

# ANNEXIN V – FITC Kit

## Apoptosis Detection Kit

PN IM3546 – 200 tests

For Research Use Only. Not for use in diagnostic procedures.

### SPECIFICITY

The ANNEXIN V-FITC Kit is an apoptosis detection kit based on the binding properties of annexin V to phosphatidylserine (PS) and on the DNA-intercalating capabilities of propidium iodide (PI).

Apoptosis (or programmed cell death) was discovered in tissues on the basis of morphological changes of the cell (1). Gradually, the morphological criteria for apoptosis, like cell shrinkage, nuclear condensation and pyknosis, were complemented with biochemical criteria such as the cleavage of DNA between the nucleosomes, resulting in the ladder appearance of DNA on agarose gels (2). Until recently, this typical feature was considered the hallmark of apoptosis. However, not all cells in apoptosis appear to cleave their DNA strands between the nucleosomes (3) and those that do, do so only late in the apoptotic pathway.

New insights in the apoptotic process led to new parameters, which can be used to detect and measure apoptosis. One of these parameters is the appearance on the surface of the cell of phosphatidylserine (PS), a negatively charged phospholipid usually located in the inner leaflet of the plasma membrane. In the early phase of apoptosis, the integrity of the cell membrane is maintained but the cells lose the asymmetry of their membrane phospholipids (4–7). PS becomes exposed at the cell surface and forms one of the specific signals for recognition and removal of apoptotic cells by macrophages (5, 8, 9).

Annexin V, a  $Ca^{2+}$ -dependent and phospholipid-binding protein, binds preferentially to PS, with high affinity. Apoptotic cell is stained by Annexin V before the dying cell changes its morphology and hydrolyzes its DNA (4, 10–13). The early detection and the ubiquity of apoptosis-associated PS exposure makes the ANNEXIN V-FITC Kit, in view of its simple and rapid protocol, a powerful tool for the study of apoptosis (10, 12, 14, 15). In the use of the ANNEXIN V-FITC Kit, the affinity of annexin V-FITC for PS in the presence of  $Ca^{2+}$  is exploited. The conjugation of annexin V with FITC in a 1:1 stoichiometric complex does not change the native phospholipid-binding properties of Annexin V. Binding kinetics show a fast association of Annexin V-FITC with the phospholipid membrane, if PS and  $Ca^{2+}$  are available. The ANNEXIN V-FITC kit is designed for the convenient, rapid measure of apoptosis in a sample of suspended cells. Apoptosis-

associated PS exposure is a phylogenetically conserved mechanism of mammalian and non-mammalian species, and its detection by annexin V has been demonstrated for human, mouse, rat, hamster, chick and drosophila cell types tested so far (5–7, 9, 10, 14, 16–18).

### REAGENT

#### Contents

- 1 vial Annexin V-FITC ready-to-use, liquid, 200  $\mu$ L.
- 6 vials 10X concentrated Binding Buffer, liquid, 1.7 mL.
- 1 vial Propidium Iodide\*, red powder, 250  $\mu$ g.

\* Warning: propidium iodide (PI) is toxic.

#### Features of Annexin V-FITC

Modified human recombinant Annexin labeled with FITC. Displays no measurable anti-coagulant activity in vitro.

- F/P ratio: 1 (stoichiometric complex)
- Purity: > 99% pure according to Fast Protein Liquid Chromatography.
- Concentration: 25  $\mu$ g/mL.

**NOTE:** Annexin V bind optimally to PS at free  $Ca^{2+}$  concentration of 1–5 mM or more. Should the medium contain less, adjust concentration by adding  $CaCl_2$  or replace the medium by the provided Binding Buffer at 1X concentration. Our experience is that RPMI1640 is less suitable for the assay. Other media such as DMEM may also be used.

### FLUORESCENCE

Annexin V-FITC :	Absorption maximum : 492 nm Emission maximum : 520 nm
Propidium iodide :	Absorption maxima : 370 nm, 550 nm Emission range : 560 – 680 nm

### APPLICATION

Detection of apoptosis by flow cytometry or fluorescence microscopy.

### STATEMENT OF WARNINGS

1. This ANNEXIN V-FITC Kit contains propidium iodide (PI) which is a potential mutagen. We recommend to avoid contact with skin and eyes, to wear suitable protective clothing and gloves, and appropriate eye / face protection. Mechanical ventilation and respiratory protection are also recommended.
2. Specimens, samples and all material coming in contact with them should be handled as if capable of transmitting

infection and disposed of with proper precautions.

3. Never pipet by mouth and avoid contact of samples with skin and mucous membranes
4. Avoid microbial contamination of reagents or incorrect results might occur.
5. Use good laboratory practices when handling these reagents.

### STORAGE CONDITIONS AND STABILITY

These reagents are stable up to the expiration date when stored at 2–8°C in the absence of light. Do not use after the expiration date. Do not freeze.

### REAGENT PREPARATION

- Dilute the 10X concentrated Binding Buffer 10 fold with distilled water and place the diluted buffer on ice. Prepare a quantity sufficient for the expected number of assays.
- Dissolve the 250  $\mu$ g PI in 1 mL of 1X Binding Buffer and place the PI solution on ice.
- After use, the solutions should be stored at 2–8°C.

### PROCEDURE

1. Wash cell samples with ice-cold culture medium or PBS and centrifuge for 5 minutes at 500 x g at 4°C. Discard supernatant, and resuspend the cell pellets in ice-cold 1X Binding Buffer to  $5 \times 10^5$  ~  $5 \times 10^6$  cells / mL. Keep tubes on ice.
2. Add 1  $\mu$ L of annexin V-FITC solution and 5  $\mu$ L of dissolved PI to 100  $\mu$ L of the cell suspensions prepared as given in step 1. Mix gently.
3. Keep tubes on ice and incubate for 15 minutes in the dark.
4. Add 400  $\mu$ L of ice-cold 1X binding buffer and mix gently.
5. Analyze cell preparations within 30 minutes by flow cytometry (or fluorescence microscopy).

### POSITIVE CONTROLS

1. Incubate cells with 3% formaldehyde-containing PBS for 30 minutes on ice. Centrifuge cells, discard the formaldehyde buffer, and resuspend cell pellets in cold 1X Binding Buffer to  $5 \times 10^5$  ~  $5 \times 10^6$  cells / mL. Proceed to staining from step 2 of the staining procedure.
2. Induction of apoptosis of Fas / CD95-expressing cells such as human Jurkat cells or mouse thymocytes.  
Add 100 ng/mL of purified agonistic anti-Fas / CD95 antibody (clone 7C11; PN

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IM2387 for human cells, or clone RK-8 ; PN IM2558 for mouse cells) to the culture medium and incubate cells for 4 ~ 24 hours at 37°C (5% CO<sub>2</sub>). Centrifuge cells, discard supernatant, and suspend cell pellets in cold 1X binding buffer to  $5 \times 10^5 \sim 5 \times 10^6$  cells / mL. Proceed to staining from step 2 of the staining procedure.

### GENERAL NOTES AND PRECAUTIONS

The flow cytometer is preferably set such that the distribution of the annexin V-negative population is in the first decade of the FITC channel and the distribution of the PI-negative population is in the first decade of the PI channel. Optimal parameter settings can be found using a positive control (see above).

The incubation with annexin V and PI should be carried out on ice so as to arrest further progress of the cells through the stages of viability  $\Rightarrow$  apoptosis  $\Rightarrow$  secondary necrosis. For rat thymocytes, when kept on ice, the population distribution (viable, apoptotic, secondary necrotic) remains stable for at least 6 hours.

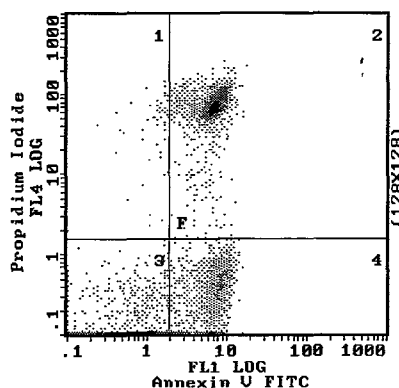
### EXAMPLE DATA

Flow cytometric analysis of apoptotic Jurkat cells after staining by the ANNEXIN V-FITC kit (performed on a COULTER® EPICS® XL™ flow cytometer). Jurkat cells have been treated by 100 ng/mL of agonistic anti-Fas (CD95) antibody (clone 7C11; PN IM2387) for 6 hours. Analysis is done with the XL System II™ Software. The biparametric representation (FL1 versus FL4) shows three distinct populations, i) the viable cells which have low FITC and a low PI signal, ii) the apoptotic cells, which have high FITC and a low PI signal, iii) the secondary necrotic cells which have high FITC and a high PI signal (see figure). Depending on the cell type and on culture and centrifugation conditions, a fourth population corresponding to the damaged viable cells with low FITC and a high PI signal may be visualized.

Quadrant 2: 40.5% (secondary necrotic cells)

Quadrant 3: 27.1% (viable cells)

Quadrant 4: 31.4% (apoptotic cells)



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### MISCELLANEOUS

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