

## Instruction Manual

Product Name	Product Description	Size	Catalog Number
Apoptotic/Necrotic Cells Detection Kit	Apoptotic/Necrotic Cells Detection Kit	50 assays	PK-CA707-30017

### Introduction

Apoptosis and necrosis are two major processes by which cells die. Apoptosis is an active, genetically regulated disassembly of the cell from within. Disassembly creates changes in the phospholipid content of the cytoplasmic membrane outer leaflet. Phosphatidylserine (PS) is translocated from the inner to the outer surface of the cell for phagocytic cell recognition. The human anticoagulant, annexin V, is a 35 kD  $\text{Ca}^{2+}$ -dependent phospholipid protein with a high affinity for PS. Annexin V labeled with fluorescein (FITC) ( $\lambda_{\text{abs}}/\lambda_{\text{em}} = 492/514$  nm) stains PS on the surface of apoptotic with green fluorescence.

Necrosis normally results from a severe cellular insult. Both internal organelle and plasma membrane integrity are lost, resulting in spilling of cytosolic and organellar contents into the surrounding environment. Ethidium homodimer III (EthD-III) is a highly positively charged nucleic acid probe, which is impermeant to live cells and early apoptotic cells, but stains necrotic cells and late apoptotic cells (entering into secondary necrosis) with red fluorescence ( $\lambda_{\text{abs}}/\lambda_{\text{em}}$  (with DNA) = 528/617 nm). EthD-III is a superior alternative to propidium iodide (PI) or ethidium homodimer I used in some of our competitors' kits due to its significantly higher affinity for DNA and higher fluorescence quantum yield.

The PromoKine Apoptotic/Necrotic Cells Detection Kit provides a convenient assay for quantifying apoptotic (green) and necrotic (red) cells within the same cell population by flow cytometry or fluorescence microscopy.

For more information, please see Frequently Asked Questions on page 3.

Please also see our Apoptotic/Necrotic/Healthy Cell Detection Kit, which includes the membrane permeant nuclear stain Hoechst 33342, which stains the nuclei of all cells with blue fluorescence.

### Kit Contents

FITC-Annexin V in TE buffer containing 0.1% BSA and 0.1%  $\text{NaN}_3$  (pH 7.5), 250  $\mu\text{l}$

Ethidium Homodimer III (EthD-III) in PBS, 250  $\mu\text{l}$

5X Binding Buffer, 15 ml

*Caution:* Sodium azide and ethidium homodimer III are hazardous substances. Handle with care and dispose properly.

### Storage and Stability

Store the kit at 4°C and protect FITC-Annexin V and EthD-III from light. Do not freeze!

### Intended Use

For in vitro research use only. Not for diagnostic or therapeutic procedures.

## Experimental Procedures

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### Suspension cells

1. Induce apoptosis in cells by a desired method.
2. Prepare 1X Binding Buffer by diluting 5X Binding Buffer 1:5 in distilled water.
3. Wash cells with PBS once and resuspend cells at  $2\text{-}3 \times 10^6$  cells /ml in 1X Binding Buffer.
4. Pipet 100  $\mu$ l cell suspension in a microcentrifuge tube.
5. Add 5  $\mu$ l of FITC-Annexin V and 5  $\mu$ l of Ethidium Homodimer III solutions to each tube.

**Note:** We recommend including two control samples, for staining with each of the probes (FITC-Annexin V and EthD-III) separately.

6. Incubate at room temperature for 15 minutes in the dark.
7. For flow cytometry analysis, add 400  $\mu$ l 1X Binding Buffer to each tube and measure fluorescence in FITC and propidium iodide channels within 1 hour of staining.
8. For fluorescence microscopy analysis, wash cells with 1X Binding Buffer, resuspend cells in 1X Binding Buffer, and observe fluorescence using FITC and Texas Red filter sets.

### Adherent cells for fluorescence microscopy

1. Induce apoptosis in cells by a desired method (Note: You may also grow cells directly on a coverslip).
2. Prepare 1X Binding Buffer by diluting 5X Binding Buffer 1:5 in distilled water.
3. Wash cells twice with 1X Binding Buffer.
4. Prepare staining solution by adding 5  $\mu$ l of FITC-Annexin V and 5  $\mu$ l of Ethidium Homodimer III to 100  $\mu$ l 1X Binding Buffer. Prepare enough staining solution to cover cells.  
Note: reducing the concentration of EthD-III may result in better signal:background ratio for some cell types.
5. Incubate samples with the staining solution for 15 minutes at RT, protected from light.
6. Wash cells with 1X binding Buffer 1-2 times.
7. Cover cells with 1X Binding Buffer and observe fluorescence using FITC and Texas Red filter sets.
8. When staining cells on coverslips: Mount coverslip onto a slide with 1X Binding Buffer, aspirate residual 1X binding Buffer and seal coverslip with nail polish or (recommended for permanent preservation of slides) mount coverslip onto a slide with a drop PromoFluor Antifade Reagent (PK-PF-AFR1), remove excess liquid and wait until the mounting medium slowly solidifies.

### Adherent cells for flow cytometry

1. Detach cells from cell culture plate or well using trypsin or other cell dissociation methods.
2. Pellet cells and discard supernatant.
3. Follow staining protocol for suspension cells.

Optional: Formaldehyde fixation may be performed for long term preservation of cell staining. Annexin V binding to PS requires calcium, therefore buffers used for washing and fixation should contain 1.25 mM calcium chloride ( $\text{CaCl}_2$ ). Fixation may increase background staining by Ethidium Homodimer III. Cells should be washed thoroughly prior to fixation to remove unbound dye.

### Expected Results

Green fluorescent plasma membrane staining identifies apoptotic cells, while necrotic cells are identified by red fluorescent nuclear staining. Late apoptotic cells may show both red and green staining.

## References

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1. Boersma A.W., et al. Quantification of apoptotic cells with fluorescein isothiocyanate-labeled annexin V in chinese hamster ovary cell cultures treated with cisplatin. *Cytometry*. 1996 Jun 1; 24(2):123-30.
2. Homburg C.H., et al. Human neutrophils lose their surface Fc gamma RIII and acquire Annexin V binding sites during apoptosis in vitro. *Blood*. 1995 Jan 15; 85(2):532-40.
3. Martin S.J., et al. Early redistribution of plasma membrane phosphatidylserine is a general feature of apoptosis regardless of the initiating stimulus: inhibition by overexpression of Bcl-2 and Abl. *J Exp Med*. 1995 Nov 1; 182(5):1545-56.

4. Vermes I., et al. A novel assay for apoptosis. Flow cytometric detection of phosphatidylserine expression on early apoptotic cells using fluorescein labelled Annexin V. *J Immunol Methods*. 1995 Jul 17; 184(1):39-51.

## Frequently Asked Questions

Question/Problem	Answer/Suggestion
Can I use the kits for staining fixed cells or tissues?	No. Fixation, freezing, sectioning, or dissociation of tissues can affect the PS on the outer leaflet and compromise membrane integrity. Both Annexin V and Ethidium Homodimer III rely upon the presence of intact membranes in healthy cells to accurately distinguish healthy cells from apoptotic or necrotic cells. To detect apoptosis in fixed cells and tissues we recommend our TUNEL based DNA Fragmentation Kits (see website). Currently we are not aware of a fluorescent probe that specifically detects necrotic cells in fixed tissues. Necrotic cells in tissue sections are identified based on morphological criteria.
Can I use the kits for staining live embryos, tissue explants or organotypic slice cultures?	The assays are designed to stain dissociated cells in culture and have not been validated for organ culture. Annexin V staining of early chicken and mammalian embryos in culture has been reported in the scientific literature. For staining of living tissues, the specimen would need to be thin enough to allow exposure of the cells to the 36 kDa Annexin V protein. Also, damage to cell membranes from dissection or sectioning of tissues could result in high background staining.
Can I fix cells after staining?	Yes, cells can be fixed with formaldehyde after staining. Because Annexin V staining is dependent on calcium, all buffers used for washing and fixation should contain 1.25 mM CaCl <sub>2</sub> . Fixation may increase the background signal from Ethidium Homodimer III. Wash the cells several times to remove unbound Ethidium Homodimer III before fixation.
What species does Annexin V cross-react with?	Annexin V is a 36 kDa protein that binds to the phospholipid phosphatidylserine. Therefore, Annexin V binding is not species-specific.
What is the source of your Annexin V?	The Annexin V protein that we use is a recombinant protein made in <i>E. coli</i> .
What is the purpose of the binding buffer?	The binding buffer is an isotonic buffer containing calcium, which is essential for the binding of Annexin V to phosphatidylserine.
I am using fewer cells than suggested in the protocol, should I adjust the amount of FITC-Annexin V and Ethidium Homodimer III in the assay?	No, the concentration of Annexin V and Ethidium Homodimer III should be kept constant regardless of cell number. However, the staining concentrations can be increased or decreased if necessary to optimize staining.
I am having trouble with low signal or high background staining.	The concentration of Annexin V and Ethidium Homodimer III can be increased or decreased if necessary to optimize staining for different cell types.
I am afraid that I'm losing some of my apoptotic cells during processing of cells from an adherent culture, how can I make sure that they are accounted for?	You may wish to process floating and detached cells separately – collect the washes and spin them down and then stain those cells using the protocol for suspension cells. Alternatively, you may collect floating cells, detach the adherent cells with trypsin (without EDTA), pool the cells together, and use the suspension cell protocol for staining.

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