Equine Annexin V-Fluorescein Apoptosis Assay Kit

Catalog #9141, 500 Tests

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

1. INTRODUCTION

Annexins are a group of cellular proteins that are mostly found in eukaryotic organisms. To date, more than 150 annexin proteins have been identified in 65 different species. To be classified as an annexin, a protein must meet the following criteria: 1) it has to be capable of binding negatively charged phospholipids in a calcium dependent manner and 2) it must contain a 70 amino acid repeat sequence called an annexin repeat.

Annexins are important in various cellular and physiological processes, such as providing a membrane scaffold which is relevant to changes in the physical shape of the cell. Annexin proteins have been shown to be involved in trafficking and organization of vesicles, exocytosis, endocytosis, and calcium ion channel formation. Annexin proteins are not exclusively intracellular proteins; they are also found in the extracellular space and have been linked to several processes, including fibrinolysis, coagulation, inflammation, and apoptosis.

Annexin V is a member of a calcium and phospholipid binding family of proteins with vascular anticoagulant activity. Results from *in vitro* experiments indicate that it may play a role in the inhibition of blood coagulation by competing for phosphatidylserine (PS) binding sites with prothrombin. In healthy cells, PS is usually kept in the inner-leaflet (the cytosolic side) of the cell membrane. When a cell undergoes apoptosis, one of the earliest detectable indicators is the loss of membrane asymmetry. No longer restricted to the cytosolic part of the membrane, PS is translocated to the outer-leaflet and becomes exposed on the surface of the cell.

ImmunoChemistry Technologies' Equine Annexin V-Fluorescein Apoptosis Assay Kit provides a proven method for quickly and easily distinguishing two populations of dying cells from viable cells using recombinant fluorescein-conjugated Equine Annexin V and Propidium Iodide (PI). Cells with intact cell membranes and surface-exposed PS, a prominent feature of apoptosis, will stain positive for Annexin V-Fluorescein. PI is included in the kit to identify dying, later stage apoptotic cells which have lost plasma membrane integrity. These cells will become dually labeled with Annexin V-Fluorescein and Propidium Iodide (green and red fluorescence). Live non-apoptotic cells with intact plasma membranes will exclude PI and will remain unstained by the Annexin V-Fluorescein probe, assuming no treatment or cell cycle-associated event temporarily exposes the normally internalized, negatively charged PS entity. The kit also includes a specially formulated, calcium-based binding buffer which is required for Annexin V binding to occur.

2. KIT CONTENTS

#9141, 500 Tests:

- 1 vial of Equine Annexin V-Fluorescein, lyophilized, #6644
- 1 vial of Annexin Reconstitution Buffer, 1X, 1 mL, #6639
- 3 bottles Annexin Binding Buffer, 10X, 10 mL, #6640
- 1 vial of Propidium Iodide, 250 µg/mL, 1 mL, #638

3. STORAGE

Store the unopened kit and each unopened component at 2-8°C until the expiration date. Reconstituted Annexin V-Fluorescein can be stored at 2-8°C for up to 6 months. Reagents do not contain preservatives. Use care to keep reagents free from contamination.

4. SAFETY DATA SHEETS (SDS)

SDS are available online at www.immunochemistry.com or by calling 1-800-829-3194 or 952-888-8788.

5. RECOMMENDED MATERIALS

- DiH_20, 270 mL to dilute Annexin Binding Buffer, 10X
- Phosphate buffered saline (PBS) pH 7.4, up to 100 mL, to wash cells prior to labeling and dilute reagents
- Cultured suspension cells treated with the experimental conditions ready to be labeled
- Reagents to create a positive control such as staurosporine (catalog #6212) or camptothecin (catalog #6210)
- Hemocytometer
- Centrifuge at 200 x g
- 15 mL polypropylene centrifuge tubes (1 per sample)
- FACS tubes

6. DETECTION EQUIPMENT

Annexin V-Fluorescein excites optimally at 494 nm and has a peak emission at 521 nm. Propidium Iodide (nucleic acid bound) excites optimally at 536 nm and has a peak emission at 617 nm.

Flow cytometer: use a standard 488 nm blue laser to excite Annexin V-Fluorescein and Propidium Iodide. Use emission filter sets that best approximate these settings:

- Filter 530/30 (typically detector FL-1) to read Annexin V-Fluorescein (FITC channel)
- Filter 670 LP (typically detector FL-3) to read Propidium Iodide.

7. EXPERIMENTAL PREPARATION & CONTROLS

Staining cells can be completed in under an hour. However, because the reagents are used to label living cells, adequate time must be allotted for the cultivation of cell samples and the experimental treatment or apoptosis induction process.

Cell concentrations used for analysis should be 5.0 x 10^5 to 1.0 x 10^6 cells/mL prior to labeling with Annexin V-Fluorescein. Avoid stressful culture conditions; cell concentrations that are too high or too low can result in the creation of false positive artifacts and erroneous interpretation of the data. Excessive (>1 hour) incubation periods in binding buffer can also lead to false positives, therefore it is important to only suspend cells in binding buffer after the experimental and control samples are ready to be labeled.

- Samples should be kept on ice at all times until they are analyzed to slow the progression of stimulated cells towards secondary necrosis.

1. Culture cells to a density optimal for the specific experiment or induction protocol. Cell density should not exceed 1.0 x 10^6 cells/mL.
2. Create experimental and control cell populations:
   a. Treated experimental population: cells exposed to the experimental condition or treatment
   b. Negative control: non-treated cells grown in a normal cell culture environment
   c. Positive control: cells stimulated using a known apoptosis induction method.
3. Flow cytometer controls: the following controls should be established for instrument compensation and gating.
   a. Unlabeled cells
   b. Negative control cells incubated with Annexin V-Fluorescein only (no PI)
   c. Positive control cells incubated with Annexin V-Fluorescein only (no PI)
   d. Negative control cells incubated with PI only (no Annexin V-Fluorescein)
   e. Positive control cells incubated with PI only (no Annexin V-Fluorescein)

8. CREATION OF POSITIVE CONTROLS

Prior to commencing the experiment, determine a reproducible method for obtaining a positive control that would involve the triggering of a PS translocation event. This process varies significantly with each cell line. For example, this membrane inversion feature may be created via the induction of apoptosis. Apoptosis may be induced by exposing cells to a 2-4 μg/mL concentration of camptothecin (catalog #6210) for >4 hours, or a 1-2 μM concentration of staurosporine (catalog #6212) for >4 hours at 37°C.

Alternatively, cells can be incubated with 3% formaldehyde in culture medium or isotonic buffer. After a 30 minute exposure period on ice, wash, and resuspend cells in ice-cold binding buffer.

- These cells will not actually become apoptotic, however they will stain positive for Annexin V-Fluorescein and Propidium Iodide due to loss of cell membrane integrity.

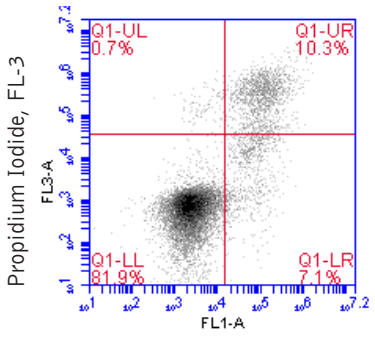
9. PREPARATION OF 1X BINDING BUFFER

ICT's calcium-based binding buffer is used for Annexin V-Fluorescein labeling. The process of Annexin V binding to PS is dependent on the presence of calcium. In the absence of calcium, Annexin V binding is reversible. Therefore samples must be suspended in binding buffer prior to labeling and remain in it until they can be analyzed (<1 hour).

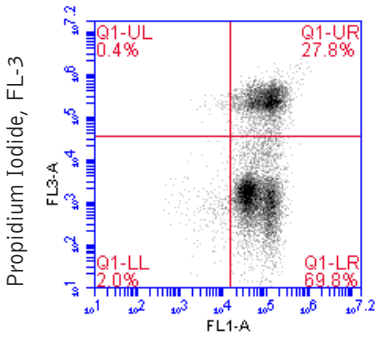
1. Dilute Annexin Binding Buffer, 10X (#6640) 1:10 in di$H_2$0 to create 1X Binding Buffer. For example, add 5 mL Annexin Binding Buffer, 10X to 45 mL di$H_2$0 for a total of 50 mL.

FIGURE 1: DUAL COLOR ANALYSIS VIA FLOW CYTOMETRY

Jurkat cells were treated with a negative control (left) or staurosporine (right), for 4 hours, washed, then stained with ICT's Equine Annexin V-Fluorescein Assay (kit #9141), for 15 min. Cells were read on an Accuri C6 flow cytometer. Treatment with the negative control induced Annexin V-Fluorescein labeling in only 17.4% of the cell population (UR + LR = 10.3% + 7.1%, left), whereas treatment with staurosporine induced Annexin V-Fluorescein labeling in 97.6% of the experimental cells (UR + LR = 27.8% + 69.8%, right). Treatment with staurosporine also significantly increased the proportion of dually stained cells in the treated population (Annexin V-Fluorescein(+)/PI(+), UR). Data courtesy of Mrs. Tracy Murphy, ICT, 227:68.



Annexin V-Fluorescein, FL-1
Negative Control, Non-Induced



Annexin V-Fluorescein, FL-1
Positive Control, Apoptosis-Induced

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- As the 10X concentrate does not contain any preservatives, prepare only enough 1X Binding Buffer for the experiment. Any unused, diluted buffer should be discarded.

2. Place 1X Binding Buffer on ice until needed.

10. PREPARATION OF ANNEXIN V-FLUORESCEIN

1. Reconstitute Equine Annexin V-Fluorescein reagent (#6644) in 1 mL Reconstitution Buffer (#6639) to form the stock concentrate. Mix well. Store the Annexin V-Fluorescein stock concentrate at 2-8°C for up to 6 months.
2. Dilute the Annexin V-Fluorescein stock concentrate 1:5 in PBS to create the Staining Solution. For example, add 100 µL Annexin V-Fluorescein stock concentrate to 400 µL PBS for a total of 500 µL.
3. The Annexin V-Fluorescein stock concentrate should be diluted just prior to use. Any unused, diluted reagent should be discarded.

11. PREPARATION OF PI STAINING SOLUTION

1. Dilute 250 µg/mL Propidium Iodide (#638) 1:10 in PBS to form the PI Staining Solution. For example, add 50 µL Propidium Iodide to 450 µL PBS for a total of 500 µL.
2. PI should be diluted just prior to use. Any unused, diluted reagent should be discarded.

12. STAINING PROTOCOL FOR SUSPENSION CELLS

1. Expose cells to the experimental condition and prepare control cell populations (Sections 7 and 8). Be certain to include all gating and compensation controls needed to set up the flow cytometer.
2. Wash cells (pellet at 200 x g for ~10 minutes) twice with ice-cold culture medium or PBS. This step is important because it removes any cellular debris containing exposed PS from the culture medium.
3. Resuspend cells in ice-cold 1X Binding Buffer (Section 9) at a concentration of 5.0 x 10^5 cells/mL to 1.0 x 10^6 cells/mL and aliquot into ~100 µL samples.
4. Add 10 µL Annexin V-Fluorescein Staining Solution as prepared from reconstituted stock reagent (Section 10) to each ~100 µL sample.
5. Add 5 µL Propidium Iodide Staining Solution as prepared from stock reagent (Section 11) to each ~100 µL sample.
6. Keep the sample tubes on ice and incubate for 15 minutes in the dark.
7. Add 400 µL 1X Binding Buffer to each sample.
8. Analyze immediately, ideally within 5 minutes. To avoid false positives, keep samples on ice until they can be analyzed and do not go longer than 1 hour before reading. Propidium Iodide is toxic to cells, therefore, it is best if PI-labeled samples are read as quickly as possible.

13. DUAL COLOR FLOW CYTOMETRY ANALYSIS OF SUSPENSION CELLS

Follow Section 12, Steps 1-8.

For meaningful dual color data analysis, fluorescence emission values must be corrected for spectral overlap through compensation techniques. Compensation will remove the contribution of Annexin V-Fluorescein to the PI fluorescence channel, and *vice versa*. When samples have been correctly compensated, the mean fluorescence intensity (MFI) values of the single-labeled positive and negative control populations should be equal in all other channels not containing the single color fluorochrome.

For example, cells stained with Annexin V-Fluorescein, regardless of whether they stain positive or negative for the green fluorochrome, should exhibit the same MFI on the PI channel. Conversely, there should be no discernible difference in MFI between PI-positive and PI-negative cells on the Annexin V-Fluorescein channel. Depending on the instrument and the software used, compensation might be set either in the instrument hardware before samples are run or within the software after data collection.

Generally, for DNA content analysis applications, PI fluorescence is detected in the FL-2 (PE) channel of most flow cytometers. However, because of the dye's broad emission peak, when used in conjunction with Annexin V-Fluorescein staining, detection of the PI signal in the FL-3 emission channel is recommended. This minimizes the amount of compensation needed to correct for signal spill-over from Fluorescein into the PI readout channel, and *vice versa*. When this strategy is employed, even novice flow cytometry users can easily and accurately analyze their samples using dual-color flow cytometry.

9. Run the unstained control. If possible, adjust voltages to place the unstained sample in the first decade of the FL dot-plots. The voltages are not changed after this step; if so, the compensation would be invalid.
10. Run each single color control and adjust compensation to remove spectral overlap from interfering FL channels. This is accomplished by subtracting a percentage of the fluorescence in the Annexin V-Fluorescein channel from the fluorescence in the PI channel, and *vice versa*.
11. Run experimental samples. Measure Annexin V-Fluorescein emission on the FL-1 channel and PI emission on FL-3. Three cell populations will be distinguishable:
    a. Annexin V-Fluorescein (+): Intact cell membranes and surface-exposed PS (Figure 1, LR)
    b. Annexin V-Fluorescein (+)/ Propidium Iodide (+): Accessible PS on the cytoplasmic side due to compromised plasma membrane integrity (Figure 1, UR)
    c. Annexin V-Fluorescein (-) / Propidium Iodide (-): Live cells, unlabeled (Figure 1, LL)

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To detect the intracellular process of apoptosis via activated caspases, use our FLICA® kits. These caspase assays are available in green, red, or far-red fluorescence for the preferred detection of individual active caspase enzymes or pan-caspase activity.

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- *Label the active apoptotic process*
- *Distinguish apoptosis from necrosis*
- *Whole cell analysis*
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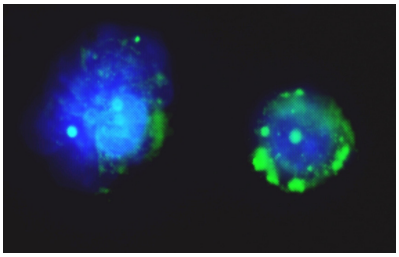

Above: Apoptotic neuroblastoma cells fluoresce green after staining with FAM-FLICA® Poly Caspase Assay (#92). Hoechst 33342 (blue in image) nucleic acid stain is included in the kit as well as propidium iodide live/dead stain.

At left: SR-FLICA® Poly Caspase Assay Kit, standard size (#917)

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