Technical Data Sheet

FITC Annexin V

Product Information

Material Number:	**556419**
Size:	200 tests
Vol. per Test:	5 µl
Storage Buffer:	Aqueous buffered solution containing BSA and ≤0.09% sodium azide.

Description

Apoptosis is a normal physiologic process which occurs during embryonic development as well as in maintenence of tissue homeostasis. The apoptotic program is characterized by certain morphologic features, including loss of plasma membrane asymmetry and attachment, condensation of the cytoplasm and nucleus, and internucleosomal cleavage of DNA. Loss of plasma membrane is one of the earliest features. In apoptotic cells, the membrane phospholipid phosphatidylserine (PS) is translocated from the inner to the outer leaflet of the plasma membrane, thereby exposing PS to the external cellular environment. Annexin V is a 35-36 kDa Ca2+ dependent phospholipid-binding protein that has a high affinity for PS, and binds to cells with exposed PS. Annexin V may be conjugated to fluorochromes including FITC. This format retains its high affinity for PS and thus serves as a sensitive probe for flow cytometric analysis of cells that are undergoing apoptosis. Since externalization of PS occurs in the earlier stages of apoptosis, FITC Annexin V staining can identify apoptosis at an earlier stage than assays based on nuclear changes such as DNA fragmentation.

FITC Annexin V staining precedes the loss of membrane integrity which accompanies the latest stages of cell death resulting from either apoptotic or necrotic processes. Therefore, staining with FITC Annexin V is typically used in conjunction with a vital dye such as propidium iodide (PI) or 7-Amino-Actinomycin (7-AAD) to allow the investigator to identify early apoptotic cells (PI negative, FITC Annexin V positive). Viable cells with intact membranes exclude PI, whereas the membranes of dead and damaged cells are permeable to PI. For example, cells that are considered viable are both FITC Annexin V and PI negative while cells that are in early apoptosis are FITC Annexin V positive and PI negative, while cells that are in late apoptosis or already dead are both FITC Annexin V and PI positive. This assay does not distinguish between cells that have undergone apoptotic death versus those that have died as a result of a necrotic pathway because in either case, the dead cells will stain with both FITC Annexin V and PI. However, when apoptosis is measured over time, cells can be often tracked from FITC Annexin V and PI negative (viable, or no measurable apoptosis), to FITC Annexin V positive and PI negative (early apoptosis, membrane integrity is present) and finally to FITC Annexin V and PI positive (end stage apoptosis and death). The movement of cells through these three stages suggests apoptosis. In contrast, a single observation indicating that cells are both FITC Annexin V and PI positive, in of itself, reveals less information about the process by which the cells underwent their demise.

FITC Annexin V is routinely tested by flow cytometric analysis. Other applications were tested at BD Biosciences Pharmingen during antibody development only or reported in the literature.

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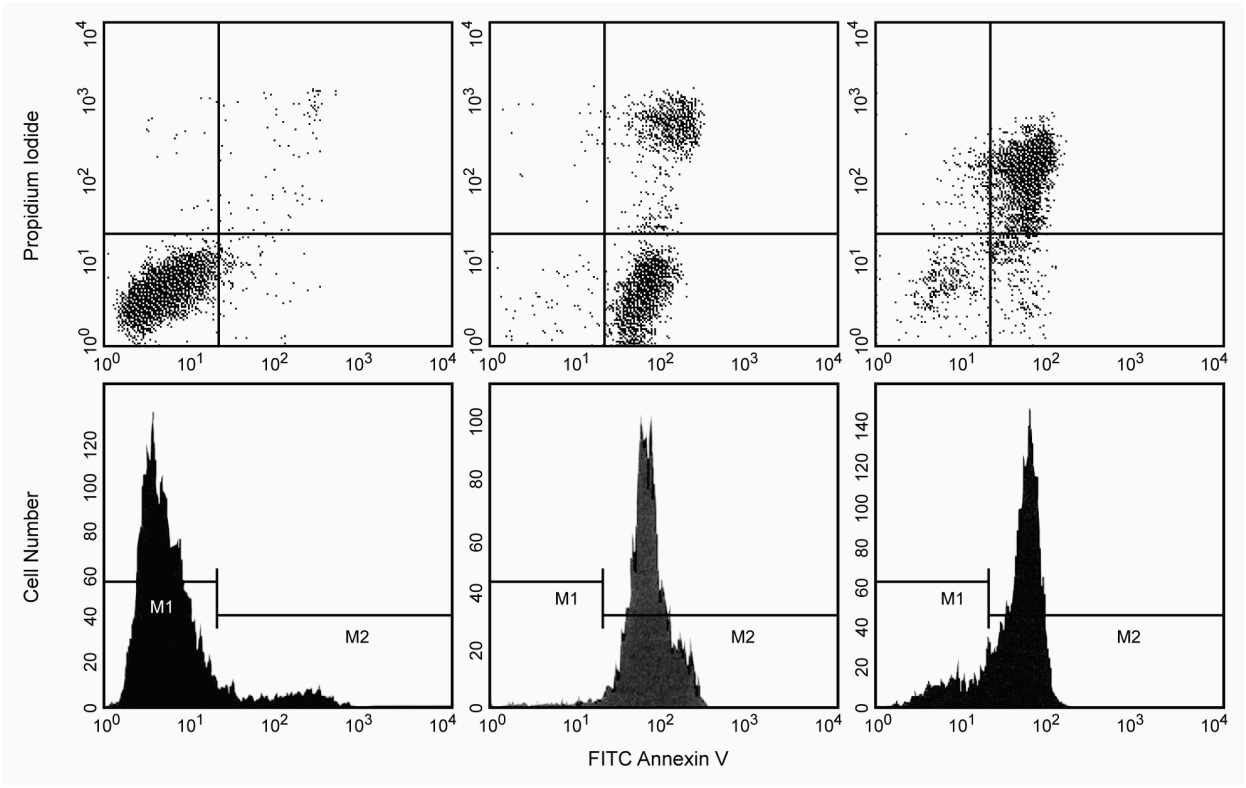

FITC Annexin V: A tool for identifying cells that are undergoing apoptosis. *HBP-ALL human leukemia cells were left untreated (top left & bottom left panels), treated for 5 hr (top middle & bottom middle panels) or 12 hr (top right & bottom right panels) with anti-human Fas antibody (Clone DX2, Cat. No. 555670) and Protein G. Cells were incubated with FITC Annexin V in a buffer containing propidium iodide (PI) and analyzed by flow cytometry. Untreated cells were primarily FITC Annexin V and PI negative, indicating that they were viable and not undergoing apoptosis. After a 5 hr treatment with DX2, the majority of the cells were either undergoing apoptosis (FITC Annexin V positive and PI negative) or had already died (FITC Annexin V and PI positive). After a 12 hr treatment with DX2, the majority of the cells had already died (FITC Annexin V and PI positive). The addition of Protein G enhances the ability of DX2 to induce apoptosis, presumably by cross-linking the Fas receptor.*

Preparation and Storage

Store undiluted at 4°C and protected from prolonged exposure to light. Do not freeze.

Application Notes

Application

Flow cytometry	Routinely Tested

Recommended Assay Procedure:

FITC Annexin V is a sensitive probe for identifying apoptotic cells, binding to negatively charged phospholipid surfaces (Kd of ~5 x 10e-2) with a higher affinity for phosphatidylserine (PS) than most other phospholipids. FITC Annexin V binding is calcium dependent and defined calcium and salt concentrations are required for optimal staining as described in the FITC Annexin V Staining Protocol. **Investigators should note that FITC Annexin V flow cytometric analysis on adherent cell types (e.g HeLa, NIH 3T3, etc.) is not routinely tested as specific membrane damage may occur during cell detachment or harvesting. Methods for utilizing Annexin V for flow cytometry on adherent cell types, however, have been previously reported (Casiola-Rosen et al. and van Engelend et al.).**

INDUCTION OF APOPTOSIS USING AN ANTI-HUMAN CD95 (FAS) ANTIBODY

The following protocol is provided as an illustration on how FITC Annexin V may be used on a human cell line.

Materials

1. A cell line or primary cells that can easily be induced to undergo apoptosis by human Fas mAb. Examples include Daudi lymphoma cells (ATCC CCL-213) and Jurkat T cells (ATCC TIB-152). It is important to note that there can be significant variation between cell lines regarding the level of apoptosis that can be induced through the Fas receptor. Also, not all cell types which express the Fas antigen will necessarily undergo Fas-mediated apoptosis. The cell lines mentioned above are good positive controls as they are strongly induced to undergo apoptosis by Fas mAb.
2. Anti-human CD95 (Fas) mAb, clone DX2 (Cat. No. 555670).
3. Recombinant Protein G (Sigma-Aldrich cat.no. P4689). We have found that the addition of Protein G to the tissue culture medium can

significantly enhance the efficiency of the DX2 clone to induce apoptosis.
4. T25 tissue culture flasks.
5. IMDM or RPMI 1640 medium with 10% heat-inactivated fetal bovine serum (FBS), 1% L-glutamine and 1% antibiotics (penicillin/ streptomycin; 100 U/ml). This supplemented medium is simply referred to as 'medium' below.

Procedure

1. Maintain the cells in culture and change the medium one day before inducing apoptosis.
2. Induction of apoptosis: Add 0.5 - 2 µg/ml of the anti-CD95 antibody (DX2 clone) and 1-2 µg/ml Protein G to a T25 flask with medium containing ~0.5 × 10e6 cells/ml. Negative controls should consist of:
   (a) ~0.5 × 10e6 cells/ml with medium alone (no mAb or Protein G), and
   (b) ~0.5 × 10e6 cells/ml with medium and 1 µg/ml Protein G alone (no mAb).
3. Incubate the cells for 2 to 12 hr at 37°C
4. Proceed with the FITC Annexin V Staining Protocol to measure apoptosis. Apoptosis can also be observed by light microscopy, gel electrophoresis (DNA fragmentation ladders) or by using a DNA fragmentation-based flow cytometry assay system such as the APO-BRDU™ Kit (Cat. No. 556405) or the APO-DIRECT™ Kit (Cat. No. 556381).

INDUCTION OF APOPTOSIS USING AN ANTI-MOUSE CD95 (FAS) ANTIBODY

The following protocol is provided as an illustration on how FITC Annexin V may be used on murine cells.

Materials

1. A cell line or primary cells that can easily be induced to undergo apoptosis by mouse Fas monoclonal antibody (mAb). Thymocytes isolated from a 4-6 week old BALB/c mouse may be used.
2. Anti-mouse CD95 (Fas) mAb, clone Jo2 (Cat. No. 554254)
3. Recombinant Protein G (Sigma-Aldrich cat.no. P4689). We have found that the addition of Protein G to the tissue culture medium can significantly enhance the efficiency of Jo2 mAb to induce apoptosis.
4. T25 tissue culture flasks
5. RPMI-1640 medium with 10% heat-inactivated fetal bovine serum (FBS), 1% L-glutamine and 1% antibiotics (penicillin/ streptomycin; 100 U/ml). This supplemented medium is simply referred to as 'medium' below.

Procedure

1. Isolate BALB/c thymocytes from the thymus of a 4-6 week old mouse.
2. Induction of apoptosis. Add 2.5-10 µg/ml Jo2 (Cat. No. 554254) and 1-2 µg/ml Protein G to a T25 flask containing ~2 × 10e6 thymocytes/ml. Negative controls should consist of
   (a) ~2 × 10e6 thymocytes/ml with medium alone (no mAb or Protein G)
   (b) ~2 × 10e6 thymocytes/ml with medium and 1-2 µg/ml Protein G alone (no mAb).
3. Incubate the cells for 2-12 hrs at 37°C.
4. Proceed with the FITC Annexin V Staining Protocol to measure apoptosis. Apoptosis can also be observed by light microscopy, gel electrophoresis (DNA fragmentation ladders) or by using a DNA fragmentation-based flow cytometry assay system such as the APO-BRDU™ Kit (Cat. No. 556405) or the APO-DIRECT™ Kit (Cat. No. 556381).

FITC ANNEXIN V STAINING PROTOCOL

FITC Annexin V is used to quantitatively determine the percentage of cells within a population that are actively undergoing apoptosis. It relies on the property of cells to lose membrane asymmetry in the early phases of apoptosis. In apoptotic cells, the membrane phospholipid phosphatidylserine (PS) is translocated from the inner leaflet of the plasma membrane to the outer leaflet, thereby exposing PS to the external environment. Annexin V is a calcium-dependent phospholipid-binding protein that has a high affinity for PS, and is useful for identifying apoptotic cells with exposed PS. Propidium Iodide (PI) is a standard flow cytometric viability probe and is used to distinguish viable from nonviable cells. Viable cells with intact membranes exclude PI, whereas the membranes of dead and damaged cells are permeable to PI. Cells that stain positive for FITC Annexin V and negative for PI are undergoing apoptosis. Cells that stain positive for both FITC Annexin V and PI are either in the end stage of apoptosis, are undergoing necrosis, or are already dead. Cells that stain negative for both FITC Annexin V and PI are alive and not undergoing measurable apoptosis.

Reagents

1. FITC Annexin V: Included. Use 5 µl per test.
2. Propidium Iodide (PI): Not Included. PI (cat.no. 556463) is a convenient, ready-to-use nucleic acid dye. Use up to 10 µl per test of a 50 µg/ml solution.
3. 10× Binding Buffer: Not Included. 0.1 M Hepes (pH 7.4), 1.4 M NaCl, 25 mM CaCl2. Store at 4°C. Alternatively, catalog number 556454 may be purchased.

Staining

1. Wash cells twice with cold PBS and then resuspend cells in 1× Binding Buffer at a concentration of 1 × 10e6 cells/ml.
2. Transfer 100 µl of the solution (1 × 10e5 cells) to a 5 ml culture tube.
3. Add 5 µl of FITC Annexin V.
4. Add 10 µl PI. The optimal concentration of PI may vary among cell lines where 10 µl of a 50 µg/ml stock is most likely the maximum to be required. Less may yield optimal results in some experimental systems.
5. Gently vortex the cells and incubate for 15 min at RT (25 °C) in the dark.
6. Add 400 µl of 1× Binding Buffer to each tube. Analyze by flow cytometry within 1 hr.

SUGGESTED CONTROLS FOR SETTING UP FLOW CYTOMETRY

The following controls are used to set up compensation and quadrants:

1. Unstained cells.
2. Cells stained with FITC Annexin V (no PI).
3. Cells stained with PI (no FITC Annexin V).

Other Staining Controls:

A cell line that can be easily induced to undergo apoptosis should be used to obtain positive control staining with FITC Annexin V and/or FITC Annexin V and PI. It is important to note that the basal level of apoptosis and necrosis varies considerably within a population. Thus, even in the absence of induced apoptosis, most cell populations will contain a minor percentage of cells that are positive for apoptosis (FITC Annexin V positive, PI negative or FITC Annexin V positive, PI positive).

The untreated population is used to define the basal level of apoptotic and dead cells. The percentage of cells that have been induced to undergo apoptosis is then determined by subtracting the percentage of apoptotic cells in the untreated population from percentage of apoptotic cells in the treated population. Since cell death is the eventual outcome of cells undergoing apoptosis, cells in the late stages of apoptosis will have a damaged membrane and stain positive for PI as well as for FITC Annexin V. Thus the assay does not distinguish between cells that have already undergone an apoptotic cell death and those that have died as a result of necrotic pathway, because in either case the dead cells will stain with both FITC Annexin V and PI.

Suggested Companion Products

Catalog Number	Name	Size	Clone
556454	Annexin V Binding Buffer, 10X concentrate	50 ml	(none)
556463	Propidium Iodide Staining Solution	2.0 ml	(none)

Product Notices

1. Since applications vary, each investigator should titrate the reagent to obtain optimal results.
2. Please refer to www.bdbiosciences.com/pharmingen/protocols for technical protocols.
3. For fluorochrome spectra and suitable instrument settings, please refer to our Fluorochrome Web Page at www.bdbiosciences.com/pharmingen/colors.
4. Source of all serum proteins is from USDA inspected abattoirs located in the United States.
5. Caution: Sodium azide yields highly toxic hydrazoic acid under acidic conditions. Dilute azide compounds in running water before discarding to avoid accumulation of potentially explosive deposits in plumbing.

References

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