L of solution:
Na_2HPO_4	58 mM	16.5 g
NaH_2PO_4	17 mM	4.1 g
NaCl	68 mM	8.0 g

Dissolve components in 1.8 L of H_2O. Adjust to pH 7.4 with 0.1 N NaOH. Add H_2O to a final volume of 2 L. Store at room temperature.

- **Centrifuge** for collecting cells

IV. Annexin V Protocol

PLEASE READ THE ENTIRE PROTOCOL BEFORE BEGINNING

Because apoptosis is a rapid and dynamic process, we recommend performing analysis immediately after staining.

If you plan to fix your cells, incubate them with annexin V **before** fixation, because cell membrane disruption can allow annexin V to bind to PS on the inner surface of the cell membrane. Rinse unbound annexin V with binding buffer before fixation.

A. Incubation of cells with Annexin V

1. Induce apoptosis by a desired method.
   a. For nonadherent cells, proceed to Step 2.
   b. For **flow cytometry** with adherent cells, trypsinize cells. Gently wash the cells once with serum-containing media before incubation with annexin V. Proceed to Step 2.
   c. For **fluorescence microscopy** with adherent cells, grow cells directly on a glass slide and proceed to Step 2.
2. Rinse 1 x 10^5–1 x 10^6 cells with 1X Binding Buffer.
3. Resuspend the cells in 200 µl of 1X Binding Buffer.
4. Add 5 µl of Annexin V and [optional] 10 µl of Propidium Iodide.
5. Incubate at room temperature for 5–15 min in the dark.

Proceed to B or C below, depending on analysis method.

B. Quantification by Flow Cytometry

Analyze cells by flow cytometry using a single laser emitting excitation light at 488 nm. Use Binding Buffer to bring the reaction volume to at least 500 µl for flow cytometry analysis.

Note: The signal generated by Annexin V can be detected in the FITC signal detector, and the signal generated by PI can be monitored by the detector reserved for phycoerythrin emission.

C. Detection by Fluorescence Microscopy

1. For analyzing nonadherent cells, place the cell suspension from Step A.5 on a glass slide. Cover with a glass coverslip.

   For analyzing adherent cells, following incubation (A.2–5), invert coverslip on glass slide and visualize cells. After incubating with annexin V, the cells can be washed and fixed in 2% formaldehyde.
2. Observe the cells under a fluorescence microscope using a dual filter set for FITC & rhodamine.

   Note: Cells with bound annexin V will show green staining in the plasma membrane. Cells that have lost membrane integrity will show red staining (PI) throughout the cytoplasm and a halo of green staining on the cell surface (plasma membrane).

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Notes

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