SIGMA

ProductInformation

Sigma TACS™ Annexin V Apoptosis Detection Kits

Instructions for Use

APO-AB	Annexin V-Biotin Apoptosis Detection Kit	100 tests

For Research Use Only. Not for use in diagnostic procedures.

Sigma TACS™ Annexin V Apoptosis Detection Kits

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I. PRINCIPLE OF THE ASSAY

Sigma TACS™ Annexin V Apoptosis Detection kit uses an Annexin V conjugate for flow cytometry or *in situ* detection to identify cell surface changes that occur early in the apoptotic process. This kit may be used to identify early apoptosis, preceding DNA fragmentation and membrane disruption. The Annexin V-Biotin offers flexibility in labeling and detection by allowing the use of streptavidin conjugated to fluorophores other than FITC.

Annexin V is an anticoagulant protein that preferentially binds negatively charged phospholipids. Early in the apoptotic process, phospholipid asymmetry is disrupted leading to the exposure of phosphatidylserine on the outer leaflet of the cytoplasmic membrane. This is thought to be important in macrophage recognition of cells undergoing apoptosis (1,2). Annexin V binding to phosphatidylserine is calcium dependent, reversible, and occurs with a k_d of approximately 5×10^{-10} M (2). It is estimated that approximately 50 exposed phospholipid monomers are bound per Annexin V molecule (3).

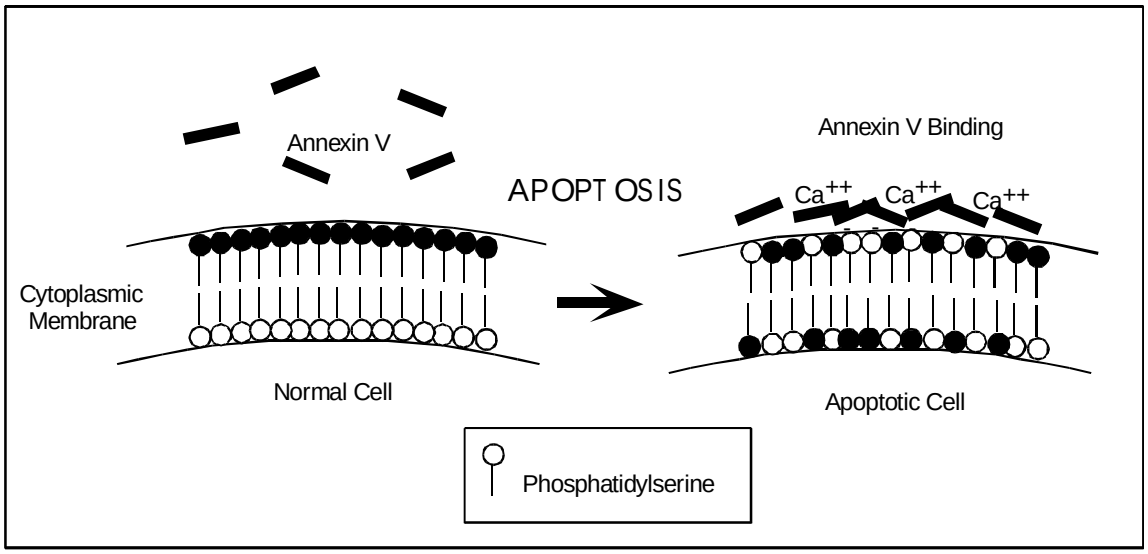

Figure 1: Schematic representation of phospholipid flipping during apoptosis and subsequent binding of Annexin V to the cell surface.

Sigma's Annexin V kit include Annexin V conjugated biotin, an optimized binding buffer and propidium iodide, allowing for a simple, three step procedure. The cells are first harvested and washed. They are then incubated for 15 minutes with the conjugated Annexin V and the propidium iodide (optional), and analyzed by flow cytometry. For *in situ* analysis the sample is prepared, washed, and then incubated with conjugated Annexin V and propidium iodide. The cells are washed again and analyzed by fluorescence microscopy. The combination of conjugated Annexin V and propidium iodide allows for the differentiation between early apoptotic cells (Annexin V positive), late apoptotic and/or necrotic cells (Annexin V and propidium iodide positive) and viable cells (unstained).

II. REAGENTS AND MATERIALS

Reagents Provided

Annexin V-Biotin (50 µg/mL)	100 µL
10X Binding Buffer	5 mL
Propidium Iodide (50 µg/mL)	1 mL

Store components at 4°C in the dark

Materials Required But Not Provided:

- 10X and 1X Phosphate-Buffered Saline (PBS)
- Aluminum foil
- Microcentrifuge tubes
- Streptavidin-fluorophore conjugates (for customizing Annexin V-Biotin)
- Fluorescence compatible mounting medium (*in situ* protocol only)
- Glass coverslips (*in situ* protocol only)
- Glass microscope slides (*in situ* protocol only)
- 50 mL tubes or Coplin jars (*in situ*, adherent cell protocol only)

Equipment Required:

- Flow cytometer or fluorescence microscope
- Microcentrifuge
- Adjustable pipettors (1-20 µL, 20-200 µL, and 200 - 1000 µL)

III. HINTS TO OPTIMIZE ACCURACY

Read this section before proceeding.

1. Phosphatidylserine flipping is an early event in apoptosis. This phenomenon may precede DNA fragmentation by several hours. When analyzing cells using this method, early time points following treatment should be investigated.
2. Cell types vary in their phosphatidylserine content, and in the amount of phosphatidylserine exposure on the cell surface after apoptosis is initiated. The following protocol is a guideline, so it may be necessary to adjust the concentration of the Annexin V conjugate. An Annexin V conjugate working concentration of 0.5 µg/mL is usually appropriate, however a concentration in the range of 0.1 to 1.0 µg/mL may be optimal.
3. Propidium iodide is light sensitive and should be kept in the dark as much as possible. Cover tubes with aluminum foil or place in a light-restrictive drawer during incubation. Keep cells in the

dark after labeling, and keep room dark during microscopy. Brief exposure to light (<30 seconds) during pipetting is acceptable.

4. Cells **must not** be fixed prior to incubation with Annexin V conjugates. Cells may be fixed following the incubation step, however, a decrease in fluorescence intensity may be observed. Fix the cells in the presence of $CaCl_2$ (1.8 mM) to prevent the Annexin V from being stripped from the cells. Use a fixative prepared in 1X binding buffer, which already contains calcium. The fixation procedure may be optimized for your system by increasing the $CaCl_2$ in 10X Binding Buffer to 25 mM (2.5 mM final).
5. Adherent cells may be released from their substrate by using trypsin. Trypsinized cells may be processed as described in the flow cytometry protocol, however, background staining may be observed. Keep trypsinized cells in the presence of 2% BSA to prevent further damage during processing.
6. If you do not have the capability to accurately pipette 1 µL of the Annexin V conjugates, you may dilute in sterile 1X PBS, pH 7.4 and use a larger volume. 1X PBS should be cooled to 2°-8°C.

Note: Dilute only the amount of Annexin V conjugate needed for immediate use.

7. Propidium iodide is toxic. Wear gloves and exercise caution. Use appropriate disposal procedures.
8. Deionized, distilled water is equivalent to water that is 18 mΩ (e.g., purified over Milli-Q system).
9. If using 50 mL tubes to wash slides (*in situ* protocol), a maximum of 2 slides can be washed per tube. The slides must have the specimens facing away from each other by placing them back to back.
10. If you find you do not have sufficient 10X Binding Buffer, prepare more using the formulation on page 10.

IV. PROCEDURES

A. Flow Cytometry Protocol (Annexin V-Biotin):

The Annexin V conjugate is provided at a concentration of 50 µg/mL, or 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. Process approximately 10^5 to 10^6 cells per 100 µL of Annexin V Incubation Reagent (see below).

1. Prepare buffers:
   a. Prepare 100 µL Annexin V Incubation Reagent for each sample of 10^5 to 10^6 cells as shown below:

10X Binding Buffer	10 µL
Propidium Iodide (optional)	10 µL
Annexin V conjugate	1 µL*
Deionized, distilled H_2O	79 µL
Total	100 µL**

Note: Keep reagent in the dark and on ice

b. Prepare 500 µL 1X Binding Buffer per sample** by diluting 50 µL of 10X Binding Buffer (1:10 dilution) in 450 µL deionized, distilled water. Keep on ice.

**This is a starting point. Many cell samples will require less Annexin V conjugate. If you find that you require less Annexin V conjugate, you may dilute the Annexin V Incubation Reagent into 1X PBS buffer and then use the diluted material in your labeling reaction. A range of 0.10 µg (1:50 dilution) to 0.01 µg (1:500 dilution) Annexin V conjugate per 100 µL reaction may be tried to optimize results.*

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

2. Collect cells by centrifugation at approximately 1000 x g for 5 to 10 minutes at room temperature (18°-24°C). See hints section, page 5, number 5 if using adherent cells that require trypsinization.
3. Wash cells by resuspending in 500 µL cold (2°-8°C) 1X PBS and then pelleting by centrifugation as in step 2.
4. Gently resuspend cells in the Annexin V Incubation Reagent prepared in step 1a, at a concentration of 10^5 to 10^6 cells per 100 µL. Incubate in the dark for 15 minutes at room temperature (18°-24°C).
5. Collect cells by centrifugation as in step 2. Resuspend cells in 100 µL 1X Binding Buffer (prepared in step 1b) that contains the fluorescent streptavidin conjugate of your choice (follow manufacturer's recommendations for concentration to use). Incubate for 15 minutes at room temperature (18°-24°C) in the dark. Proceed with step 6.
6. If the number of cells is lower than the recommended 10^5 cells per 100 µL, wash cells once by adding 300 µL of 1X Binding Buffer, pellet cells at 1000 x g for 5 to 10 minutes, resuspend cells in 100 µL 1X Binding Buffer and then process the samples. If the cells are within the recommended range of 10^5 to 10^6, add 400 µL 1X Binding Buffer (prepared in step 1b) to each sample.
7. Process by flow cytometry within one hour for maximal signal.

B. *in situ* Detection Protocols (Annexin V-Biotin):

Suspension Cells

The Annexin V conjugate is provided at a concentration of 50 µg/mL, or 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. Process approximately 10^5 to 10^6 cells per 100 µL of Annexin V Incubation Reagent as shown:

1. Prepare buffers:
   a. Prepare 100 µL Annexin V Incubation Reagent for each sample of 10^5 to 10^6cells as shown below:

10X Binding Buffer	10 µL
Propidium Iodide (optional)	10 µL
Annexin V conjugate	1 µL*
Deionized, distilled H_2O	79 µL
Total	100 µL**

Keep reagent in the dark and on ice.

b. Prepare 500 µL 1X Binding Buffer per sample** by diluting 50 µL 10X Binding Buffer (1:10 dilution) in 450 µL deionized distilled water. Keep on ice.

**This is a starting point. Many cell samples will require less Annexin V conjugate. If you find that you require less Annexin V conjugate, you may dilute the Annexin V Incubation Reagent into 1X PBS buffer and then use the diluted material in your labeling reaction. A range of 0.10 µg (1:50 dilution) to 0.01 µg (1:500 dilution) Annexin V conjugate per 100 µL reaction may be tried to optimize results.*

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

2. Collect cells by centrifugation at approximately 1000 x g for 5 to 10 minutes at room temperature (18°-24°C).
3. Wash cells by resuspending in 500 µL cold (2°-8°C) 1X PBS and then pelleting by centrifugation as in step 2.
4. Gently resuspend cells in the Annexin V Incubation Reagent (prepared in step 1a) at a concentration of 10^5 to 10^6 cells per 100 µL. Incubate in the dark for 15 minutes at room temperature (18°-24°C).
5. Collect cells by centrifugation as in step 2. Resuspend cells in 100 µL 1X Binding Buffer (prepared in step 1b.) that contains the fluorescent streptavidin conjugate of your choice (follow manufacturer's recommendations for concentration to use). Incubate for 15 minutes at room temperature (18°-24°C) in the dark. Proceed with step 6.
6. Collect cells by centrifugation at 1000 x g for 5 minutes at room temperature (18°-24°C). Wash cells once by resuspending in 300 µL of 1X Binding Buffer (prepared in step 1b) and then centrifuging again. Resuspend cells in 100 µL of 1X Binding Buffer.
7. Cells may be viewed immediately by fluorescence microscopy by placing 25 µL of the resuspended cells onto a glass microscope slide and covering with a glass coverslip. Alternatively, place 25 µL of cells onto a glass microscope slide and allow to dry for a few minutes. *BEFORE* the cells are completely dry, place a drop of fluorescence compatible mounting medium and coverslip onto the cells. The mounting medium will mix the cells to assure even distribution.

Adherent Cells:

Note: See Appendix for 10 X Binding Buffer formulation to prepare the wash volumes required for this protocol.

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 µL to 500 µL of cold 1X PBS (per 5 cm^2 area) onto cells.
2. Prepare 100 µL Annexin V Incubation Reagent per sample of approximately 5 cm^2:

10X Binding Buffer	10 µL
Propidium Iodide (optional)	10 µL
Annexin V conjugate	1 µL*
Deionized, distilled H_2O	79 µL
Total	100 µL**

Note: Keep reagent in the dark and on ice

**This is a starting point. Many cell samples will require less Annexin V conjugate. If you find that you require less Annexin V conjugate, you may dilute the Annexin V Incubation Reagent into 1X PBS buffer and then use the diluted material in your labeling reaction. A range of 0.10 µg (1:50 dilution) to 0.01 µg (1:500 dilution) Annexin V conjugate per 100 µL reaction 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 1X PBS wash from the slide and gently blot around the edges of the sample using a lab wipe.
4. Place 100 µL of Annexin V Incubation Reagent onto the sample. You may need to gently spread the reagent with the side of a pipette tip in order to completely cover the sample. Incubate the slide for 15 minutes at room temperature (18°-24°C) in the dark.
5. Wash cells in 50 mL 1X Binding Buffer. Remove slide(s) from buffer and gently blot around the edges of the sample using a lab wipe. Place 100 µL 1X Binding Buffer containing the fluorescent streptavidin conjugate of your choice onto the sample (follow manufacturer's recommendations for concentration to use). Incubate for 15 minutes at room temperature temperature (18°-24°C) in the dark.
6. Wash cells twice for 2 minutes each wash in 50 mL 1X Binding Buffer.
7. Place a drop of fluorescence compatible mounting medium and coverslip onto the sample.
8. View cells immediately by fluorescence microscopy.

Analysis of *in situ* results
Cells that are apoptotic should fluoresce brightly when viewed through a fluorescein compatible filter (Annexin V-Biotin with fluorophore). 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 both the Annexin V-conjugate positive and the propidium iodide positive cells in the same field, however there may be significant signal overlap between fluorophore and propidium iodide making the interpretation of results difficult. It is normal to see bright propidium iodide cells, as well as lightly counterstained propidium iodide cells. Decreasing the propidium iodide concentration in the labeling reaction may give better results in some cells. For this procedure, a time course experiment is the best way to help distinguish between apoptosis and necrosis.

V. REFERENCES

1. Fadock *et al., The Journal of Immunology,* **148:**2207 (1992).
2. Tait and Gibson, *Archives of Biochemistry and Biophysics.,* **298**:187 (1992).
3. Meers and Mealy, *Biochemistry,* **32**:11711 (1993).
4. Koopman *et al., Blood.* **84**:1415 (1994).
5. Marin *et al., Journal of Experimental Medicine,* **182:**1545 (1995).
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7. Kaetzel and Dedman, *NIPS,* **10:**171 (1993).

VI. APPENDIX

10X Annexin V Binding Buffer:

	For 100 mL of 10X Binding Buffer
100 mM HEPES pH 7.4 (pH with NaOH)	10 mL of 1M HEPES/NaOH, pH 7.4
1.5 M NaCl	30 mL of 5 M NaCl
50 mM KCl	5 mL of 1 M KCl
10 mM $MgCl_2$	1 mL of 1 M $MgCl_2$
18 mM $CaCl_2$	1.8 mL of 1 M $CaCl_2$
	52.2 mL deionized, distilled H_2O
	100 mL

The 10X Binding Buffer should be filter sterilized (0.22 µM filter) and stored at 2°-8°C for a stability of at least 2 months. If dilution is required, use deionized, distilled water.

VII. WARNINGS

Refer to MSDS for hazard information.

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