

Annexin V Kits

TACS[®] Annexin V-FITC Apoptosis Detection Kit

Catalog Numbers: 4830-01-K (125 tests)
4830-250-K (250 tests)

Apoptosis detection by flow cytometry or *in situ* labeling.

This package insert must be read in its entirety before using this product.
For research use only. Not for use in diagnostic procedures.

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INTRODUCTION

Apoptosis is often defined by morphological criterion accompanied by biochemical analysis. Apoptosis is characterized by a number of intracellular phenomena such as membrane blebbing, chromatin condensation, and nuclear DNA fragmentation. Early in the apoptosis process, phosphatidylserine (PS) becomes exposed on the cell surface by flipping from the inner to outer leaflet of the cytoplasmic membrane (Figure 1). This event is thought to be important for macrophage recognition of cells undergoing apoptosis, thus allowing the cells to be rapidly phagocytosed. The binding of Annexin V to phosphatidylserine is calcium-dependent and reversible, but very tight, with a K_d of approximately 5×10^{-10} M. At low PS concentration, a binding ratio of eight Annexins to one PS has been reported. These features make Annexin V conjugates ideal for identifying membrane changes associated with apoptosis, by using either flow cytometric or *in situ* labeling methods. Annexin V allows for the identification of cell surface changes that occur early during the apoptotic process.

The TACS[®] Annexin V-FITC Apoptosis Detection Kit facilitates rapid fluorometric quantitation of apoptotic cells either by flow cytometry or *in situ* detection.

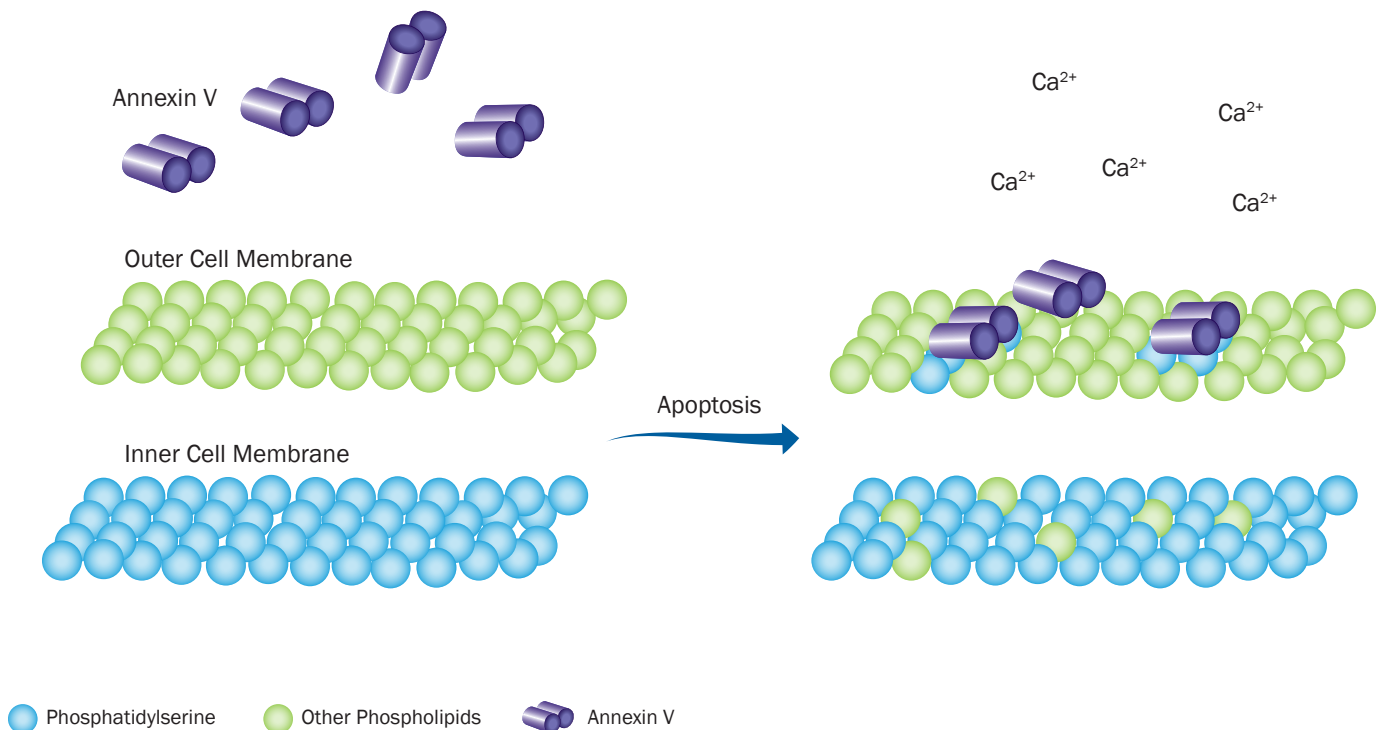


Figure 1 - Schematic depiction of phosphatidylserine flipping during apoptosis and subsequent detection using Annexin V.

PRINCIPLE OF THE ASSAY

The TACS Annexin V-FITC Apoptosis Detection Kit is supplied with TACS Annexin V-FITC, an optimized binding buffer, and Propidium Iodide. Cells are harvested and washed, then incubated with an TACS Annexin V-FITC in binding buffer for 15 minutes at room temperature, after which cells can be directly analyzed.

Propidium Iodide is included in the incubation mix to identify cells that have lost membrane integrity (i.e. late apoptotic/necrotic cells). As cells disintegrate, greater access to the inner cell membrane allows for additional Annexin V binding. Thus, double labeling is used to help differentiate between early and late apoptotic/necrotic events.

TACS Annexin V-FITC is provided at a 100X concentration with a 1:1 molar ratio of Annexin V to FITC. The binding of Annexin V will vary depending upon cell type and assay method, therefore the concentration of Annexin V used may require optimization.

TACS Annexin V-FITC may also be used for *in situ* detection of apoptosis. After incubation with Annexin V-FITC and Propidium Iodide, cells may be visualized *in situ* by fluorescence microscopy.

LIMITATIONS OF THE PROCEDURE

- FOR RESEARCH USE ONLY. NOT FOR USE IN DIAGNOSTIC PROCEDURES.
- Do not mix or substitute reagents with those from other lots or sources.
- Variations in sample collection, processing, and storage may cause sample value differences.

TECHNICAL HINTS

- When mixing or reconstituting solutions, always avoid foaming.
- To avoid cross-contamination, change pipette tips between additions of each standard level, between sample additions, and between reagent additions. Also, use separate reservoirs for each reagent.

PRECAUTIONS

The acute and chronic effects of overexposure to reagents of this kit are unknown. Safe laboratory procedures should be followed, and protective clothing should be worn when handling kit reagents.

Wear protective gloves, clothing, eye, and face protection. Wash hands thoroughly after handling. Refer to the SDS on our website prior to use.

MATERIALS PROVIDED & STORAGE CONDITIONS

Do not use past kit expiration date.

| CATALOG # | PART | PART # | AMOUNT PROVIDED | STORAGE OF UNOPENED MATERIAL |
|------------|---------------------|-----------|------------------------|------------------------------|
| 4830-01-K | TACS Annexin V-FITC | 4830-01-1 | 125 µL | Store 2-8 °C. |
| | 10X Binding Buffer | 4830-01-2 | 10 mL | |
| | Propidium Iodide | 4830-01-3 | 1.25 mL | |
| 4830-250-K | TACS Annexin V-FITC | 4830-01-1 | 2 vials (125 µL/vial) | |
| | 10X Binding Buffer | 4830-01-2 | 2 vials (10 mL/vial) | |
| | Propidium Iodide | 4830-01-3 | 2 vials (1.25 mL/vial) | |

OTHER MATERIALS REQUIRED

Equipment:

- Fluorescence microscope or flow cytometer (capable of FITC and Propidium Iodide detection)
- Microcentrifuge
- Pipette and pipette tips
- Ice bucket and ice

Reagents:

- 10X Phosphate Buffered Saline (PBS)
- Distilled water
- Fluorescence compatible mounting media

Disposables:

- Microcentrifuge tubes
- Aluminum foil
- Gloves
- Glass coverslips
- Glass microscope slides

ASSAY PROTOCOL

Early in the apoptosis process, phosphatidylserine (PS) becomes exposed on the cell surface by flipping from the inner to outer leaflet of the cytoplasmic membrane. This phenomenon may precede DNA fragmentation by several hours. When analyzing cells using this method, early time points following treatment should be investigated.

Different cell types vary in their concentration of phosphatidylserine as well as in the amount of its exposure on the cell surface after apoptosis is initiated. The following protocol is a guideline, however, it may be necessary to adjust the concentration of the TACS Annexin V-FITC. Typically, a 1:100 dilution of the TACS Annexin V-FITC is appropriate, dilutions of 1:50 up to 1:1000 may be needed. Cells should not be fixed prior to incubating with an TACS Annexin V-FITC.

FLOW CYTOMETRY PROTOCOL

It is recommended that the X-axis of the dot plot reflects the log of the Annexin V-FITC fluorescence, and the Y-axis reflects the Propidium Iodide fluorescence. Apoptotic cells have been observed to have varying light scattering properties which must be compensated for during flow cytometry. Untreated, unlabeled cells should appear in the lower left quadrant of a log dot plot. When setting up an experiment, it is necessary to calibrate the flow cytometer to avoid spectral overlap between the two Photomultiplier Tube (PMT) channels.

CONTROLS

Three control samples should be used to calibrate the instrument prior to sample analysis. First, cells resuspended in 1X Binding Buffer only, should be assessed to evaluate the level of auto-fluorescence and to adjust the instrument accordingly. Then, treated cells should be stained separately with either TACS Annexin V-FITC or Propidium Iodide to define the boundaries of each population. Apoptotic cells labeled with TACS Annexin V-FITC only should appear in the lower half of the dot plot, with no events accumulating in the upper left or upper right quadrants. Similarly, cells labeled with Propidium Iodide alone should show no events in the upper or lower right quadrants.

TACS Annexin V-FITC is provided at 100X final concentration. The following steps should be followed as a general guide. A higher or lower final concentration of TACS Annexin V-FITC may be required. Approximately 1×10^5 - 1×10^6 cells should be processed per 100 μ L of labeling reagent.

Adherent cells may be released from their substrate using 0.25% Trypsin or 0.02-2% EDTA in PBS or HBSS. Care should be taken during trypsinization in order to prevent excessive cell damage. It can help to keep trypsinized cells in the presence of 2% BSA to prevent further damage when processing these samples. When using EDTA, it is necessary to remove all EDTA by washing twice in 1X PBS or 1X Binding Buffer prior to labeling to avoid chelating the calcium necessary for Annexin binding.

FLOW CYTOMETRY PROTOCOL *CONTINUED*

FLOW CYTOMETRY PROCEDURE

1. Collect cells by centrifugation at approximately 300 x g for 5-10 minutes at room temperature.
2. Wash cells once by resuspending them in 500 μ L of cold 1X PBS and then pelleting by centrifugation at 300 x g for 5-10 minutes at room temperature.
3. Prepare 400 μ L of 1X Binding Buffer per sample for washing cells after incubation by diluting 10X Binding Buffer (1:10 dilution) in distilled water. Keep on ice.
4. Prepare Annexin V Incubation Reagent. Per sample of 1×10^5 - 1×10^6 cells, prepare 100 μ L Annexin V Incubation Reagent by combining:

| Reaction Component | Volume |
|---|-------------|
| 10X Binding Buffer | 10 μ L |
| Propidium Iodide (optional, wear gloves) | 10 μ L |
| TACS Annexin V-FITC * | 1.0 μ L |
| Distilled water | 79 μ L |
| Total volume** (keep reagent in the dark and on ice) | 100 μ L |

*This is a starting point. Many cell samples will require less TACS Annexin V-FITC. If so, dilute the TACS Annexin V into 1X binding buffer and use the diluted material in your labeling reaction. Typically a 1:100 dilution of the TACS Annexin V-FITC is appropriate, dilutions of 1:50 up to 1:1000 may be used to optimize results.

**Prepare sufficient reagent to process all samples. This reagent is stable for at least 2 hours.

5. Gently resuspend cells in the Annexin V Incubation Reagent at a concentration of 1×10^5 - 1×10^6 cells/100 μ L. Incubate in the dark for 15 minutes at room temperature.
6. Add 400 μ L 1X Binding Buffer to samples (per 100 μ L reaction) and process by flow cytometry within one hour for maximal signal. If the number of cells is lower than the recommended 1×10^5 cells/100 μ L, wash cells once by adding 300 μ L of 1X Binding Buffer (room temperature), pellet cells at 1000 x g for 5-10 minutes, resuspend cells in 100 μ L 1X Binding Buffer and then process the samples.

DATA INTERPRETATION

The results obtained should show a distinct population of cells that have bound Annexin V (lower right quadrant of a dot or density plot). These cells are early apoptotic. Annexin V positive cells that also take up Propidium Iodide are either late apoptotic or necrotic (upper right quadrant of dot plot). There may also be a population of cells that are negative for both Annexin V and Propidium Iodide (lower left quadrant of dot plot). These are normal viable cells.

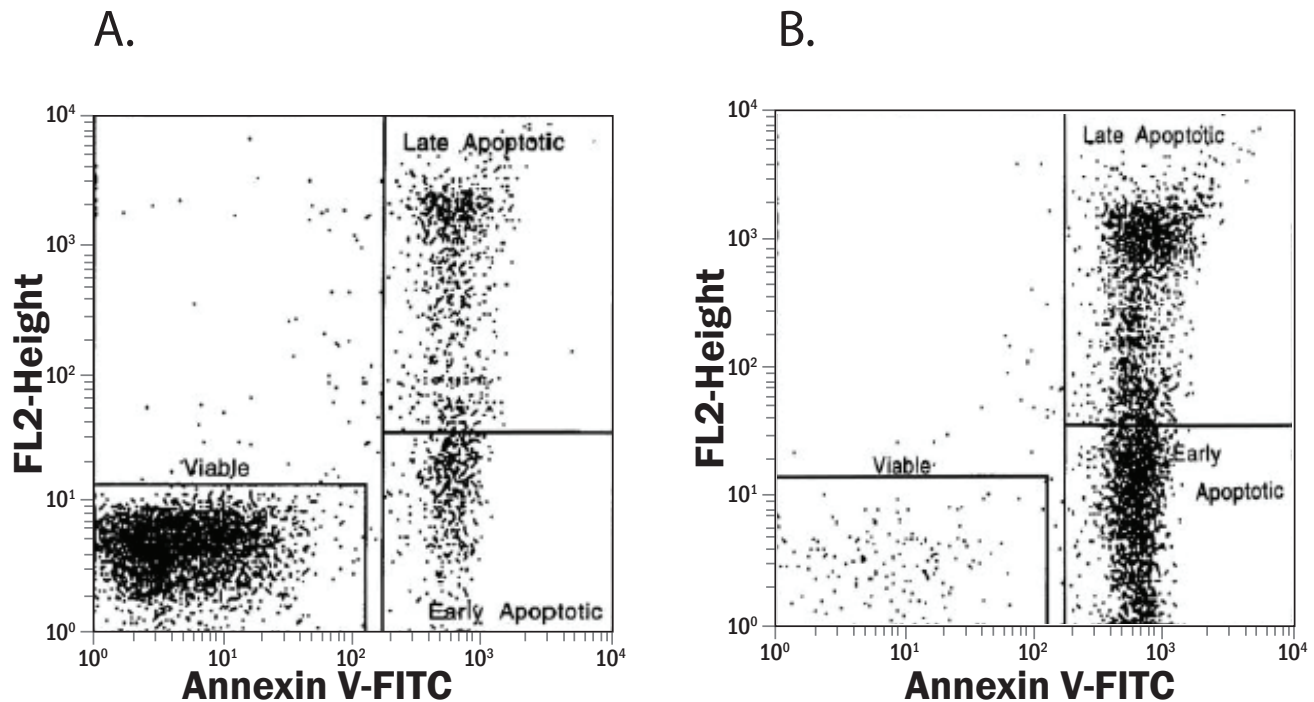


Figure 2. Analysis of Dexamethasone-Treated thymocytes using TACS Annexin V-FITC Apoptosis Detection Kit. Dot plot of untreated (A) and treated (B) thymocytes. Shown are viable, early apoptotic (Annexin V-FITC positive) and late apoptotic or necrotic cells. Cells were treated with 100 nM dexamethasone for 15.5 hours.

Analysis courtesy of Dr. C.M. Knudson, Howard Hughes Medical Institute, St. Louis, MO.

IN SITU DETECTION PROTOCOL

Suspension Cells

1. Collect cells by centrifugation at approximately 300 x g for 5-10 minutes at room temperature. Approximately 1×10^5 - 1×10^6 cells should be processed per 100 μ L of Annexin V Incubation Reagent (see step 3 below).
2. Wash cells once in cold (2-8 °C) 1X PBS by resuspending cells in 1.0 mL cold 1X PBS and then pelleting by centrifugation at 300 x g for 5-10 minutes at room temperature.
3. Prepare Annexin V Incubation Reagent. Per sample of 1×10^5 - 1×10^6 cells, prepare 100 μ L Annexin V Incubation Reagent by combining:

| Reaction Component | Volume |
|---|-------------|
| 10X Binding Buffer | 10 μ L |
| Propidium Iodide (optional, wear gloves) | 10 μ L |
| TACS Annexin V-FITC* | 1.0 μ L |
| Distilled water | 79 μ L |
| Total volume** (keep reagent in the dark and on ice) | 100 μ L |

*This is a starting point. Many cell samples will require less TACS Annexin V-FITC. If so, dilute the TACS Annexin V into 1X binding buffer and use the diluted material in your labeling reaction. Typically a 1:100 dilution of the TACS Annexin V-FITC is appropriate, dilutions of 1:50 up to 1:1000 may be used to optimize results.

**Prepare sufficient reagent to process all samples. This reagent is stable for at least 2 hours.

4. Prepare 400 μ L 1X Binding Buffer per sample for washing cells after incubation by diluting 10X Binding Buffer (1:10 dilution) in deionized or distilled water. Keep on ice.
5. Gently resuspend cells in the Annexin V Incubation Reagent prepared in step 3 at a concentration of 1×10^5 - 1×10^6 cells/100 μ L prepared. Incubate **in the dark** for 15 minutes at room temperature.
6. Collect cells by centrifugation at 300 x g for 5 minutes and discard supernatant. Wash cells once by resuspending in 500 μ L of 1X Binding Buffer at room temperature and pellet cells at 300 x g for 5-10 minutes.
7. Resuspend cells in 100 μ L of 1X Binding Buffer. Cells may be viewed by spotting on a glass microscope slide and covering with a glass coverslip for a few minutes. Before the cells completely dry, place a drop of fluorescent mounting media onto the cells, and coverslip. The mounting medium will mix with the cells to assure even distribution upon mounting.

IN SITU DETECTION PROTOCOL *CONTINUED*

Adherent Cells:

Note: Additional 10X Binding Buffer may be required for the in situ protocol for adherent cells.

1. Remove culture medium from cells, and immerse slide into cold (2-8 °C) 1X PBS. If cells are grown on chamber slides, remove media and wash by placing 300-500 µL of cold 1X PBS (per 5 cm² area) onto cells.

Note: Apoptosis cells may round up and lift off plate, therefore, cells from the supernatant should also be harvested and analyzed according to the protocol for suspension cells.

2. Prepare Annexin V Incubation Reagent. Per sample of approximately 5 cm², prepare 100 µL Annexin V Incubation Reagent

| Reaction Component | Volume |
|--|--------|
| 10X Binding Buffer | 10 µL |
| Propidium Iodide (optional, wear gloves) | 10 µL |
| TACS Annexin V-FITC* | 1.0 µL |
| Distilled water | 79 µL |
| Total volume** (keep reagent in the dark and on ice) | 100 µL |

*This is a starting point. Many cell samples will require less TACS Annexin V-FITC. If so, dilute the TACS Annexin V-FITC into 1X Binding Buffer and use the diluted material in your labeling reaction. Typically, a 1:100 dilution of the TACS Annexin V-FITC is appropriate, however dilutions of 1:50 up to 1:1000 may be used to optimize results.

**Prepare sufficient reagent to process all samples. This reagent is stable for at least 2 hours.

3. Prepare 400 µL of 1X Binding Buffer per sample for washing cells after incubation by diluting 10X Binding Buffer (1:10 dilution) in distilled water. Keep on ice.
4. Remove the PBS wash from the slide or culture dish by gently tapping the edge of the slide or culture dish on a paper towel placed on the bench top.
5. Wash cells twice for 2 minutes in excess volume of 1X Binding Buffer at room temperature. Cells may be viewed immediately by fluorescence microscopy using a fluorescent mounting medium and coverslip (if needed).
6. Wash cells twice for 2 minutes in excess volume of 1X Binding Buffer at room temperature. Cells may be viewed immediately by fluorescence microscopy using a fluorescent mounting medium and coverslip (if needed).

DATA INTERPRETATION

Cells that are apoptotic should fluoresce brightly when viewed through a fluorescein compatible filter. It should be possible to identify patches of fluorescence on the cell surface. Cells may be viewed through a dual pass filter allowing you to visualize the TACS Annexin V-FITC positive and the Propidium Iodide positive cells in the same field, however, there may be significant signal overlap between FITC and Propidium Iodide making the interpretation of results difficult. It is normal to see bright cells that have taken up Propidium Iodide as well as lightly counterstained Propidium Iodide cells. Decreasing the Propidium Iodide concentration in the labeling reaction may give better results.

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