

Annexin-V-Alexa 568

Alexa 568-conjugated anticoagulant for the detection of phosphatidylserine on the outer leaflet of apoptotic cells
This product is a replacement for Cat. No. 1 985 485, the handling and performance are the same.

Cat. No. 03 703 126 001

500 µl (250 tests)

Version August 2005

Store at +2 to +8°C
Protect from light!

1. Product overview

Introduction

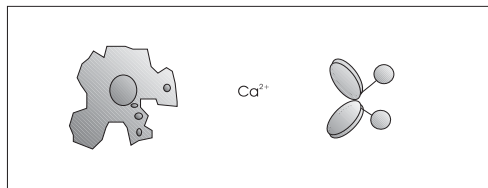
In the early stages of apoptosis, changes occur at the cell surface (1, 2, 3). One of these plasma membrane alterations is the translocation of phosphatidylserine (PS) from the inner part of the plasma membrane to the outer layer, by which PS becomes exposed at the external surface of the cell (4). Fadok et al. showed that macrophages specifically recognize PS exposed on the surface of lymphocytes during the development of apoptosis (2). The recognition and phagocytosis of apoptotic cells and bodies protects organisms from the exposure to cellular compounds leading to inflammation, which mostly accompanies necrosis.

Assay principle

The analysis of phosphatidylserine on the outer leaflet of apoptotic cell-membranes is performed by using Annexin-V- Alexa 568 in combination with a DNA stain [e.g., BOBO-1 (B-3582)] to differentiate apoptotic cells from necrotic cells or can be combined with a cell surface marker for cell characterization.

The procedure involves:

Stage	Description
1	Washing the cells in PBS.
2	Incubation of cells with Annexin-V-Alexa 568 in a HEPES buffer containing BOBO-1 (B-3582) or labeling reagent for cell surfaces (e.g., CD-marker).
3	If CD-marker antibodies are used, a washing step with HEPES buffer is needed (not necessary when BOBO-1 (B-3582) staining is applied).
4	Analysis of the samples under a fluorescence microscope or on a flow cytometer.



apoptotic cell with phosphatidylserine exposed on the outer leaflet of the membrane Annexin-V-Alexa 568

Fig. 1: Test principle.

Application

Annexin-V is a Ca^{2+} -dependent, phospholipid-binding protein with high affinity for PS (4). This protein can hence be used as a sensitive probe for PS exposure on the outer leaflet of the cell membrane, and is therefore suited to detect apoptotic cells (4, 5, 6, 7).

Since necrotic cells also expose PS as a result of lost membrane integrity, apoptotic cells have to be differentiated from these necrotic cells. The simultaneous application of a DNA stain (used for dye exclusion tests) allows the discrimination of necrotic cells from the Annexin-V positively-stained cell cluster.

Additional secondary labeling is also possible. For example, membrane surface can be stained with fluorescein-labeled monoclonal antibodies for further cellular characterization.

Sample material

- Cell lines
- Freshly isolated cells

Number of tests

For 250 tests.

Preparation

Recombinant Annexin-V is produced in *E. coli* (strain NB42). The GST-tagged protein is purified by standard purification protocols.

Specificity

Annexin-V-Alexa 568 binds in a Ca^{2+} -dependent manner to negatively charged phospholipid surfaces and shows high specificity to phosphatidylserine.

Characteristics

Alexa 568 is a fluorescent label with the following characteristics:

excitation	in the range of 488 – 596 nm
emission	above 600 nm

The Annexin-V-Alexa 568-conjugate can be easily analyzed on a 488 nm laser Fluorescent Analytical Cell Sorter (FACS) machine, or using a fluorescence microscope.

Storage/stability

Stable at +2 to +8°C until the expiration date printed on the label.

Note: Protect from light! Aliquot if long-term stability is required, store aliquots at -15 to -25° , avoid repeated freezing and thawing.

2. Procedures and required material

Additional solutions required

- PBS
- Incubation buffer
- BOBO-1 stock solution

Solution	Composition/Preparation	Storage/Stability
Incubation buffer	Prepare a solution containing 10 mM HEPES/NaOH, pH 7.4, 140 mM NaCl, 5 mM CaCl ₂	3 month at +2 to +8°C
BOBO-1 stock solution	Prepare a stock solution of 50 µg/ml. BOBO-1 (e.g., Molecular Probes, Cat.-No. B-3582) in double-distilled water.	3 month at +2 to +8°C

Preparation of Annexin-V-Alexa 568 labeling solution

Predilute 20 µl Annexin-V-Alexa 568 in 1 ml Incubation buffer and add 20 µl BOBO-1 stock solution.

Note: Prepare freshly before use! 1 ml is enough for 10 samples.

Staining of cell suspensions

In the following table please find the staining procedure for cell suspensions.

Step	Action
1	Before staining grow cells in suspension, induce apoptosis.
2	Wash 10 ⁶ cells with PBS and centrifuge cells at 200 × g for 5 min.
3	<ul style="list-style-type: none"> • Resuspend the cell pellet in 100 µl of Annexin-V-Alexa 568 labeling solution. • Incubate 10-15 min at +15 to +25°C.
4	Analyze by fluorescence microscopy or on a flow cytometer (see 3. Analysis).

Staining of adherent cells

In the following table please find the staining procedure for adherent cells.

Step	Action
1	Before staining grow cells on chamber slides, induce apoptosis.
2	Remove chambers and silicon borders of cells grown on chamber slides.
3	Remove medium and cover slides with Annexin-V-Alexa 568 labeling solution (100 µl/chamber).
4	Put coverslips on slides and incubate for 10 – 15 min at +15 to +25°C.
5	Analyze by fluorescence microscopy or on a flow cytometer (see 3. Analysis). Note: Adherent cells are difficult to analyze in Flow cytometry.

2.2 Double labeling with monoclonal antibodies

Additional solutions required

- PBS
- Incubation buffer
- Directly labeled antibody solutions (e.g. anti-CD4-Fluorescein) or reconstituted according to the manufacturer.

Solution	Composition/Preparation	Storage/Stability
Incubation buffer	Prepare a solution containing 10 mM HEPES/NaOH, pH 7.4, 140 mM NaCl, 5 mM CaCl ₂	3 month at +2 to +8°C

Preparation of Annexin-V-Alexa 568 labeling solution

Predilute 20 µl Annexin-V-Alexa 568 in 1 ml Incubation buffer.

Note: Prepare freshly before use! 1 ml is enough for 10 assays.

Staining of cell suspensions

In the following table please find the staining procedure for cell suspensions.

Step	Action
1	Before staining grow cells in suspension, induce apoptosis.
2	Wash 10 ⁶ cells with PBS and centrifuge cells at 200 × g for 5 min.
3	Resuspend the cell pellet in 100 µl of Annexin-V-Alexa 568 labeling solution. Add up to 10 µl antibody solution in a concentration recommended by the manufacturer.
4	Incubate 10–15 min at +15 to +25°C.
5	Add 2 ml Incubation buffer. Centrifuge at 200 × g for 5 min.
6	Resuspend pellet in 100 µl Incubation buffer.
7	Analyze by fluorescence microscopy or on a flow cytometer (see 3. Analysis).

3. Analysis

Fluorescence microscopy

For evaluation by fluorescence microscopy use the following:

Fluorochrome	Filter used
BOBO-1 or Fluorescein	use 450 – 490 nm excitation in combination with a 515 nm bandpass filter
Annexin-V-Alexa 568	use 530 – 585 nm excitation in combination with a 615 nm longpass filter

Flow cytometry

Add 0.4 - 0.8 ml Incubation buffer to dilute the cells. Analyze on a flow cytometer using 488 nm laser excitation in combination with a 515 nm band-pass filter for BOBO-1 or Fluorescein detection, and a filter > 600 nm for Annexin-V-Alexa 568 detection.

Note: Electronic compensation of the instrument is required to exclude overlapping of the two emission spectra.

Figure 2

Differentiation of apoptotic versus non-apoptotic and necrotic cells are shown in figure 2.

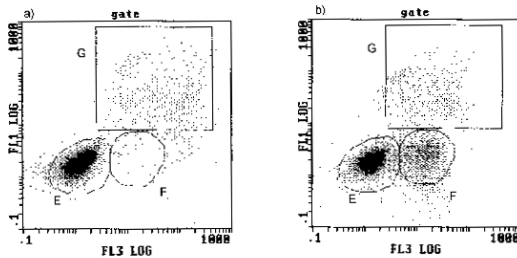
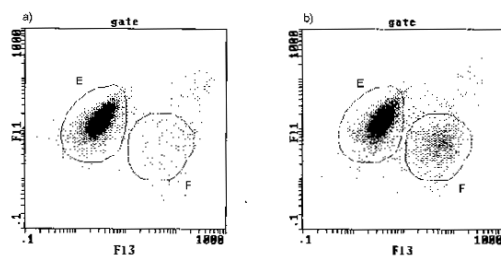


Fig. 2: FACS analysis of U937 cells stained with Annexin-V-Alexa 568 and BOBO-1. Cell cultivation for 4 h in the absence (a) or presence (b) of 4 µg/ml camptothecin.

Fluorescence	Cluster
FL1 = BOBO-1	E = living cells
FL3 = Annexin-V-Alexa 568	F = apoptotic cells
	G = necrotic cells

Figure 3

Double staining of apoptotic cells and control cells with monoclonal antibodies are shown in figure 3.

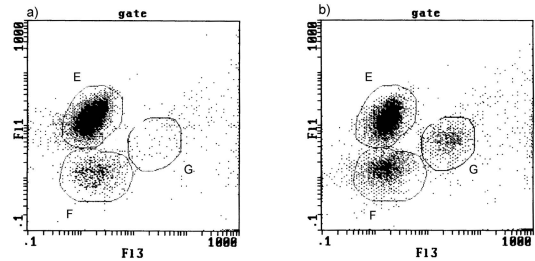


FACS analysis of U937 cells stained with Annexin-V-Alexa 568 and anti-CD4-fluorescein. Cell cultivation for 4 h in the absence (a) or presence (b) of 4 µg/ml camptothecin. The FL1 decrease in labeled Annexin-positive cells is due to the shedding of CD4 surface receptors on apoptotic cells.

Fluorescence	Cluster
FL1 = anti-CD4-fluorescein	E = living cells
FL3 = Annexin-V-Alexa 568	F = apoptotic cells

Figure 4

Non-apoptotic Raji cells were mixed with either apoptotic U937 cells or untreated U973 cells, and double labeled with the Annexin-V-Alexa 568 conjugate and a monoclonal antibody against CD4.



FACS analysis of cells stained by Annexin-V-Alexa 568 and anti-CD4-fluorescein.

Cell cultivation of U937 cells for 4 h in the absence (a) or presence (b) of 4 µg/ml camptothecin and mixed with untreated Raji cells.

The decreased CD4 labeling of annexin positive cells is due to shedding of surface receptors on apoptotic cells.

Fluorescence	Cluster
FL1 = anti-CD4-fluorescein	F = living Raji cells (low annexin and low anti-CD4 binding)
FL3 = Annexin-V-Alexa 568	E = living U937 cells (low annexin and high anti-CD4 binding)
	G = apoptotic U937 cells (high annexin and high anti-CD4 binding)

4. References

- 1 Andree, H.A.M. et al. (1990), *J. Biol. Chem.* **265**, 4923.
- 2 Fadok, V. et al. (1992) *J. Immunology* **148**, 2207.
- 3 Creutz, C.E. (1992) *Science* **258**, 924.
- 4 Vermes, I. et al. (1995) *J. Immunol. Methods* **184**, 39.
- 5 Koopman, G. et al (1994) *Blood* **84**, 1415.
- 6 Homburg, C.H.E. et al (1995) *Blood* **85**, 532.
- 7 Verhoven, B. et al (1995) *J. Exp. Med.* **182**, 1597.

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Ordering Information

Roche Applied Science offers a large selection of reagents and systems for life science research. For a complete overview of related products and manuals, please visit and bookmark our home page <http://www.roche-applied-science.com> and our Apoptosis Special Interest Site: <http://www.roche-applied-science.com/apoptosis>

Apoptosis-specific physiological change DNA fragmentation

Detection Mode/ Product	Pack Size	Cat. No.
Gel Electrophoresis		
Apoptotic DNA-Ladder Kit	20 tests	11 835 246 001
<i>In situ</i> assay		
In Situ Cell Death Detection Kit, TMR red	1 kit (50 tests)	12 156 792 910
In Situ Cell Death Detection Kit, Fluorescein	1 kit (50 tests)	11 684 795 910
In Situ Cell Death Detection Kit, AP	1 kit (50 tests)	11 684 809 910
In Situ Cell Death Detection Kit, POD	1 kit (50 tests)	11 684 817 910
Single reagents for TUNEL and supporting reagents		
TUNEL AP	70 tests (3.5 ml)	11 772 457 001
TUNEL POD	70 tests (3.5 ml)	11 772 465 001
TUNEL Enzyme	2 × 50 µl (20 tests)	11 767 305 001
TUNEL Label	3 × 550 µl (30 tests)	11 767 291 910
TUNEL Dilution Buffer	20 ml	11 966 006 001
ELISA		
Cell Death Detection ELISA	1 kit	11 544 675 001
Cell Death Detection ELISA ^{PLUS}	1 kit (96 tests)	11 774 425 001
Cell Death Detection ELISA ^{PLUS} , 10×	1 kit	11 920 685 001
Cellular DNA Fragmentation ELISA	1 kit (500 tests)	11 585 045 001
Microscopy or FACS		
Annexin-V-Alexa 568	250 tests	03 703 126 001
Annexin-V-Biotin	250 tests	11 828 690 001
Annexin-V-FLUOS	250 tests	11 828 681 001
Annexin-V-FLUOS Staining Kit	50 tests 250 tests	11 858 777 001 11 988 549 001
Western Blot		
Anti-Poly (ADP-Ribose) Polymerase	100 µl	11 835 238 001
FIENA		
Caspase 3 Activity Assay	1 kit	12 012 952 001
Homogenous Caspases Assay, fluorometric	100 tests 1000 tests	03 005 372 001 12 236 869 001
<i>In situ</i> Assay		
M30 CytoDEATH (formalin grade)	50 tests 250 tests	12 140 322 001 12 140 349 001
M30 CytoDEATH, Fluorescein	250 tests	12 156 857 001
Apoptosis Induction		
Anti-Fas (CD95/APO-1)	1000 tests	11 922 432 001
ELISA		
p53 pan ELISA	1 kit	11 828 789 001
Single reagents		
DNase I, grade I	20,000 U	10 104 132 001
Pepsin	1 g	10 108 057 001
Trypsin Solution	100 ml	10 210 234 001

Cell membrane alterations

Enzymatic activity

Expression of apoptosis-related proteins

Single reagents

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