TACS™ Annexin V-FITC

Apoptosis Detection Kits

Catalog Number: TA4638 - 100 tests

Catalog Number: TA5532 - 250 tests

This package insert must be read in its entirety before using this product.

FOR RESEARCH USE ONLY.
NOT FOR USE IN DIAGNOSTIC PROCEDURES.

TABLE OF CONTENTS

DISTRIBUTED BY:

R&D Systems, Inc.
614 McKinley Place N.E.
Minneapolis, MN 55413
United States of America

TELEPHONE: (800) 343-7475
(612) 379-2956
FAX: (612) 379-6580
E-MAIL: info@RnDSystems.com

R&D Systems Europe
19 Barton Lane
Abingdon Science Park
Abingdon, Oxon OX14 3NB
United Kingdom

TELEPHONE: (0)1235 529449
FAX: (0)1235 533420
E-MAIL: info@RnDSystems.co.uk

R&D Systems GmbH
Borsigstrasse 7
65205 Wiesbaden-Nordenstadt
Germany

FREEPHONE: (0)800 909 4455
TELEPHONE: (0)6122 90980
FAX: (0)6122 909819
E-MAIL: infogmbh@RnDSystems.co.uk

PRINCIPLE OF THE ASSAY

TACS™ Annexin V-FITC Apoptosis Detection kits use Annexin V-FITC conjugates for flow cytometry or *in situ* detection of cell surface changes that occur early in the apoptotic process. The Annexin V-FITC conjugate facilitates rapid fluorimetric detection of apoptotic cells. 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 (PS) on the outer leaflet of the cytoplasmic membrane. This event is thought to be important for macrophage recognition of cells undergoing apoptosis. Annexin V binding to PS is calcium dependent, reversible, and occurs with a kd of approximately 5×10^{-10} M. It is estimated that approximately 50 exposed phospholipid monomers bind per Annexin V molecule.

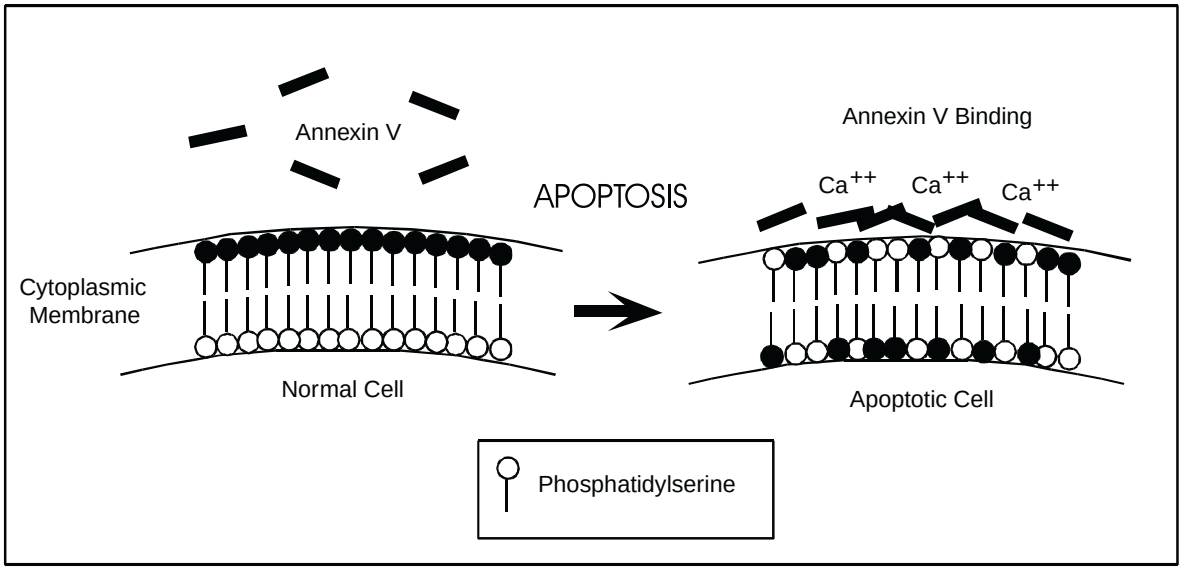

Figure 1: Schematic representation of phospholipid flipping during apoptosis and subsequent binding of Annexin V to the cell surface.

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

REAGENTS PROVIDED

Store components at 2 - 8° C in the dark.

Product Code	Description	Size
TA4638	**Annexin V-FITC**	**100 Tests**
4830-01-01	Annexin V-FITC (25 µg/mL)	100 µL
4830-01-02	10X Binding Buffer	5 mL
4830-01-03	Propidium Iodide (50 µg/mL)	1 mL
TA5532	**Annexin V-FITC**	**250 Tests**
4830-250-01	Annexin V-FITC (25 µg/mL)	250 µL
4830-250-02	10X Binding Buffer	20 mL
4830-250-03	Propidium Iodide (50 µg/mL)	2.5 mL

MATERIALS REQUIRED BUT NOT PROVIDED

- 10X and 1X Phosphate-Buffered Saline (PBS) containing calcium
- Aluminum foil
- Microcentrifuge tubes
- 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)
- Flow cytometer or fluorescence microscope
- Microcentrifuge
- Adjustable pipettors (1 - 20 µL, 20 - 200 µL, and 200 - 1000 µL)

HINTS TO OPTIMIZE ACCURACY

1. PS 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 induction of apoptosis should be investigated.
2. Cell types vary in their PS content, and in the amount of PS exposure on the cell surface after apoptosis is initiated. The following protocol is a guideline. It may be necessary to adjust the concentration of Annexin V-FITC. An Annexin V-FITC working concentration of 0.25 µg/mL is usually appropriate, however, a concentration in the range of 0.05 - 0.5 µg/mL may be optimal.
3. Annexin V-FITC and propidium iodide are light sensitive and should be kept in the dark. 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 or following incubation with Annexin V-FITC. Fixation is not recommended for cells labeled with Annexin V-FITC as the fluorescence may be quenched.
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-FITC, you may dilute it in sterile 1X Binding Buffer, pH 7.4 and use a larger volume. 1X Binding Buffer should be cooled to 2 - 8° C.

   Note: *Dilute only the amount of Annexin V-FITC needed for immediate use.*
7. Use deionized, distilled water.
8. 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.
9. If you do not have a sufficient amount of 10X Binding Buffer, prepare more using the formulation on page 9.

Caution: *Propidium iodide is toxic. Wear gloves and exercise caution. Use appropriate disposal procedures.*

PROCEDURES

Flow Cytometry Protocol

Annexin V-FITC is provided at a concentration of 25 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-FITC may be required. Process approximately 1 x 10^5 - 1 x 10^6 cells/100 µL of Annexin V Incubation Reagent (see below).

Note: *See Appendix (page 9) for 10X Binding Buffer formulation to prepare the wash volumes required for this protocol.*

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

10X Binding Buffer	10 µL
Propidium Iodide (optional)	10 µL
Annexin V-FITC	1 µL*
dH_2O	79 µL
Total volume	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 dH_2O. Keep on ice.

   **This is a starting point. Many cell samples will require less Annexin V-FITC. If you find that you require less Annexin V-FITC, 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 0.05 µg (1:50 dilution) to 0.005 µg (1:500 dilution) Annexin V-FITC 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 500 x g for 5 - 10 minutes at room temperature (18 - 24° C). Refer to page 4, 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 pellet by centrifugation as in step 2.
4. Gently resuspend cells in the Annexin V Incubation Reagent prepared in step 1a, at a concentration of 1 x 10^5 - 1 x 10^6 cells/100 µL. Incubate in the dark for 15 minutes at room temperature.
5. If the number of cells is lower than the recommended 1 x 10^5 cells/100 µL, wash cells once by adding 300 µL of 1X Binding Buffer, pellet cells at 500 x g for 5 - 10 minutes, resuspend cells in 100 µL 1X Binding Buffer and then process the samples. If the cells are within the recommended range of 1 x 10^5 - 1 x 10^6, add 400 µL 1X Binding Buffer (prepared in step 1b) to each sample.
6. Analyze samples by flow cytometry within one hour for maximal signal.

Analysis of Flow Cytometry Results

- The X-axis (FL1) of the dot plot should reflect log Annexin V-FITC fluorescence and the Y-axis (FL2) should reflect the propidium iodide fluorescence.
- Apoptotic cells have been observed to have varying light scattering properties that 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, calibrate the flow cytometer to avoid spectral overlap between the two photomultiplier tube (PMT) channels. 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 V-FITC should appear in the lower right quadrant of the dot plot, with no events accumulating in the upper left or upper right quadrants.

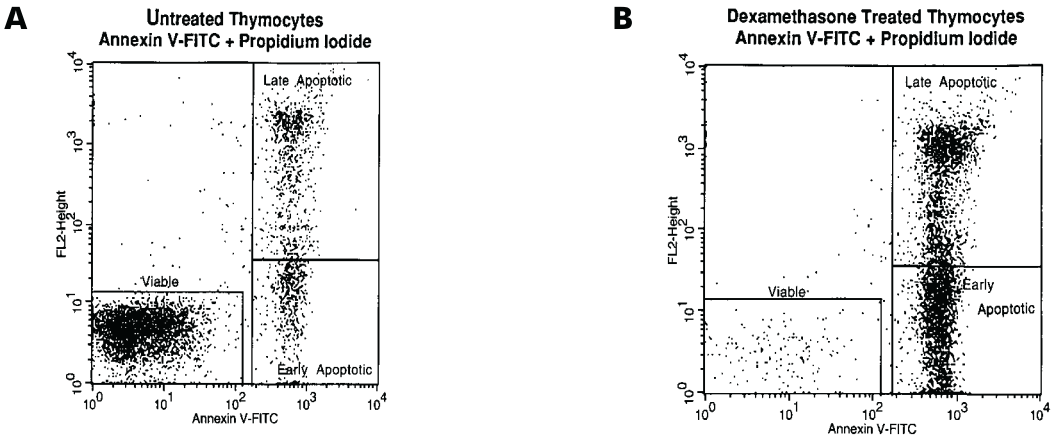

Figure 2: Analysis of dexamethasone treated thymocytes using Annexin V-FITC and propidium iodide. Dot plot of untreated (A) and treated (B) thymocytes. Figure shows viable, early apoptotic (Annexin V-FITC positive), and late apoptotic or necrotic cells. Treatment of cells was with 100 nM dexamethasone, for 15.5 hours. Analysis courtesy of Dr. C.M. Knudson, Howard Hughes Medical Institute, St. Louis, MO.

The results obtained with apoptotic cells should show a distinct population of cells that have bound Annexin V (lower right quadrant of dot plot). These are early apoptotic cells. 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.

In Situ Detection Protocols

Suspension Cells

Annexin V-FITC is provided at a concentration of 25 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-FITC may be required. Process approximately 1 x 10^5 - 1 x 10^6 cells/100 µL of Annexin V Incubation Reagent (see below).

Note: *See Appendix (page 9) for 10X Binding Buffer formulation to prepare the wash volumes required for this protocol.*

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

10X Binding Buffer	10 µL
Propidium Iodide (optional)	10 µL
Annexin V-FITC	1 µL*
dH_2O	79 µL
Total Volume	100 µL

   Note: *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 dH_2O. Keep on ice.

   **This is a starting point. Many cell samples will require less Annexin V-FITC. If you find that you require less Annexin V-FITC, 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 0.05 µg (1:50 dilution) to 0.005 µg (1:500 dilution) Annexin V-FITC 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 500 x g for 5 - 10 minutes at room temperature.
3. Wash cells by resuspending in 500 µL cold (2 - 8° C) 1X PBS and then pellet by centrifugation as in step 2.
4. Gently resuspend cells in the Annexin V Incubation Reagent (prepared in step 1a) at a concentration of 1 x 10^5 - 1 x 10^6 cells/100 µL. Incubate in the dark for 15 minutes at room temperature.
5. Collect cells by centrifugation as in step 2. Wash cells once by resuspending in 300 µL of 1X Binding Buffer (prepared in step 1b) then centrifuging again. Resuspend cells in 100 µL of 1X Binding Buffer.
6. 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 a coverslip onto the cells. The mounting medium will mix the cells to assure even distribution.

Adherent Cells

Note: *See Appendix (page 9) for 10X 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 - 500 µL of cold 1X PBS (per 5 cm^2) 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-FITC	1 µL*
dH_2O	79 µL
Total volume	100 µL

Note: *Keep reagent in the dark and on ice*.

**This is a starting point. Many cell samples will require less Annexin V-FITC. If you find that you require less Annexin V-FITC, 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 0.05 µg (1:50 dilution) to 0.005 µg (1:500 dilution) Annexin V-FITC 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 to completely cover the sample. Incubate the slide for 15 minutes at room temperature in the dark.
5. Wash cells twice for 2 minutes each wash in 50 mL 1X Binding Buffer.
6. Place a drop of fluorescence compatible mounting medium and coverslip onto the sample.
7. View cells immediately by fluorescence microscopy.

Analysis of *In Situ* Results

Cells that are apoptotic should fluoresce brightly when viewed through a fluorescein 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 both the Annexin V-FITC positive and propidium iodide positive cells in the same field. However, there may be significant signal overlap 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 produce better results in some cells. For this procedure, a time course experiment is recommended as the best way to help distinguish between apoptosis and necrosis.

APPENDIX

10X 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 dH_2O

The 10X Binding Buffer should be filter sterilized (0.22 µm filter) and stored at 2 - 8° C (stable for a minimum of 2 months). If dilution is required, use sterile dH_2O.

WARNINGS

Hazardous Ingredients

The acute and chronic effects of overexposure to reagents of this kit are unknown. Some kit reagents contain minute amounts of propidium iodide and other substances, which as concentrated solutions are poisonous and may be fatal if swallowed, absorbed through the skin, or inhaled. We recommend following the handling, emergency, and disposal procedures indicated below for all kit reagents.

Handling Precautions

Safe laboratory procedures should be followed when handling all kit reagents. It is recommended that protective laboratory clothing (gloves, laboratory coat, safety glasses) be worn when handling kit reagents.

Emergency Exposure Procedures

In case of exposure to reagent solutions, we recommend following these emergency first-aid procedures:

Skin or eye contact:
Wash with water for at least 15 minutes. Remove any contaminated clothes.

Inhalation:
Remove individual to fresh air. If breathing is difficult, give oxygen and call a physician.

Ingestion:
Rinse mouth with copious amounts of water and call a physician.

Reactivity Data

This kit contains propidium iodide, which is a carcinogen. Wear gloves, eye protection, and protective clothing when handling. Dispose of according to local regulations.

USA patent: US5834196
European patent: 0755516
Japanese patent: 2824155
Australian patent: 689248
New Zealand patent: 283171
Norway patent: 303513
This product is manufactured for Trevigen, Inc. By NeXins Research - The Netherlands.

NOTES