# Annexin V-FITC/PI Apoptosis Detection Kit

## A211

Vazyme

Instruction for Use

Version 22.1

Vazyme

# Contents

## 01/Product Description

Apoptosis is a normal physiological process in embryonic development and maintenance of body homeostasis. Apoptosis has distinct morphological features, including loss of cell membrane asymmetry and attachment, cytoplasmic and nucleoplasmic condensation, and DNA fragmentation between nucleosomes. Disruption of cell membranes is an early feature of apoptosis. Under normal physiologic conditions, phosphatidylserine (PS) is predominantly located in the inner leaflet of the plasma membrane. Upon initiation of apoptosis, PS loses its asymmetric distribution across the phospholipid bilayer and is translocated to the extracellular membrane leaflet marking cells as targets of phagocytosis. Annexin V is a 35 - 36 kDa $Ca^{2+}$-dependent phospholipid-binding protein with high affinity to PS, so it can bind to the cell membrane of early apoptotic cells through PS exposed on the outside of the cell. Therefore, Annexin V is recognized as one of the sensitive indicators for detecting early apoptosis of cells.

Annexin V is labeled with green fluorescent FITC, and the labeled Annexin V retains a high affinity for PS and can be used as a probe to detect apoptosis by fluorescence microscopy or flow cytometry. Since PS eversion occurs in the early stage of apoptosis, Annexin V-FITC staining can identify the occurrence of apoptosis in the early stage of apoptosis. Propidium Iodide (PI) is a nucleic acid dye. PI cannot penetrate the intact cell membrane of normal cells or early apoptotic cells, but can penetrate the cell membrane of membrane-damaged cells such as late apoptotic cells or necrotic cells, and bind to their DNA, causing their nuclei to become red. It can be used to distinguish early apoptotic cells from late apoptotic cells or necrotic cells. Therefore, by co-staining Annexin V-FITC with PI, cells in different apoptotic stages can be distinguished. On the scattergram of dual-color flow cytometry, Annexin V-FITC and PI double negative are normal cells, Annexin V-FITC positive and PI negative are early apoptotic cells, and Annexin V-FITC and PI double positive cells are late apoptotic cells or necrotic cells.

## 02/Components

| Components | A211-01 (50 rxns) | A211-02 (100 rxns) |
|---|---|---|
| Annexin V-FITC | 250 μl | 500 μl |
| PI Staining Solution | 250 μl | 500 μl |
| 1 × Binding Buffer | 25 ml | 2 × 25 ml |

## 03/Storage

Store at 2 ~ 8℃ and protect Annexin V-FITC and PI Staining Solution from light. If not in use for a long time, store Annexin V-FITC at -30 ~ -15℃ to avoid repeated freezing and thawing. Adjust the shipping method according to the destination.

## 04/Applications

It is applicable for the apoptosis detection of adherent cells and suspension cells.

## 05/Notes

For research use only. Not for use in diagnostic procedures.

1. The apoptosis is a rapid process. It is recommended that samples should be analyzed within 1 h after staining.
2. The occurrence and stage of apoptosis are determined by detecting changes in the cell membrane. Therefore, before Annexin V-FITC and PI staining, fixatives and penetrants that destroy the integrity of the cell membrane cannot be used to fix or penetrate the membrane.
3. During the operation, the action is gentle to avoid mechanical damage to the cells.
4. For adherent cells, digestion is a critical step. If there are floating cells when the adherent cells induce apoptosis, the floating cells and the adherent cells should be collected and stained together. Care should be taken when handling adherent cells to avoid artificial damage. If the trypsin digestion time is too short, the cells need to be vigorously blown and sucked to fall off, which will easily cause damage to the cell membrane and lead to excessive intake of PI; if the digestion time is too long, the cell membrane is also easy to be damaged, and even affect the bond between PS and Annexin V-FITC on the cell membrane. During digestion, spread the trypsin all over the bottom of the well plate, shake gently to make the trypsin fully contact with the cells, then pour out most of the trypsin, and use the remaining small amount of trypsin to digest for a period of time until the space between cells increases, and the bottom of the plate is The variegated can be terminated. Try to use trypsin without EDTA, EDTA will affect the binding of Annexin V to PS.
5. If the sample is from blood, must be sure to remove platelets from the blood. This is because platelets contain PS, which can bind to Annexin V, thereby interfering with the experimental results. Platelets can be washed away by centrifugation at 1,400 rpm (200 × g) using a buffer containing EDTA.
6. Briefly centrifuge the reagent before opening the cap, and shake the liquid on the inner wall of the cap to the bottom of the tube to avoid spilling the liquid when opening the cap.
7. Annexin V-FITC and PI are photosensitive substances, please avoid light during operation.
8. For your safety and health, please wear a lab coat and disposable gloves.

## 06/Experiment Process

### 06-1/Sample Staining

1. Induce apoptosis according to experimental requirements. Untreated cell samples should be included in the test sample as a negative control. In addition, when performing flow detection, the experimental group needs to be individually stained with Annexin V-FITC and PI to adjust the compensation.
2. Harvest cells: collect 1 - 5 × $10^5$ cells
   Suspension cells: centrifuge at 4℃ 1,800 rpm (300 × g) for 5 min each time, discard the culture supernatant;
   Adherent cells: digest the cells with trypsin without EDTA, collect the cells after terminating the digestion, centrifuge at 4℃ 1,800 rpm (300 × g) for 5 min each time, and discard the supernatant.
3. Wash cells: wash cells twice with pre-cooled PBS, centrifuge at 4℃ 1,800 rpm (300 × g) for 5 min each time, discard the supernatant.
4. Cell resuspension: add 100 μl of 1 × Binding Buffer, and gently mix the single cell suspension.
5. Cell staining: add 5 μl Annexin V-FITC and 5 μl PI Staining Solution, and mix gently; incubate in the dark at room temperature (20 ~ 25℃) for 10 min; add 400 μl 1 × Binding Buffer and mix gently. The samples were detected by flow cytometry within 1 h after staining.

▲ In order to avoid losing cells when washing cells, the front end of the large pipette tip can be added the small pipette tip to aspirate solution.

### 06-2/Sample Analysis

**A. Flow cytometry analysis**

The excitation wavelength of the flow cytometer is 488 nm; the green fluorescence of FITC is detected in the FL1 channel; the red fluorescence of PI is detected in the FL2 or FL3 channel, FL3 is recommended, and 10,000 events are collected for each sample. Use software such as FlowJo for data analysis. FL1 is the abscissa and FL3 is the ordinate. According to FITC and PI fluorescence values, the cut-off between positive and negative cells can be given to set the gate. In a typical experiment, cells can be divided into three subpopulations: living cells are double negative (Annexin V-$FITC^-$/$PI^-$); early apoptotic cells are Annexin V-FITC single positive (Annexin V-$FITC^+$/$PI^-$); Late apoptotic cells are double positive for Annexin V-FITC and PI (Annexin V-$FITC^+$/$PI^+$).

**B. Fluorescence microscopy analysis**

Place a drop of the stained cell suspension on a glass slide, cover the cells with a coverslip, and place it under a fluorescence microscope for observation. Observed with a bichromatic filter under a fluorescence microscope: the fluorescence signal of Annexin V-FITC is green, and the fluorescence signal of PI is red.

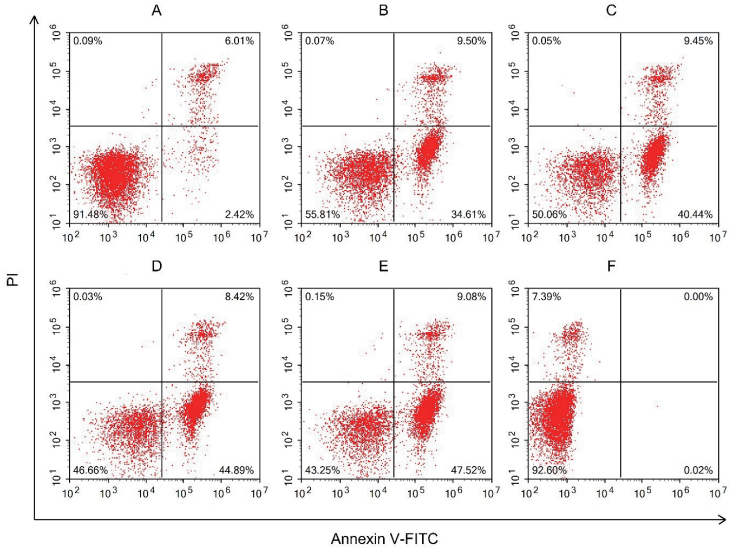
▲ For adherent cells, the coverslip can be directly used to culture cells and induce apoptosis.

## 07/Examples

### Apoptosis of Jurkat cells induced by camptothecin detected by flow cytometry

The apoptosis of Jurkat cells (human T lymphoma cells) was induced with camptothecin (CPT) at the induction concentrations of 0, 4, 8, 12, and 16 μM, respectively. After induction for 4 h, the staining was carried out according to the 06/Experimental Process. The results of flow cytometry are shown in the following figure.

(A) 0 μM. (B) 4 μM. (C) 8 μM. (D) 12 μM. (E) 16 μM. (F) Competition blocking experiment. Jurkat cells were induced with 16 μM CPT for 4 h, and the cells were collected and washed according to this instruction. Before staining, unlabeled Annexin V protein was added to incubate for 10 min, and then Annexin V-FITC/PI staining was performed. The dye-free Annexin V bound PS on the cells, and when re-stained, Annexin V-FITC could not bind PS, indicating the specificity of the staining.



## 08/FAQ & Troubleshooting

◇ Annexin V-FITC Staining Failed or The Positive Rate Was Low.

① The first step: determine whether the inducer used in the experiment can produce apoptosis. This can be excluded by setting positive drug control with clear apoptosis-inducing effect.

② Annexin V-FITC staining fail, the most common cause of experimental operation is improper digestion of adherent cells.

③ The binding of Annexin V to PS requires $Ca^{2+}$. The Binding Buffer contains 2.5 mM $Ca^{2+}$. Trypsin digestion with EDTA will affect the staining. It is recommended to use trypsin without EDTA. If trypsin with EDTA is used, the EDTA must be completely removed by washing steps.

④ After washing cell pellets with PBS, should remove residual liquid as much as possible. Phosphate radicals in the residual PBS will form calcium phosphate precipitates.

⑤ The cap of the Binding Buffer should be tightly closed to prevent the formation of calcium carbonate precipitation after the entry of $CO_2$ in the air. This leads to a decrease in free $Ca^{2+}$ and then the experiment fails.

⑥ The density of PS on the cell membrane of some cells is low, and the staining effect is poor. Therefore, it is recommended to change the cell line or use the TUNEL method to detect apoptosis.

⑦ If it is adherent cells, the floating cells after drug induction should also be collected. These cells are often apoptosis-positive cells, and discarding them will result in lower positive results.

◇ False Positive.

① In the experiment, it's found that the double positive ratio of Annexin V-FITC/PI is too high after staining of the control cells without induced apoptosis. The reason for this result may be the low viability of the cells themselves. It is recommended to use trypan blue staining to calculate the cell viability, and the negative control trypan blue-rejected cells without drug treatment should be greater than 95%. If the cell viability is low, it is recommended to resuscitate the cells. In general, the resuscitated cells should be passaged at least 2 - 3 times before the experiment can be performed.

② Another possibility is that the cells are improperly operated:

a. During the operation, the cells are blown and sucked too vigorously.

b. The digestion time of the adherent cells is too long during the digestion process. These may lead to the destruction of the cell membrane and false positives.

③ In addition, early apoptosis can occur within a few hours of induction, so the operation should usually be completed within 48 h; if the induction time is too long, nutrients will be exhausted, resulting in poor cell status and high false-positive results.

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