

Apoptotic/Necrotic/Healthy Cells Detection Kit



Instruction Manual

Product Name	Size	Catalog Number
Apoptotic/Necrotic/Healthy Cells Detection Kit	50 assays	PK-CA707-30018

Introduction

Apoptosis and necrosis are the two major processes leading to cell death. Apoptosis is an active, genetically regulated “Cell suicide” induced by diverse factors. This disassembly of the cell from within creates multiple events and is associated e.g. with changes in the phospholipid content of the outer leaflet of the cytoplasmic membrane. Phosphatidylserine (PS) is translocated from the inner to the outer surface of the cell for phagocytic cell recognition.

Apoptotic cells can be easily and reliably identified with Annexin V, a 35 kD Ca^{2+} -dependent phospholipid protein with a high affinity for PS, that binds specifically to PS exposed on the outer membrane leaflet of apoptotic cells. When Annexin V is labeled e.g. with fluorescein (FITC; $\lambda_{\text{abs}}/\lambda_{\text{em}} = 492/514$ nm) apoptotic cells are stained brightly green.

Necrosis normally results from a severe cellular insult. Since both internal organelle and plasma membrane integrity are lost, this results 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 or apoptotic cells, but stains necrotic cells intensively with red fluorescence ($\lambda_{\text{abs}}/\lambda_{\text{em}} = 528/617$ nm). Due to its significantly higher affinity for DNA and higher fluorescence quantum yield, EthD-III is a superior alternative to propidium iodide (PI) or ethidium homodimer I (EthD-I) commonly used in some other cell staining kits.

Membrane-permeable Hoechst 33342 is a minor groove-binding DNA stain that emits bright blue fluorescence upon binding to DNA ($\lambda_{\text{abs}}/\lambda_{\text{em}} = 350/461$ nm). It is used for blue-fluorescent staining of the entire cell population.

The Apoptotic/Necrotic/Healthy Cells Detection Kit provides a convenient assay for quantifying apoptotic (green), necrotic (red) cells and healthy (blue only) within the same cell population by flow cytometry or fluorescence microscopy. Membrane permeable Hoechst 33342 stains the nuclei of both apoptotic and necrotic cells. However, healthy cells are stained by Hoechst only, not by FITC-Annexin V or EthD-III. Late apoptotic cells (entering into secondary necrosis) are stained both green and blue. Necrotic cells are stained both red and blue. Cells stained with triple colors blue, green and red are dead cells progressing from apoptotic cell population.

Kit Contents

FITC-Annexin V in TE buffer containing 0.1% BSA and 0.1% NaN_3 (pH 7.5), 250 μl

Ethidium Homodimer III (EthD-III) in PBS, 250 μl

Hoechst 33342 in PBS, 250 μl

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. Do not freeze. Protect FITC-Annexin V, EthD-III and Hoechst 33342 from light.

Intended Use

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

Experimental Procedures

This assay must be used on unfixed cells. Both Annexin V and EthD-III rely upon the presence of intact membranes in healthy cells to accurately distinguish healthy cells from apoptotic or necrotic cells. The dyes cannot be used for live/dead discrimination in fixed cells or tissues and cannot withstand fixation after staining.

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 (mix 1 volume of 5X buffer with 4 volumes of dH₂O).
3. Wash cells with PBS once and resuspend cells at 2-3 x 10⁶ cells /ml in 1X Binding Buffer.
4. Pipet 100 µl cell suspension in a microcentrifuge tube.
5. Add 5 µl of FITC-Annexin V, 5 µl of Ethidium Homodimer III and 5 µl Hoechst 33342 solutions to each tube.

Note: We recommend including two control samples, for staining with each of the probes (FITC-Annexin V, EthD-III, Hoechst 33342) separately. Reducing the concentration of EthD-III may result in better signal:background ratio for some cell types.

6. Incubate at room temperature for 15 minutes in the dark.
7. For flow cytometry analysis, add 400 µl 1X Binding Buffer to each tube and measure fluorescence in FITC, propidium iodide and DAPI 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, Texas Red or Cy3 and DAPI filter sets.

Adherent cells for microscope viewing

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 (mix 1 volume of 5X buffer with 4 volumes of dH₂O).
3. Wash cells twice with 1X Binding Buffer.
4. Prepare staining solution by adding 5 µl of FITC-Annexin V, 5 µl of Ethidium Homodimer III and 5 µl of Hoechst 33342 to 100 µl 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, Texas Red or Cy3 and DAPI 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 (CaCl₂). 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 green and red staining. All cell nuclei will be stained with blue fluorescence. Dead cells may stain more intensely with Hoechst 33342 than live cells.

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 ₂ . 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.

References

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. 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.
3. 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.
4. Belloc F., et al. A flow cytometric method using Hoechst 33342 and propidium iodide for simultaneous cell cycle analysis and apoptosis determination in unfixed cells. *Cytometry*. 1994 Sep 1; 17(1):59-65.

PromoCell GmbH

Sickingenstr. 63/65
69126 Heidelberg
Germany

Email: info@promocell.com
www.promocell.com

USA/Canada

Phone: 1 – 866 – 251 – 2860 (toll free)
Fax: 1 – 866 – 827 – 9219 (toll free)

Deutschland

Telefon: 0800 – 776 66 23 (gebührenfrei)
Fax: 0800 – 100 83 06 (gebührenfrei)

France

Téléphone: 0800 90 93 32 (ligne verte)
Téléfax: 0800 90 27 36 (ligne verte)

United Kingdom

Phone: 0800 – 96 03 33 (toll free)
Fax: 0800 – 169 85 54 (toll free)

Other Countries

Phone: +49 6221 – 649 34 0
Fax: +49 6221 – 649 34 40