TACS® Annexin V Kits
Apoptosis Detection by Flow Cytometry or *In Situ* labeling

Annexin V-FITC
100 tests Catalog# 4830-01-K
250 tests Catalog# 4830-250-K

Annexin V-Biotin
100 tests Catalog# 4835-01-K
250 tests Catalog# 4835-250-K

I. Background

Please read the *Instructions for Use* prior to using this kit. 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. Annexin V allows identification of cell surface changes that occur early during the apoptotic process either by flow cytometry or *in situ* detection. The Annexin V-FITC conjugate facilitates rapid fluorometric quantitation of apoptotic cells. The Annexin V-Biotin conjugate offers flexibility in labeling by allowing the use of streptavidinconjugated fluorophores other than FITC for detection. 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 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, reversible, but very tight, with a Kd of approximately 5 x 10-10 M. At low PS concentration, a binding ratio of 8 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. TACS® Annexin V-conjugates are supplied with an optimized binding buffer and propidium iodide. Cells are harvested and washed, then incubated with an Annexin V conjugate in binding buffer, for 15 minutes at room temperature prior to direct analysis. Alternatively, a secondary streptavidin conjugate can be used for detection. 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. TACS® Annexin V-Biotin is provided at 100X concentration with a 1:2.5 molar ratio of Annexin V to biotin. The binding of Annexin V will vary depending upon cell type and assay method, therefore, the concentration of Annexin V used may require optimization. Annexin V conjugates may also be used for *in situ* detection of apoptosis. Cells may be visualized *in situ*

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directly, after incubation with Annexin V-conjugates and propidium iodide, by fluorescence microscopy.

II. Precautions and Limitations

1. For Research Use Only. Not for use in diagnostic procedures.
2. Propidium iodide is toxic. Wear gloves and exercise caution when using.
3. Annexin V-FITC and propidium iodide are light sensitive and should be kept in the dark as much as possible during labeling and handling. Keep tubes covered with aluminum foil or place in a drawer during incubation steps. Keep cells in the dark after labeling, and keep the room dark during microscopy. Brief exposure to light (<30 seconds) during pipetting is acceptable.
4. The physical, chemical and toxicological properties of these products may not yet have been fully investigated therefore Trevigen recommends the use of gloves, lab coats and eye protection while using these chemical reagents. Trevigen assumes no liability for damage resulting from handling or contact with these products.

III. Materials Supplied

Component	Quantity	Storage	Catalog #
4830-01-K			
TACS® Annexin V-FITC	100 µl	4 oC	4830-01-1
10X Binding Buffer	8 ml	4 oC	4830-01-2
Propidium Iodide	1 ml	4 oC	4830-01-3
4835-01-K			
TACS® Annexin V-Biotin*	100 µl	4 oC	4835-01-1
10X Binding Buffer	8 ml	4 oC	4830-01-2
Propidium Iodide	1 ml	4 oC	4830-01-3
4830-250-K			
TACS® Annexin V-FITC	250 µl	4 oC	4830-250-1
10X Binding Buffer	20 ml	4 oC	4830-250-2
Propidium Iodide	2.5 ml	4 oC	4830-250-3
4835-250-K			
TACS® Annexin V-Biotin*	250 µl	4 oC	4835-250-1
10X Binding Buffer	20 ml	4 oC	4835-250-2
Propidium Iodide	2.5 ml	4 oC	4835-250-3

* The separate purchase of a fluorescent streptavidin-conjugate is required for detection when using Annexin V-Biotin for enhanced sensitivity.

IV. Materials/Equipment Required But Not Supplied Equipment

1. Fluorescence Microscope or Flow Cytometer (Capable of FITC and Propidium Iodide Detection)
2. Microcentrifuge
3. Adjustable Pipettors (100-1000 µl, 10-200 µl, and 1-20 µl)
4. Ice Bucket and Ice

Reagents and Disposables

1. 10X PBS Buffer (Cat# 4870-500-6)
2. Microcentrifuge Tubes
3. Aluminum Foil
4. Gloves

5. Fluorescence Compatible Mounting Media (*In Situ* Protocol Only) (Cat # 4866-20)
6. Glass Coverslips (*In Situ* Protocol Only) (Cat# 4862-10)
7. Glass Microscope Slides (*In Situ* Protocol Only) (Cat# 4861-72)
8. Streptavidin-conjugate (for Annexin V-Biotin only, e.g. Strep-Fluorescein cat# 4800-30 14)

V. **Assay Protocols**

Before you get started, note that phosphatidylserine flipping is an early event in apoptosis. This phenomenon may precede DNA fragmentation by several hours, therefore, when analyzing cells using this method, early time points following treatment should be investigated. Different cell types vary in their phosphatidylserine content and, similarly, in the amount of exposure on the cell surface after apoptosis is initiated. The following protocol is a guideline for getting started, however, it may be necessary to adjust the concentration of the Annexin V conjugate. Typically a 1 to 100 dilution of the Annexin V conjugate is appropriate, however, dilutions 1:50 up to 1:1000 may be needed. Cells should not be fixed **prior** to incubating with an Annexin V conjugate. (Refer to Appendix B. *Sample fixation*)

A. Flow Cytometry Protocol:

It is recommended that the X axis of the dot plot reflects the log of the Annexin VFITC 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:
Before proceeding, 3 control samples should be used to calibrate the instrument. First, cells resuspended in binding buffer only, should be assessed to evaluate the level of autofluorescence and to adjust the instrument accordingly. Then, treated cells should be stained separately with Annexin V-FITC and Propidium Iodide to define the boundaries of each population. Apoptotic cells labeled with Annexin V4 FITC 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. 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 Annexin V conjugate may be required. Approximately 105 to 106 cells should be processed per 100 µl of labeling reagent (see below). Adherent cells may be released from their substrate using 0.25% trypsin or 0.02 to 2% EDTA in PBS or HBSS. Care should be taken when trypsinizing 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.

a. Flow cytometry procedure
1. Collect cells by centrifugation at approximately 300 x g for 5 to 10 minutes at room temperature.
2. Wash cells once in cold (4 oC) 1X PBS buffer by resuspending cells in 500 µl cold PBS and then pelleting by centrifugation as in step 1.

3. Prepare 400 µl 1X Binding Buffer per sample for washing cells after incubation by diluting 10X Binding Buffer (1:10 dilution) in deionized distilled H2O. Keep on ice.
4. Prepare 100 µl Annexin V Incubation Reagent by combining per sample of 105 to 106 cells:
10 µl 10X Binding Buffer
10 µl Propidium Iodide (OPTIONAL, wear gloves)
1 µl Annexin V-FITC or -biotin*****
79 µl Deionized, Distilled H2O
100 µl Total****** (*KEEP REAGENT IN THE DARK AND ON ICE*)

*This is a starting point. Many cell samples will require less Annexin V-FITC or conjugate. If so, dilute the Annexin V into 1X binding buffer and use the diluted material in your labeling reaction. Typically a 1 to 100 dilution of the Annexin V-FITC or -biotin is appropriate, however dilutions of 1 to 50 up to 1 to 1000 may be used to optimize results. **Prepare sufficient reagent to process all your samples. This reagent is stable for at least 2 hours.
5. Gently resuspend cells in the Annexin V Incubation Reagent prepared in step
4 at a concentration of 105 to 106 cells per 100 µl prepared. Incubate in the dark for 15 minutes at room temperature.
6. **Annexin V-FITC**: Proceed to step 7. **Annexin V-Biotin**: Add 400 µl of 1X Binding Buffer to each sample, collect cells by centrifugation as above (step 1). Resuspend cells in 100 µl 1X binding buffer containing fluorescent Streptavidin conjugate of your choice (follow manufacturer's recommendations
for the concentration to use). Incubate for 15 minutes at room temperature in the dark.
7. Add 400 µl 1X Binding Buffer to samples (per 100 µl reaction) and process by flow cytometry within one hour for maximal signal. If the number of cells is lower than the recommended 105 cells per 100 µl, wash cells once by adding 300 µl of 1X binding buffer (room temperature), pellet cells at 1000 x g for 5 to 10 minutes, and resuspend cells in 100 µl 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.

B. In Situ Detection (Annexin V-FITC and Annexin V-Biotin)*:*
a. Suspension Cells. Note: *Annexin V-Biotin colorimetric detection using* ***Alkaline Phosphatase*** *should not be used on suspension cells:*
1. Collect cells by centrifugation at approximately 300 x g for 5 to 10 minutes at room temperature. Approximately 105 to 106 cells should be processed per 100 µl of Annexin V Incubation Reagent (see step 3 below).
2. Wash cells once in cold (4 oC) 1X PBS buffer by resuspending cells in 1 ml cold PBS and then pelleting by centrifugation as in step 1.
3. Per sample of 105 to 106 cells, prepare 100 µl Annexin V Incubation Reagent by combining:
10 µl 10X Binding Buffer
10 µl Propidium Iodide (OPTIONAL, wear gloves)

1 µl Annexin V-conjugate*****
79 µl Deionized, Distilled H2O
100 µl Total****** (*KEEP REAGENT IN THE DARK AND ON ICE*)

*This is a starting point. Many cell samples will require less Annexin V. If so, dilute the Annexin V-conjugate into 1X binding buffer and use the diluted material in your labeling reaction. Typically a 1 to 100 dilution of the Annexin Vconjugate is appropriate, however dilutions of 1 to 50 up to 1 to 1000 may be used to optimize results.
**Prepare sufficient reagent to process all your samples. This reagent is stable for at least 2 hours. Also prepare 400 µl 1X Binding Buffer per sample for washing cells after incubation by diluting 10X Binding Buffer (1:10 dilution) in deionized or distilled H2O. Keep on ice.
4. Gently resuspend cells in the Annexin V Incubation Reagent prepared in step 3 at a concentration of 105 to 106 cells per 100 µl prepared. Incubate in the dark for 15 minutes at room temperature.

5. **Annexin V-FITC**: Proceed to step 6. **Annexin V-Biotin**: Add 400 µl of 1X Binding Buffer to each sample, collect cells by centrifugation as above (step 1). Resuspend cells in 100 µl of 1X Binding Buffer containing a fluorescent Streptavidin conjugate of your choice (follow manufacturer's recommendations for concentration use). Incubate for 15 minutes at room temperature in the dark.
6. Collect cells by centrifugation at 300 x g for 5 minutes and discard supernatant. Wash cells once by resuspending in 500 µl of 1X Binding Buffer (room temperature) and pellet cells at 300 x g for 5 to 10 minutes.
7. Resuspend cells in 100 µl 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.
b. Adherent Cells:
Note: 10X Binding Buffer is also available in 100 ml volumes (Cat# 4830-100-
2) to prepare the 1X buffer required for the *in situ* protocol for adherent cells.
1. Remove culture medium from cells, and immerse slide into cold (4 oC) 1X PBS. If cells are grown on chamber slides, remove media and wash by placing 300 to 500 µl of cold 1X PBS (per 5 cm2 area) onto cells. Note: apoptotic 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 100 µl Annexin V Incubation Reagent per sample of approximately 5 cm2:
10 µl 10X Binding Buffer
10 µl Propidium Iodide (OPTIONAL)
1 µl Annexin V Conjugate*
79 µl Deionized, Distilled H2O
100 µl Total** (KEEP REAGENT IN THE DARK AND ON ICE)

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

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

3. 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.
4. Carefully wipe the slide around the sample with a lab wipe. Place 100 µl of Annexin V Incubation Reagent onto the sample. You may need to gently spread the reagent with the side of a pipet tip to completely cover the sample. Incubate the slide in the dark for 15 minutes at room temperature.
5. **Annexin V-FITC**: Proceed to step 6. **Annexin V-Biotin**: Wash cells by covering sample with an excess of 1X Binding Buffer or by immersing slides in 50 ml of buffer. Place 100 µl of 1X Binding Buffer containing fluorescent or alkaline phosphatase streptavidin conjugate onto sample (see manufacturer's recommendations for concentration to use). Incubate for 15 minutes at room temperature in the dark.
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).

c. Tissues for Annexin V- Biotin ONLY:
Apoptosis of cells in tissues, which are either cultured or freshly isolated can be detected using Annexin V-Biotin. Both early and late apoptotic cells are detected with this method:
1. Immerse the tissue in a small volume of culture medium or 1X Binding Buffer.

Note: If you are using culture medium, the Ca2+ level should be 1.5 mM or more. Avoid using IMDM.
2. Add 10 µl of Annexin V-Biotin stock solution to 100 µl of medium, and incubate for 30 minutes at room temperature or 37 oC.
3. Fix the tissue in formaldehyde and embed in paraffin using standard protocols.
4. Cut thin sections and visualize cell-bound Annexin V-Biotin in the section by performing standard procedures to stain for biotin-labeled compounds in tissue sections (deparaffinize, block, etc.).

NOTE: The boundaries of the tissue may bind Annexin V-Biotin because of damage to the viable cells at these edges during tissue isolation. If you wish to quantitate, use radiolabeled streptavidin. This will provide semiquantitative information as to the extent of apoptosis in a certain section (not the number of apoptotic cells.

C. In Situ Analysis
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 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.

VI. References
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