Advion Interchim scientific

Fluorescent Annexin V

To measure swiftly Apoptosis in a variety of suspended cell types

Product Information

Name :	cat. #:	$\lambda_{exc./em.}$ (nm)
Annexin V – FITC	FP-M19651, 500µl	494 / 518
Annexin V – R-Phycoerythrin	FP-AH191A, 500µl (50 tests)	496,546,565 / 578
Annexin V – APC	FP-AK194A, 100 tests	650 / 660
Annexin V – Biotin	FP-FX8471, 200µl (100 tests)	to be used with labeled (strep)avidins

See superior Annexin V- FluoProbes®, and other related products

Storage: +4°C **DO NOT FREEZE**. Protect from light and moisture. (K)

Introduction

AnnexinV detects apoptotic cells by the fact that early after the cell has decided to execute apoptosis, it exposes phosphatidylserine (PS) at its plasma membrane surface. Annexin V recognizes the cell surface-exposed PS and binds with high affinity to it, before the dying cell changes morphology and cleaves its DNA (Vermes 1995). Apoptotic cells are stained with a fluorescent conjugate of Annexin-V by a simple and quick one-step staining procedure. No fixation of the cells and no washing procedures are necessary. The stained cells can be measured by flow cytometry, microscopy or other fluorescent based instruments.

AnnexinV is available labeld with various conventionnal fluorochromes, and biotin:
FITC label is a popular green label. For superior result, ask our alterantive FluoProbes488 label, that improves signal and phtostability for better discrimination by FCM and more contrsted images by microscopy.
The **R-PE** and **APC** labels are great phycobiliproteins red/NIR labels, with very intense fluorescence.
The **biotin** label allows versatile detections, using the second reagent (strep)avidin that is available with various other labels, fluorochromes or enzymes (please inquire).

As a result, our labeled AnnexinV provide a simple, rapid and ubiquitous method for early detection of apoptosis.

Directions for use

General protocol

1- Wash the cells of interest with culture medium or PBS and finally suspend them in 1x binding buffer at 10^6 - 10^7 cells/ml.

2- Add 5 µl labeled Annexin-V to 100 µl of the cell suspension prepared as given by step 1.

3- Incubate for 15-30 minutes in the dark.

4- Add 400 µl 1x binding buffer.

5- Wash cells with 1x binding buffer, or use as washing buffer (25 mM Hepes/NaOH pH 7.4, 140 mM NaCl, 5 mM KCl, 1 mM $MgCl_2$ and 2.5 mM $CaCl_2$; if necessary add 1 mg/ml BSA).

6- Measure the cell sample by flow cytometry or microscopy with settings suitable to the label.

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Simultaneous of mammalian cells using annexin V, PhiPhiLux G1D2, 7-AAD and immunophenotypic labeling

Viable cells can be simultaneously labeled with fluorochrome-conjugated annexin V (detecting PS asymmetry in the plasma membrane, an early marker of apoptosis), PhiPhiLux G1D2 (allowing the detection of functional caspase 3), 7-aminoactinomycin D (7-AAD) (detecting increased membrane permeability associated with both apoptosis and necrosis) and one or more fluorochrome-conjugated antibodies. A dual laser flow cytometer can be used for the simultaneous detection of the PhiPhiLux reagent (which emits in the FITC range), allophycocyanin-conjugated annexin V (which is excited by a HeNe or diode laser emitting at 632 nm and emits at 660 nm), 7-AAD (excited at 488 nm and emitting at 670 nm) and a PE-conjugated antibody. Fluorochrome compatibility is good, although careful intralaser compensation is required for simultaneous use of PE and 7-AAD. Little or no interlaser compensation is required for APC and 7-AAD.

The annexin V and PhiPhiLux reagents are usually available in kit form, with buffers provided. This method does not make use of any of the buffers provided in these kits, but attempts to replicate the optimal reaction conditions in the buffer system used. Do not use the kit buffers for this method.

Materials

- Allophycocyanin (APC)-conjugated annexin V: Annexin V has been conjugated to a variety of fluorochromes. APC-conjugated annexin V is used for multicolor flow applications.
- PhiPhiLux G1D2: The PhiPhiLux system is designed to detect caspase 3 activity using the consensus substrate peptide DEVD. PhiPhiLux G1D2 is excited by a standard 488 nm laser and emits at a wavelength range similar to FITC.
- 7-aminoactinomycin (7-AAD) is a DNA binding dye that excites at 488 nm and emits in the far red, with a peak at 670 nm. 7-AAD should be dissolved in EtOH at 1 mg/ml.
- Dulbecco's PBS (containing calcium and magnesium).

Method

- If the cells of interest are in in vitro tissue culture, harvest them and transfer them to 12 x 75 mm tubes. Centrifuge at 800 g and completely decant the supernatant. Nearly complete removal of the supernatant is critical for the following steps; the amount of remaining sup should be as low as possible (less than 50 µl if possible). It is assumed that the cell medium is complete for normal cell growth (containing serum and other growth factors); if cells are obtained from clinical or other in vivo sources, they should be washed in complete medium (such as RPMI containing 10% FBS) prior to use.
- Tap each tube to resuspend the cell pellet in the remaining supernatant. Add 50 µl of the PhiPhiLux reagent to each tube and shake. Incubate the tubes for 45 minutes at 37oC.
- Remove the tubes from the incubator and add 5 µl APC-conjugated annexin V per tube (from 0.2 mg/ml stock solution). The cells should not be washed between PhiPhiLux and annexin V addition. Incubate the cells at room temperature for 15 minutes.
- If subsequent immunohenotyping is desired, place the tubes on ice for 5 minutes (still no washing). Add the fluorochrome-conjugated antibody of interest and incubate for the necessary time interval (usually for 30 minutes). It should be noted that the tubes still contain the PhiPhiLux and annexin V reagents in the original supernatant; try to add the antibody in as minimal a volume as possible so as to not disrupt PhiPhiLux concentration equilibrium and loading. If no immunophenotyping is desired, skip this and go on to the next step.
- Resuspend the cells in 3 mls Dulbecco's PBS and centrifuge at 800 g. PBS containing calcium is critical for this single wash step, since annexin V will reversibly dissociate from PS moieties in the absence of divalent cations.
- Decant the supernatant and resuspend cells in a solution of 7-AAD at 5 µg/ml in Dulbecco's PBS. Allow the samples to sit at room temperature for 10 minutes, then analyze. All samples should be analyzed within 30 minutes of 7-AAD addition.
- PhiPhiLux can be analyzed through a standard FITC filter. 7-AAD can be analyzed through a Cy5 filter (such as a 675 nm narrow bandpass). APC can be detected through a 660 nm narrow bandpass filter.

Technical information

Apoptosis

Apoptosis is a well-organized process of cell death by suicide. It is physiological and it forms a fundamental process to the healthy organization of multicellular organisms (Wyllie *et al.* 1980). Recently it has been recognized that apoptosis or aberrant forms of apoptosis are involved in the pathophysiology of diseases such as congenital malformations, infections, malignancies, lymphoproliferative disorders, neurodegenerative disorders, alopecia, psoriasis and atherosclerosis. Apoptosis was first discovered in tissues on basis of its morphological appearance (Kerr *et al.* 1972). Gradually the morphological criteria for apoptosis, like cell shrinkage, nuclear condensation and pyknosis, were extended with biochemical criteria like the cleavage of DNA between the nucleosomes resulting in the ladder appearance of DNA on agarose gels (Wyllie *et al.* 1984). Until recently this typical feature was considered as the Hallmark for apoptosis. However, not all cells in apoptosis appeared to cleave their DNA strands between the nucleosomes (Oberhammer *et al.* 1993) and those which did,
cleaved not until late in the apoptotic pathway. New insights in the apoptotic process have come up with new parameters, which can be used to detect and measure apoptosis. One of these parameters is the phosphatidylserine (PS).During apoptosis the cell changes the structure of its plasma membrane (PM) to signal its suicide to the environment. Phagocytes pick up this signal and remove the dying cell by phagocytosis (Savill 1996). The appearance of PS at the cell surface is one of the structural changes which occurs and which is recognized by phagocytes (Fadok *et al.* 1992a,b).

Phosphatidylserine (PS)

The viable cell treats PS in a specific manner by localizing PS predominantly in the membrane leaflet facing the cytosol. The outer leaflet of the PM facing the environment is almost devoid of PS. Aminophospholipid translocases, the activity of which has been measured in all viable cell types tested so far, are thought to be responsible for this asymmetric distribution of PS. The molecular identity(ies) of the translocases have not been resolved so far (Diaz and Schroit 1996).
During apoptosis the equilibrium distribution of PS changes by an increased appearance of PS in the outer leaflet of the PM. It has been postulated that as yet unknown specific intrinsic membrane proteins facilitate this movement (Verhoven *et al.* 1996, Zwaal and Schroit 1997). This exposed PS serves the function of removal of the dying cell from the tissues. This functionality and the fact that specific molecular machineries regulate the localisation of PS in the plasma membrane strongly suggest that cell surface exposed PS constitutes a functionally important part of the apoptotic pathway.

Annexin-V properties

Annexin-V is a phospholipid binding protein that belongs to the Annexin family (Van Heerde 1995) . In the presence of calcium ions it exhibits a high affinity for binding selectively to phosphatidylserine (PS). Annexin-V displays very low affinity for phospholipid species like phosphatidylethanolamine, sphingomyelin and phosphatidylcholine. This was firstly demonstrated for model membranes (Tait 1989; Andree 1990) and later for blood platelets, which expose PS at their cell surface under certain activating conditions (Thiagarajan and Tait 1992; Dachary-Prigent 1993) . It is this phospholipid binding property which makes Annexin-V a powerful and selective tool to detect apoptotic cells.

Detection of apoptosis using Annexin-V

Annexin-V was used successfully to measure apoptosis of various cell types by flow cytometry (Koopman 1994, Homburg 1995, Vermes 1995, Martin 1995, Van Engeland 1996) . These studies showed that PS exposure starts soon after the cell has decided to execute apoptosis. The cell does so well before other features of apoptosis, like nuclear condensation and DNA cleavage, become visible. Cell surface exposure of PS also proceeds while the integrity of the plasma membrane remains un-compromised. These findings strongly suggest that specific machineries are being activated to translocate PS from the inner to the outer leaflet of the plasma membrane during the early phase of apoptosis. Once exposed at the surface PS remains there during the rest of the apoptotic pathway (see figure 1). Recently it was suggested that cell surface exposure of PS occurs downstream of the point of no return (Castedo 1996).

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As depicted by the figure the different phenotypes of apoptosis are characterized by the cell surface exposure of PS. Execution of apoptosis *in vitro* is eventually accompanied by a loss of plasma membrane integrity during the late phase. This phenotype is designated as secondary necrotic.

When conjugated to a fluorochrome, Annexin-V is a powerful tool to measure apoptosis by flow cytometry and fluorescence microscopy.

The phenomenon of PS exposure appears to be ubiquitous in the sense that all cell types tested so far exhibit this phenomenon under the action of all apoptosis inducing stimuli used so far (see table 1) (Martin *et al.* 1995) .

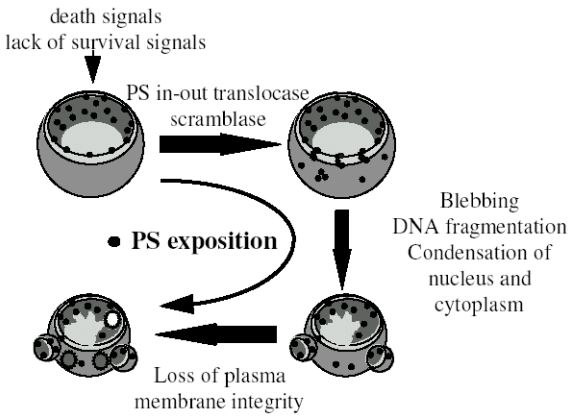

Figure 1: Schematic presentation of PS exposure during apoptosis

Table 1: Ubiquity of PS exposure during apoptosis

Cell Types	*Initiating stimulus*
Leukocytes	Plasma membrane receptor/ligand
neutrophils	lack of growth factors
T-lymphocytes	Fas/Fas ligand interaction
B-lymphocytes	Intracelulare receptor/ligand
monocytes	glucocorticoids
Tissue cells	Intracelluar signallling
endothelial cells	C2-ceramide
smooth muscle cells	Macromolecular synthesis
smooth muscle cells	actinomycin D
neurons	cycloheximide
Tumors	DNA
leukemia cells	inhibition topoisomerase II
carcinoma cells	damage by UV irradiation

Ordering information

To be used *in vitro* for research purposes only, not for diagnostic or therapeutic procedures!

Related products

- AnnexinV binding buffer (10x), FP-BU2080
- AnnexinV, recombinant, FP-JQ7610
- 7-Aminoactinomycin (7-AAD), FP-132303
- PhiPhiLux G1D2, U29491
- Propidium iodide, 1 mg/ml, FP-36774A
- Streptavin-Fluoprobes®488, FP-BA2221
- Annexin V- FluoProbes®488, *FCM* Grade, FP-BH4140, *Microscopy* Grade, FP-BH9390, or *in vivo* Grade, FP-BY6820
- Annexin V- FluoProbes®782, FP-DV7150
- Annexin V – FITC (25mg/ml) + Binding Buffer, FP-M1965C

See Products Highlights, BioSciences Innovations catalogue and e-search tool.

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