Technical Data Sheet

Purified Recombinant Annexin V

Product Information

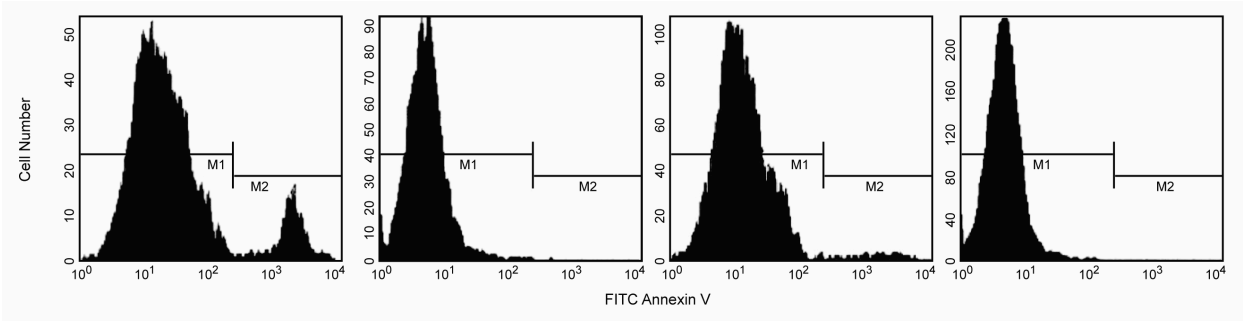
Material Number:	**556416**
Size:	100 µg
Concentration:	0.5 mg/ml
Storage Buffer:	Aqueous buffered solution containing ≤0.09% sodium azide.

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, such as fluorescein isothiocyanate (FITC), to serve 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, Annexin V staining can identify apoptosis at an earlier stage than assays based on nuclear changes such as DNA fragmentation. In addition, Annexin V binding sites may be blocked by incubating cells with purified recombinant Annexin V prior to incubation with one of the fluorochrome labeled formats of Annexin V.

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 Annexin V is typically used in conjunction with a vital dye such as 7-Amino-Actinomycin (7-AAD) to allow the investigator to identify early apoptotic cells (7-AAD negative, Annexin V positive). Viable cells with intact membranes exclude 7-AAD, whereas the membranes of dead and damaged cells are permeable to 7-AAD. For example, cells that are considered viable are both Annexin V and 7-AAD negative while cells that are in early apoptosis are Annexin V positive and 7-AAD negative, while cells that are in late apoptosis or already dead are both Annexin V and 7-AAD 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 Annexin V and 7-AAD. However, when apoptosis is measured over time, cells can be often tracked from Annexin V and 7-AAD negative (viable, or no measurable apoptosis), to Annexin V positive and 7-AAD negative (early apoptosis, membrane integrity is present) and finally to Annexin V and 7-AAD 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 Annexin V and 7-AAD positive, in of itself, reveals less information about the process by which the cells underwent their demise.

Purified 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.



Flow cytometric analysis of Annexin V staining and blocking. *Jurkat T cells were induced to undergo apoptosis by treatment with anti-Fas antibody, clone DX2 (Cat. No. 555670) and Protein G for three hours (first & second panels). Other cultures were left untreated (third & fourth panels). Cells were incubated with FITC Annexin V (first & third panels) or with recombinant Annexin V to block Annexin V binding sites, and then with FITC Annexin V (second & fourth panels). After a three hour treatment with Fas mAb, a population of cells was FITC Annexin V positive (first panel, M2 gate). FITC Annexin V staining was blocked when cells were first incubated with purified recombinant Annexin V (second panel). As expected, the cell population that was not treated with Fas mAb were primarily Annexin V negative (third panel). The small number of Annexin V positive cells in the untreated population likely represents a basal level of apoptosis. This was blocked when cells were first incubated with purified recombinant Annexin V (fourth panel). The M1 and M2 gates demarcate FITC Annexin V negative and positive populations, respectively. The addition of Protein G enhances the ability of DX2 to induce apoptosis, presumably by crosslinking the Fas receptor.*

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Preparation and Storage

Store product at -80°C prior to use or for long term storage of stock solutions.
Avoid multiple freeze-thaws of product.
Recombinant Annexin V was expressed in bacteria and is ≥95% pure as determined by SDS/PAGE stained with Coomassie Blue.

Analysis of purified recombinant Annexin V (1 µg) by SDS/PAGE. *Gel was stained with Commasie Blue.*

Application Notes

Application

Flow cytometry	Routinely Tested
Blocking	Routinely Tested

Recommended Assay Procedure:
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. Annexin V binding is calcium dependent and defined calcium and salt concentrations are required for optimal staining as described in the Annexin V Staining Protocol. **Investigators should note that 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 BY CAMPTOTHECIN
The following protocol is provided as an illustration on how Annexin V may be used on a cell line (Jurkat).

Materials
1. Prepare Camptothecin stock solution (Sigma-Aldrich Cat. No. C-9911): 1 mM in DMSO.
2. Jurkat T cells (ATCC TIB-152).

Procedure
1. Add Camptothecin (final conc. 4-6 µM) to 1 x 10e6 Jurkat cells.
2. Incubate the cells for 4-6 hr at 37°C.
3. Proceed with the Annexin V Staining Protocol to measure apoptosis.

INDUCTION OF APOPTOSIS USING AN ANTI-HUMAN CD95 (FAS) ANTIBODY
The following protocol is provided as an illustration on how 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 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).

ANNEXIN V STAINING & BLOCKING PROTOCOL

Reagents

1. Biotin (Cat.No. 556418), FITC (Cat.No. 556420), PE (Cat.No. 556422), APC (Cat.No. 550474), Cy5 (Cat.No. 559933), or Cy5.5 (Cat.No. 559935) conjugated Annexin V reagents: Not Included. Use 5 µl per test.
2. 7-Amino-Actinomycin D (7-AAD): Not included. 7-AAD (Cat.No. 559925) is a convenient, ready-to-use nucleic acid dye with fluorescence detectable in the far red range of the spectrum. Use 5 µl per test.
3. 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. 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.
4. 10X 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.
5. Purified Recombinant Annexin V (Cat.No. 556416): Included.

Staining

1. Wash cells twice with cold PBS and then resuspend cells in 1X Binding Buffer at a concentration of 1 x 10e6 cells/ml.
2. Transfer 100 µl of the solution (1 x 10e5 cells) to a 5 ml culture tube.
3. Add 5 µl of fluorochrome conjugated-Annexin V (for one and two color analysis) and/or 5 µl of 7-AAD or 10 µl PI (for two color analysis only).
4. Gently vortex the cells and incubate for 15 min at RT (25°C) in the dark.
5. Add 400 µl of 1X Binding Buffer to each tube. Analyze by flow cytometry within 1 hr.

Blocking

1. Wash cells twice with cold PBS and then resuspend cells in 1X Binding Buffer at a concentration of 1 x 10e6 cells/ml.
2. Transfer 100 µl of the solution (1 x 10e5 cells) to a 5 ml culture tube.
3. Add 5-15 µg of purified recombinant Annexin V. The amount of purified recombinant Annexin V required to saturate binding sites may vary according to cell type and stage of apoptosis. In some cases, investigators may need to reduce the number of cells to 0.5 x 10e5 and still add 5-15 µg of recombinant Annexin V to obtain optimal results. Titration is strongly recommended.
4. Gently vortex the cells and incubate for 15 min at room temperature.
5. Add 5 µl of fluorochrome conjugated-Annexin V (for one and two color analysis) and/or 5 µl of 7-AAD or 10 µl PI (for two color analysis only).
6. Gently vortex the cells and incubate for 15 min at room temperature in the dark.
7. Add 400 µl of 1X Binding Buffer to each tube. Analyze by flow cytometry as soon as possible (within 1 hr).

Suggested Companion Products

Catalog Number	Name	Size	Clone
556454	Annexin V Binding Buffer, 10X concentrate	50 ml	(none)
556420	FITC Annexin V	100 tests	(none)
559925	7-AAD	2.0 ml	(none)

Product Notices

1. Since applications vary, each investigator should titrate the reagent to obtain optimal results.
2. 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.

3. For fluorochrome spectra and suitable instrument settings, please refer to our Fluorochrome Web Page at www.bdbiosciences.com/pharmingen/colors.
4. Please refer to www.bdbiosciences.com/pharmingen/protocols for technical protocols.

References

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