

Version 1 Last updated 26 March 2018

ab219909 Annexin V- iFluor 750 Detection Reagent

For the rapid, sensitive and accurate measurement of PS exposure in live cells

This product is for research use only and is not intended for diagnostic use.

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1. Overview

Annexin V-iFluor 750 Detection Reagent (ab219909) is a cell-impermeable reagent designed to bind to phosphatidylserine (PS) residues exposed on the outer cell surface of cells with a flow cytometer or fluorescence microscopy at Ex/Em = 750/778 nm.

We recommend using an impermeable nuclear stain such as Propidium Iodide (ab14083) together with Annexin V-iFluor 750 Detection Reagent to discriminate necrotic and dead cells: plasma membrane is disrupted in these cells and therefore the Annexin V reagent will bind to PS found in the interior of cells.

Apoptosis is a regulated process of cell death that occurs during embryonic development as well as maintenance of tissue homeostasis. Inappropriately regulated apoptosis is implicated in different disease states, such as neurodegeneration disease and cancer. The apoptosis program is characterized by morphologic features, including loss of plasma membrane asymmetry and attachment, condensation of the cytoplasm and nucleus, and compaction and fragmentation of the nuclear chromatin. Exposure of phosphatidylserine (PS) on the external surface of the cell membrane has been reported to occur in the early phases of apoptotic cell death, during which the cell membrane remains intact. In leukocyte apoptosis, PS on the outer surface of the cell marks the cell for recognition and phagocytosis by macrophages. The human vascular anticoagulant, annexin V, is a 35-36 kDa Ca^{2+} dependent phospholipid binding protein that has a high affinity for PS, and shows minimal binding to phosphatidylcholine and sphingomyelin. Changes in PS asymmetry, which can be analyzed by measuring annexin V binding to the cell membrane, are generally observed before morphological changes associated with apoptosis occurred and before membrane integrity is lost.

2. Protocol Summary

Induce apoptosis in cells



Add Annexin V-iFluor 750 Reagent assay solution



Incubate at room temperature for 30-60 minutes



Analyse with a flow cytometer or a fluorescence microscope
(Ex/Em = 750/778 nm)

3. Precautions

Please read these instructions carefully prior to beginning the assay.

- We understand that, occasionally, experimental protocols might need to be modified to meet unique experimental circumstances. However, we cannot guarantee the performance of the product outside the conditions detailed in this protocol booklet.
- Reagents should be treated as possible mutagens and should be handled with care and disposed of properly. Please review the Safety Datasheet (SDS) provided with the product for information on the specific components.
- Observe good laboratory practices. Gloves, lab coat, and protective eyewear should always be worn. Never pipet by mouth. Do not eat, drink or smoke in the laboratory areas.
- All biological materials should be treated as potentially hazardous and handled as such. They should be disposed of in accordance with established safety procedures.

4. Storage and Stability

Store reagent at -20°C in the dark immediately upon receipt. Kit has a storage time of 1 year from receipt.

5. Limitations

- Assay kit intended for research use only. Not for use in diagnostic procedures.
- Do not mix or substitute reagents or materials from other kit lots or vendors. Kits are QC tested as a set of components and performance cannot be guaranteed if utilized separately or substituted.

6. Materials Supplied

Item	Quantity	Storage temperature (before prep)	Storage temperature (after prep)
Annexin V-iFluor 750 conjugate	200 µL	-20°C	-20°C

7. Materials Required, Not Supplied

These materials are not included in the kit, but will be required to successfully perform this assay:

- Flow cytometer or fluorescence microscope capable of measuring fluorescence at Ex/Em = 750/778 nm
- PBS
- Annexin V binding assay buffer [10 mM HEPES, 140 mM NaCl, 2.5 mM CaCl_2 , pH 7.4].
- Pipettes and pipette tips, including multi-channel pipette
- Assorted glassware for the preparation of reagents and buffer solutions
- Tubes for the preparation of reagents and buffer solutions
- Impermeable DNA labeling reagent with different excitation/emission spectra to Annexin V-iFluor 750: we recommend Propidium Iodide (ab14083)

For flow cytometry assay:

- 12 x 75 mm tubes for flow cytometry

For fluorescence microscopy assay:

- Sterile 96-well plate with clear flat bottom, preferably black. Use a poly-D-lysine coated plate for suspension cells

8. Technical Hints

- This kit is sold based on number of tests. A “test” simply refers to a single assay well. The number of wells that contain sample, control or standard will vary by product. Review the protocol completely to confirm this kit meets your requirements. Please contact our Technical Support staff with any questions.
- Selected components in this kit are supplied in surplus amount to account for additional dilutions, evaporation, or instrumentation settings where higher volumes are required. They should be disposed of in accordance with established safety procedures.
- Avoid foaming or bubbles when mixing or reconstituting components.
- Avoid cross contamination of samples or reagents by changing tips between sample and reagent additions.
- Ensure all reagents and solutions are at the appropriate temperature before starting the assay.
- Make sure all necessary equipment is switched on and set at the appropriate temperature.

9. Reagent Preparation

Briefly centrifuge small vials at low speed prior to opening.

9.1 Annexin V-iFluor 750 conjugate:

Ready to use as supplied. Equilibrate to room temperature before use. Aliquot so that you have enough reagent to perform the desired number of assays. Keep on ice while in use. Store at -20°C protected from light.

10. Assay Procedure – flow cytometry

- Equilibrate all materials and prepared reagents to room temperature prior to use.
- We recommend that you assay all controls and samples in duplicate.

10.1 Prepare and incubate cells:

10.1.1 Grow and treat cells of interest with appropriate compounds to induce apoptosis.

Treatment times may vary depending on the agent and cell line. Suggested positive controls:

- Jurkat cells treated with 1 μM staurosporine for 5 hours
- Jurkat cells treated with 6 μM camptothecin for 4 hours

Δ Note: a negative control with untreated cells should be included in the analysis. The negative control is used to define the basal level of apoptotic and necrotic or dead cells.

10.1.2 Additional controls necessary to set up flow cytometer compensation and quadrants (first time use only):

- Untreated unstained cells
- Untreated cells stained with Annexin V-iFluor 750 only
- Untreated cells stained with propidium iodide (or other DNA dye) alone

10.2 Harvest and stain cells:

10.2.1 Harvest untreated and treated cells ($1-5 \times 10^5$ cells/tube).

- Suspension cells: transfer cells to collection tube directly.
- Adherent cells: gently trypsinize and wash cells once with serum-containing media. Be aware that membrane damage may occur during cell detachment or harvesting which can lead to an increase in background signal.

10.2.2 Wash cells twice at room temperature in PBS.

10.2.3 Prepare Annexin V binding assay buffer (10 mM HEPES, 140 mM NaCl, and 2.5 mM CaCl_2 , pH 7.4).

10.2.4 Resuspend cells in 200 μL of Annexin V binding assay buffer.

10.2.5 Add 2 μL of Annexin V-iFluor 750 conjugate to the cells. If using, add propidium iodide (or other viability stain) to cells for dead/necrotic cells.

Δ Note: alternatively, for an easier procedure, Annexin V conjugate and propidium iodide can be added to assay buffer before cells resuspension. In that case, add 202 μ L (+ propidium iodide volume) of the staining mix to each tube of cells.

10.2.6 Incubate at room temperature for 30 to 60 minutes, protected from light.

10.2.7 Add 300 μ L of Annexin V binding assay buffer to increase volume before analyzing the cells with a flow cytometer.

10.3 Analyze cells by flow cytometry:

Δ Note: we recommend analyzing cells within one hour of staining.

10.3.1 Set appropriate FSC vs SSC gates to exclude debris and cell aggregates.

10.3.2 Use set up controls (Step 10.1.2) to set up necessary laser compensations.

10.3.3 Collect Annexin V-iFluor 750 and cell dead dye fluorescence in the appropriate filters.

11. Assay Procedure – fluorescence microscopy

- Equilibrate all materials and prepared reagents to room temperature prior to use.
- We recommend that you assay all controls and samples in duplicate.
- The procedure described in this section has been optimized for 96-well microplates. Volumes can be scaled up to adapt protocol for larger culture plates.

11.1 Prepare and grow cells:

11.1.1 Suspension cells:

- Grow $1-5 \times 10^5$ cells/well in a 96-well microplate (black wells/clear flat bottom)
- Cells can be attached to the bottom of plates by centrifuging plates in an appropriate plate-adapted centrifuge.

11.1.2 Adherent cells: number of cells depend on the cell type (general recommendation below):

- CHO-K1 cells: $5-8 \times 10^4$ cells/well
- HeLa cells: $3-5 \times 10^4$ cells/well

Δ Note: cells can also be grown on coverslips in 12-well/24-well culture plates. Volumes should be adjusted accordingly to ensure cells are always covered.

11.1.3 Treat cells with appropriate compounds to induce apoptosis. Treatment times may vary depending on the agent and cell line. Suggested positive controls:

- Jurkat cells treated with 1 μ M staurosporine for 5 hours
- Jurkat cells treated with 6 μ M camptothecin for 4 hours

Δ Note: A negative control with untreated cells should be included in the analysis. The negative control is used to define the basal level of apoptotic and necrotic or dead cells.

11.2 Stain cells:

11.2.1 Prepare Annexin V binding assay buffer (10 mM HEPES, 140 mM NaCl, and 2.5 mM CaCl_2 , pH 7.4).

11.2.2 Wash cells twice at room temperature in Annexin V binding assay buffer.

- 11.2.3 Add 200 μ L of Annexin V binding assay buffer to cells.
- 11.2.4 Add 2 μ L of Annexin V-iFluor 750 conjugate to the cells. If using, add propidium iodide (or other viability stain) to cells for dead/necrotic cells.

Δ Note: alternatively, for an easier procedure, Annexin V conjugate and propidium iodide can be added to assay buffer before cells resuspension. In that case, add 202 μ L (+ propidium iodide volume) of the staining mix to each well.

- 11.2.5 Incubate at room temperature for 30 to 60 minutes, protected from light.

11.3 Analyze cells by fluorescence microscope:

- 11.3.1 If cells are on cover slips, invert cover slip on a glass slide.

Δ Note: you can add a drop of anti-fading solution and before inverting cover slip onto glass slide. Edges can be sealed with rubber cement or clear nail polish to ensure cells do not dry out.

- 11.3.2 Analyze cells by fluorescence microscopy using the appropriate filters as soon as possible.

12. Data Analysis

FOR FLOW CYTOMETRY

The following are general guidelines. Specific methods of analysis will vary with different flow cytometer analysis programs.

- Establish appropriate FSC vs SSC gates to exclude debris and cell aggregates.
- Collect Annexin V conjugate and PI (or viability dye used) fluorescence in the appropriate channels.
- Using fluorescence intensity, determine fold change between control and treated cells.

FOR FLUORESCENCE MICROSCOPY

- We recommend acquiring several images per well.
- We recommend data analysis after coding and mixing images to ensure unbiased results.
- For manual analysis, if you do not have a specific software installed in your microscope, you can download ImageJ, an open source image processing designed for scientific multidimensional images by the National Institute of Health (NIH).

The table below can be used as guidance for interpretation of results:

	↓ Annexin V	↑ Annexin V
↓ Propidium Iodide	No apoptosis / viable cells	Early apoptosis
↑ Propidium Iodide	Dead cells	Late apoptosis / necrotic cells

Δ Note: elevated Annexin V signal and/or PI staining: apoptosis is an ongoing process so that cells stained with Annexin V should not be kept for long before measurement. Viable cells which still maintain membrane integrity may become positive for PI since the dye can enter intact cells through slow diffusion. Analyze cells as soon as you finish staining.

13. Typical Data

Data provided for **demonstration purposes** only.

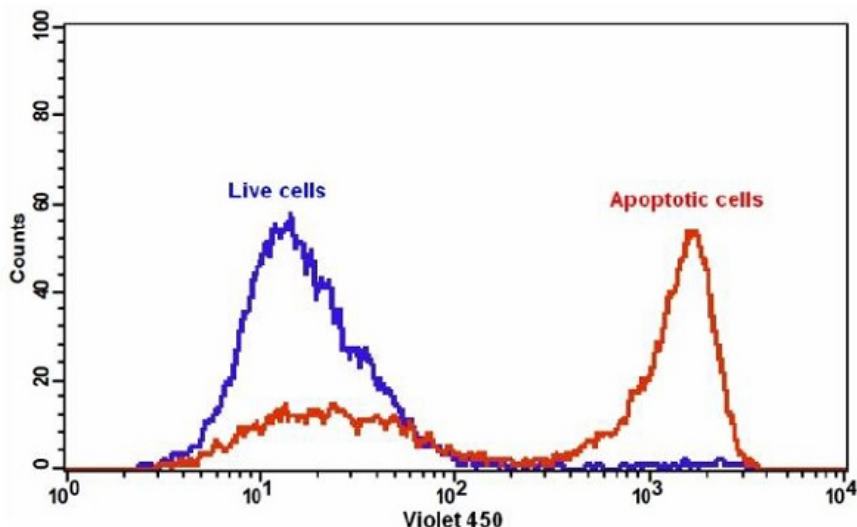


Figure 1. Detection of phosphatidylserine (PS) exposure in Jurkat cells using Annexin V-mFluor Violet450 Detection Reagent (ab219911), which has similar PS binding properties to Annexin V-iFluor 750 conjugate. Jurkat cells were left untreated (blue) or treated with 1 μ M staurosporine (red) in a 37°C, 5% CO₂ incubator for 5 hours. Cells were then incubated with Annexin V Detection Reagent for 30 minutes. The fluorescence intensity of Annexin V-mFluor 450 Detection Reagent was measured with a FACSCalibur (BD systems) flow cytometer using violet laser at Ex/Em = 405/450 nm.

In live non-apoptotic cells, Annexin V-iFluor 750 conjugate detects innate apoptosis in non-induced cells, which is typically 2-6% of all cells. In apoptotic cells Annexin V-iFluor 750 conjugate binds to phosphatidylserine, which is located on the outer leaflet of the cell membrane, resulted in increased staining intensity.

14. Notes

Technical Support

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