Annexin V-FITC Apoptosis Assay

Catalog #9124

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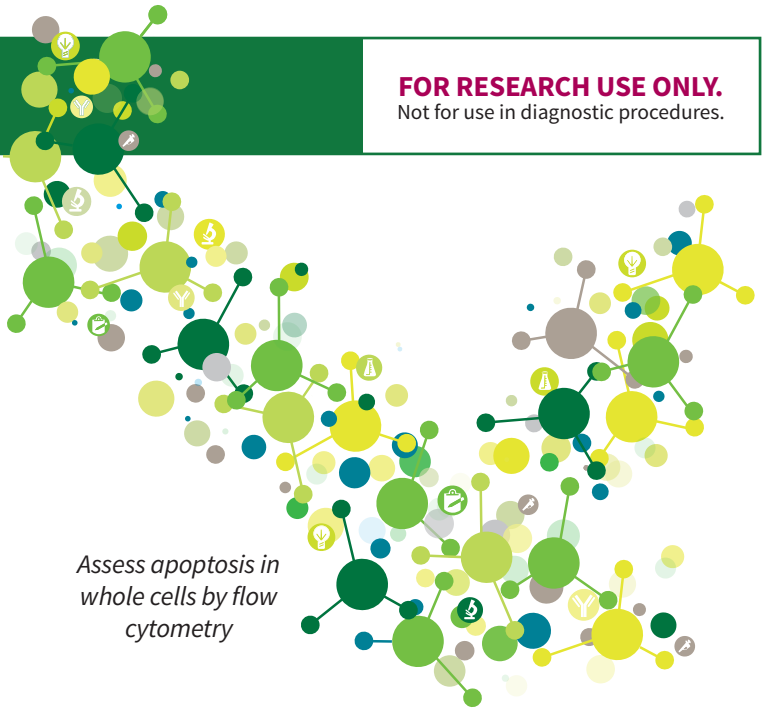


Assess apoptosis in whole cells by flow cytometry

1. INTRODUCTION

Apoptosis is a carefully controlled process of programmed cell death that is biochemically and morphologically distinct from necrosis, a form of traumatic cell death that results from acute cellular injury or cytotoxicity. Morphologically, apoptosis is characterized by compaction of nuclear chromatin, shrinkage of the cytoplasm, and loss of membrane asymmetry[1]. Unlike necrosis, the process is typified by the formation of membrane-enclosed apoptotic bodies that phagocytic cells are able to engulf and quickly remove. This highly conserved programmed cell death process prevents the contents of the cell from spilling out onto surrounding cells, potentially leading to inflammation-associated tissue damage[2]. Biochemically, apoptosis is distinguished by DNA fragmentation and structured cleavage of cellular proteins.

Although comparable in their final outcome, necrosis and apoptosis are markedly different processes, as is evident from the divergent triggers necessary to initiate and carry out the two death-generating mechanisms. Necrosis is often the result of an accumulation of toxic reagents within cells or the end result of acute cellular injury. Depleted levels of ATP favor the necrotic cell death pathway. Apoptosis is an energy-dependent cell death pathway that requires adequate levels of ATP[3]. It can be initiated by various internal and external environmental stimuli, which leads to the activation of endogenous endonuclease activity. Disruption of plasma membrane integrity is a normal initial consequence of necrosis. In contrast, loss of membrane integrity is a very late apoptotic event that is usually preceded by the destructive action of endogenous cellular enzymes.

Research in and around apoptosis has increased substantially in recent years. In addition to its importance in normal growth and development, defective regulation of apoptotic processes has been implicated in an extensive variety of diseases. For example, conditions such as atrophy and ischemia[4] result in excessive apoptosis, whereas the failure of normal apoptosis induction leads to uncontrolled cell proliferation management situations as evidenced in cancerous tumor development[5] and autoimmune disease onset[6].

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[7].

ICT's Annexin V-FITC Apoptosis Assay provides a proven method for quickly and easily distinguishing two populations of dying cells from viable cells using Annexin V-FITC and Propidium Iodide (PI). Cells in the early stages of apoptosis with intact cell membranes and surface-exposed PS will stain positive for Annexin V-FITC. PI is included in the kit to identify late apoptotic and necrotic cells, which have lost plasma membrane integrity. These cells will become dually labeled with Annexin V-FITC and Propidium Iodide (green and red fluorescence). Live cells with intact plasma membranes will exclude PI and will remain unstained by the Annexin V-FITC 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

- 1 vial of Annexin V-FITC (200X), 50 µL, #6340
- 3 vials of 10X Binding Buffer, 1.7 mL, #6341
- 1 vial of Propidium Iodide, 1 mL, 250 µg/mL, #638

3. STORAGE

Store the unopened kit and each unopened component at 2-8°C until the expiration date. 1X Binding Buffer may be stored at 2-8°C in the dark for up to 6 months.

4. SAFETY DATA SHEETS (SDS)

SDS are available online at www.immunochemistry.com or by calling 1-800-829-3194 or 530-758-4400.

5. RECOMMENDED MATERIALS

- DiH_20, 50 mL to dilute 10X Binding Buffer
- 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 induce apoptosis, such as staurosporine (catalog #6212) or camptothecin (catalog #6210), to create a positive control
- Hemocytometer
- Centrifuge at 200 x g
- 15 mL polypropylene centrifuge tubes (1/sample)
- FACS tubes

6. DETECTION EQUIPMENT

Annexin V-FITC excites optimally at 494 nm and has a peak emission at 519 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-FITC and Propidium Iodide.

Use emission filter sets that best approximate these settings:

- Filter 530/30 (typically detector FL1) to read Annexin V-FITC (FITC channel)
- Filter 670 LP (typically detector FL2 or FL3) to read Propidium Iodide.

7. EXPERIMENTAL PREPARATION & CONTROLS

Staining apoptotic and necrotic 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-FITC. 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 with Annexin V-FITC.

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

1. Culture cells to a density optimal for the specific experiment or apoptosis induction protocol. Cell density should not exceed 10^6 cells/mL. Cells cultivated in excess of this concentration may begin to naturally enter apoptosis.
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 induced to undergo apoptosis using a known apoptosis induction method.
3. Flow cytometer setup: The following controls should be established for instrument compensation and gating.
   a. Unlabeled cells
   b. Negative control cells incubated with Annexin V-FITC only (no PI)
   c. Positive control cells incubated with Annexin V-FITC only (no PI)
   d. Negative control cells incubated with PI only (no Annexin V-FITC)
   e. Positive control cells incubated with PI only (no Annexin V-FITC)

8. APOPTOSIS INDUCTION

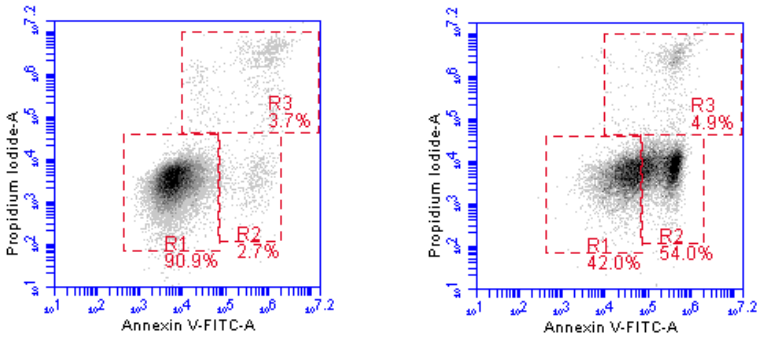
Prior to commencing the experiment, determine a reproducible method for obtaining a positive control that would involve the triggering of a PS translocation event. This process varies significantly with each cell line. For example, this membrane inversion feature may be created via the induction of apoptosis. 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-FITC and Propidium Iodide due to loss of cell membrane integrity.

FIGURE 1: DUAL COLOR ANALYSIS VIA FLOW CYTOMETRY

Jurkat cells were treated with a negative control (left) or staurosporine, an apoptosis-inducing agent (right), for 4 hours, washed, and then stained with ICT's Annexin V-FITC Apoptosis Assay (catalog #9124) for 10 min. Cells were read on an Accuri C6 flow cytometer. Treatment with the negative control induced Annexin V-FITC labeling in only 2.7% of the cell population (R2, left), whereas treatment with staurosporine induced Annexin V-FITC labeling in 54% of the experimental cells (R2, right). This is a ratio of 20:1. Treatment with staurosporine did not significantly increase the proportion of late apoptotic/necrotic cells (R3) at 4 hours. Data courtesy of Mrs. Tracy Murphy, ICT, 213:87.



Negative Control, Non-induced

Positive Control, Apoptosis-induced

9. PREPARATION OF 1X BINDING BUFFER

ICT's calcium-based binding buffer is used for Annexin V-FITC 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 this buffer prior to labeling and remain in it until they can be analyzed (< 1 hour).

1. Dilute 10X Binding Buffer (#6341) 1:10 in diH_20 to create 1X BB. For example, add 1.7 mL 10X Binding Buffer to 15.3 mL diH_20 for a total of 17 mL. 1X Binding Buffer solution may be stored at 2-8°C in the dark for up to 6 months.
2. Place buffer on ice until needed.

10. PREPARATION OF ANNEXIN V-FITC

1. Dilute the 200X Annexin V-FITC (#6340) 1:10 in PBS to prepare the Annexin V-FITC Staining Solution. For example, add 10 µL Annexin V-FITC to 90 µL PBS for a total of 100 µL.
2. Annexin V-FITC should be diluted just prior to use. Any unused, diluted reagent should be discarded.

11. PREPARATION OF PI STAINING SOLUTION

1. Dilute the 250 µg/mL Propidium Iodide Reagent (#638) 1:2 in PBS to prepare the PI Staining Solution. For example, add 50 µL Propidium Iodide to 50 µL PBS for a total of 100 µL.
2. PI should be diluted just prior to use. Any unused, diluted reagent should be discarded.

12. STAINING PROTOCOL

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 for flow cytometer set-up.
2. Wash cells (pellet at 200 x g for ~ 10 minutes) 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 Annexin V-FITC Staining Solution (prepared from stock reagent, Section 10) to each sample at a ratio of 1:20 (5 µL per ~100 µL aliquot of cultured cells).
5. Add Propidium Iodide Staining Solution (prepared from stock reagent, Section 11) to each sample at a ratio of 1:20 (5 µL per ~100 µL aliquot of cultured cells).
6. Keep the sample tubes on ice and incubate for 10 minutes in the dark.
7. Dilute the samples by adding 250 µL 1X Binding Buffer to each.
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. FLOW CYTOMETRY ANALYSIS

For meaningful bi-color data analysis, fluorescence emission values must be corrected for spectral overlap through compensation techniques. Compensation will remove the contribution of Annexin V-FITC 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-FITC, regardless of whether they stain positive or negative for the green fluorochrome, should exhibit the same MFI on the PI channel. Similarly, there should be no discernable difference in MFI between PI-positive and PI-negative cells on the Annexin V-FITC 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 FL2 (PE) channel of most flow cytometers. However, because of the dye's broad emission peak, when used in conjunction with Annexin V-FITC staining, we recommend detection of the PI signal in the FL3 emissions channel. This minimizes the amount of compensation needed to correct for signal spillover from FITC into the PI read-out channel, and *vice versa*. When this strategy is employed, flow cytometry can be used to easily and accurately identify apoptotic vs. necrotic cells within a sample.

1. Run an 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.
2. 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-FITC channel from the fluorescence in the PI channel, and vice versa.
3. Run experimental samples. Measure Annexin V-FITC emission on the FL1 channel and PI emission on FL3. Three cell populations will be distinguishable:
   a. Annexin V-FITC / Propidium Iodide (-): Live cells, unlabeled (Figure 1, R1)
   b. Annexin V-FITC (+): Early apoptotic cells, intact cell membranes and surface-exposed PS (Figure 1, R2)
   c. Annexin V-FITC / Propidium Iodide (+): Late apoptotic and necrotic cells, accessible PS on the cytoplasmic side due to compromised plasma membrane integrity (Figure 1, R3)

14. REFERENCES

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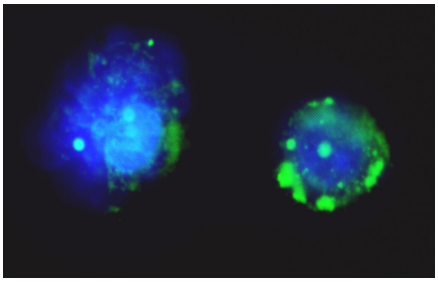

ABOVE: Apoptotic neuroblastoma cells fluoresce green after staining with FAM-FLICA® Poly Caspase Assay (#92). Hoechst 33342 (blue in image) nucleic acid stain is included in the kit as well as Propidium Iodide live/dead stain (not shown).
BELOW: SR-FLICA® Poly Caspase Assay, Standard Size (#917)

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ImmunoChemistry Technologies, LLC gratefully acknowledges the significant contributions made by one of its founders, Brian W. Lee, Ph.D in the development of this product, including the creation and illustration of its strategy and protocol.