CELL TECHNOLOGY

APO ACTIVE 3™

Antibody to active caspase 3 FITC / PE Detection Kits

Apoptosis Detection and in situ labeling of active caspase in live cells.

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Background

Apoptosis is an evolutionarily conserved form of cell suicide, which follows a specialized cellular process. The central component of this process is a cascade of proteolytic enzymes called caspases. These enzymes participate in a series of reactions that are triggered in response to pro-apoptotic signals and result in cleavage of protein substrates, causing the disassembly of the cell (1).

Caspases have been identified in organisms ranging from C. *elegans* to humans. The mammalian caspases play distinct roles in apoptosis and inflammation. In apoptosis, caspases are responsible for proteolytic cleavages that lead to cell disassembly (effector caspases), and are involved in upstream regulatory events (initiator caspases). An active caspase consists of two large (~20 kD) and two small (~10 kD) subunits to form two heterodimers which associate in a tetramer (2-4). As is common with other proteases, caspases are synthesized as precursors that undergo proteolytic maturation, either autocatalytically or in a cascade by enzymes with similar specificity (5).

Caspase 3, also known as CPP-32, Apopain or Yama, is a key effector caspase in the apoptotic pathway (6). It is present in many different cell lineages and is responsible for the cleavage of a variety of molecules such as poly ADP-ribose polymerase (PARP), protein kinase Cd, actin and DNA-dependent protein kinase (7,8).

Assay Principle

Cell Technology's highly specific APO ACTIVE 3 $_{TM}$ Kit utilizes a primary rabbit affinity purified polyclonal antibody raised against amino acid 163-175 of murine caspase 3 (9). This neo epitope is present on the p18 subunit of cleaved caspase 3 (9). Cell undergoing apoptosis are fixed and permeabilized prior to the addition of the primary antibody. A secondary FITC or Phycoerythrin (PE) labeled goat anti rabbit antibody is used to visualize the bound rabbit anti caspase 3 polyclonal antibody.

A **B**

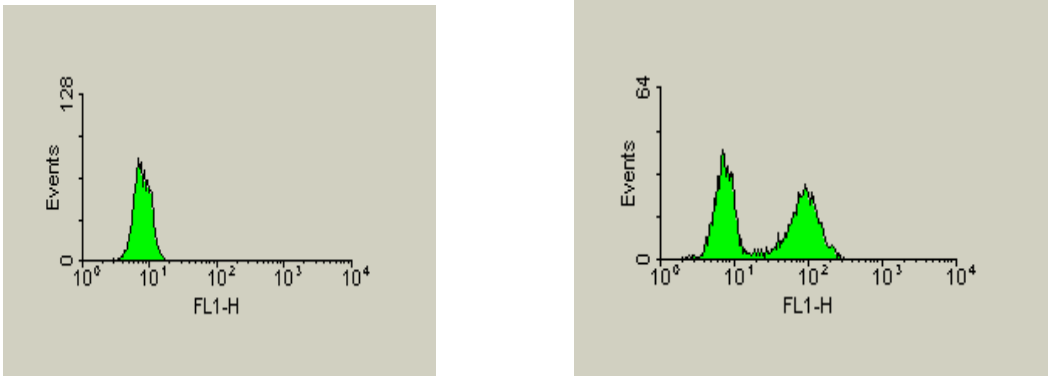

Figure 1: Jurkat Cells were stimulated with staurosporine (B) or DMSO (A) for 2 hours. The cells were washed and fixed for 15 minutes. The cells were then permeabilized with saponin and stained with Rabbit anti active caspase 3 antibody for 1 hour. The samples were then stained with FITC labeled Goat anti Rabbit antibody

II. Warnings and Precautions

1. For Research Use Only. Not for use in diagnostic procedures.
2. Reagents contains sodium azide; harmful if swallowed or absorbed through the skin. Sodium azide can react with lead and copper-containing sink drains forming explosive compounds. When disposing of excess Wash Buffer, flush sink drain with large amounts of water.
3. Practice safe laboratory procedures by wearing protective clothing and eye ware.

III. Storage and Shelf Life

1. Store primary and **FITC** labeled secondary antibodies at -20^{0}C. Store **PE** labeled secondary antibody at 4-8^{0}C.
2. Store 10X Fixative at room temperature. 1X fixative is stable for 1 week at 4^{0}C.
3. Avoid multiple freeze-thaw cycles of the antibodies.
4. Keep FITC or PE labeled secondary antibody protected from light at all times.

IV. Kit Content

A. Catalog#: FAB200-1 Tests: 25

1. 1 Vial of Rabbit anti active affinity purified polyclonal antibody……. Part # 1002
2. 1 Vial of FITC Goat anti Rabbit affinity purified polyclonal antibody…..Part # 2002
3. 10X Fixative solution…… Part # 3001

B. Catalog#: FAB200-2 Tests: 100

1. 1 Vial of Rabbit anti active affinity purified polyclonal antibody……. Part # 1001
2. 1 Vial of FITC Goat anti Rabbit affinity purified polyclonal antibody…..Part # 2001
3. 10X Fixative solution…… Part # 3001

C. Catalog#: PAB200-1 Tests: 25

1. 1 Vial of Rabbit anti active affinity purified polyclonal antibody……. Part # 1002
2. 1 Vial of PE Goat anti Rabbit affinity purified polyclonal antibody……..Part # 2004
3. 10X Fixative solution…… Part # 3001

D. Catalog#: PAB200-2 Tests: 100

1. 1 Vial of Rabbit anti active affinity purified polyclonal antibody……. Part# 1001
2. 1 Vial of PE Goat anti Rabbit affinity purified polyclonal antibody…….Part # 2003
3. 10X Fixative solution…… Part # 3001

V. Materials Required But Not Supplied

1. Solutions

a. Phosphate-Buffered Saline (PBS) + 1% Saponin (Sigma Cat#: S7900)
b. Phosphate-Buffered Saline (PBS) + 2% BSA: Wash Buffer or other buffer of choice.

2. Equipment

a. A flow cytometer, equipped with a 15 mW, 488 nm argon excitation laser, with appropriate filters.
b. Fluorescence microscope or plate reader with appropriate filters. Ex=488nm , FITC Em= 515-530nm or PE Em= 578nm

VI. Experimental Preparation and Setup

1X Working Dilution of Rabbit anti active caspase 3

1.Make a 1X working concentration of the antibody by diluting it 1:20 in PBS + 2%BSA Buffer:

A. For 25 Test Kits: Each vial contains 13.75 uL of antibody. Spin down the vial and add 261.25 uL of PBS + 2%BSA Buffer. Gently vortex to mix contents of vial. This is a 1:20 dilution. This 1X concentration is stable for several months at 4ºC. For long term storage aliquated and freeze below –20^{0}C.

B. For 100 Test kit: Each vial contains 55uL of antibody. Spin down the vial and add 1.045 mL of PBS + 2%BSA Buffer. This 1X concentration is stable for several months at 4ºC. For long term storage aliquated and freeze below –20^{0}C.

2. Avoid repeated freezing and thaw cycles.

1X Working Dilution of Secondary Antibody: FITC or PE Labeled Goat anti Rabbit

1. Make a 1X working concentration of the antibody by diluting it 1:10 in PBS + 2%BSA Buffer. For example to 20 mL of antibody add 180 mL of PBS + 2%BSA Buffer.

2. Mix thoroughly at room temperature. We recommend using the 1x dilution within 24 hours however contents may be stored for up to one week at 4ºC or at -20ºC for 6 months. Store PE labeled antibodies at 4ºC. Avoid repeated freezing and thaw cycles.

1X Working Dilution of Fixative Solution

1. Make a 1X working concentration of the fixative by diluting it 1:10 in PBS. For example to 1 mL of 10X fixative add 9 mL of PBS. Store 1X fixative at 4^{0}C.

VII. Staining of Activated and Control Cells With Rabbit anti Active Caspase 3.

1. Induce cells and negative control samples at time points according to your specific protocol. Cells maybe cultured in a tissue culture plate or culture tube. If you are using adherent cells, detach them after the required activation time point before fixing them.

Note: For adherent cells, keep the media to recover cells that may have spontaneously detached due to apoptosis.

2. Fixing Samples

a. After the required activation time, wash the cells 2 times in PBS.

b. After the final wash, decant the supernatant and gently vortex the cell pellet. Re-suspend the cells at a concentration of 1x 10^6 to 3x 10^6 cells/mL in 500 mL of 1X fixative solution. Incubate the samples at room temperature for 15-20 minutes.

c. Wash cells 2 times in PBS.

d. After the final wash decant the supernatant and gently vortex the cell pellet. If you are going to label your samples right away proceed to step 3. If you are going to store the cells, adjust the cell concentration, with PBS + 2% BSA, to 1 x 10^6 to 3x 10^6 cells /mL and store at 4^{o}C for up to 48 hours.

3. Labeling Samples

a. Adjust the cells concentration to a **maximum** of 1 x 10^6 to 3 x 10^6 cells /mL with PBS + 1% Saponin.

b. Pipette out 100 mL to 200 mL of cells into a 12x75 mm culture tube or any suitable tube that will hold 2mls.

c. Add 10 mL of the 1X Rabbit anti active caspase 3. Vortex the tube and incubate for 30 to 60 minutes at room temperature.

d. Wash the samples 2X (2mL washes) in PBS + 1% saponin. After the final wash decant the supernatant and add 10 mL of the 1X FITC or PE labeled Goat anti Rabbit antibody. Vortex the samples and incubate for 30 to 60 minutes at room temperature in the dark.

4. Washing Samples
a. To each tube add 2 ml of PBS + 1% Saponin and centrifuge the samples at 300g for 5 minutes.
b. After the centrifuge step, decant the supernatant and gently vortex the cells pellet.
c. Add 2 mL of PBS + 2% BSA and centrifuge the samples at 300g for 5 minutes.
d. After the centrifuge step, decant the supernatant and gently vortex the cells pellet. Add 400 to 500 mL of PBS + 2% BSA per tube. If you are going to analyze the samples, proceed to **step VIII.** Otherwise, add 40-50mL of **10X** fixative and store the samples at 4^0C for up to 24 hours prior to analysis.

VIII. Cell Analysis

1. Single color Flow cytometry
Flow cytometry analysis is done using a 15 mW argon ion laser at 488 nm.
Measure FITC on the FL1 channel or PE (Phycoerythrin) on the FL2 channel. Generate a log FL1 (X-axis) or FL2 (X-axis) versus number of cells (Y-axis) histogram.
2. Bicolor Flow cytometry
Controls For Flow Cytometry
Three types of control samples are recommended for flow cytometry to aid in setting up electronic compensation and quadrant statistics. For bicolor experiments, these are: 1) cells stained with *APO ACTIVE 3* only; 2) cells stained with *other antibodies or stains only*; and 3) unstained cells.
3. **Fluorescence microscope**
After step 4c, Washing Samples, re-suspend the cells in 100mL to 200mL PBS + 2% BSA.
Place one drop of the cell suspension onto a microscope slide and cover with a cover slip. To view FITC fluorescence, observe cells under a fluorescence microscope using the following filters: excitation 490 nm, emission 520 nm. To view PE fluorescence, observe cells under a fluorescence microscope using the following filters: excitation 488 nm, emission 578 nm.

IX. Adherent Non Detached Cells

a. Seed your cells in black clear bottom 96 well plates or any other suitable culture dish.
Note: Take into account that the total volume of 1X Rabbit anti active caspase 3, or incubation time with 1X Rabbit anti active caspase 3 will increase if larger culture dishes are used.
b. Induce cells at time points according to your specific protocol.
c. After step **a**. Spin down culture plates. Adherent cells undergoing apoptosis will detach and float in the medium.
d. Gently aspirate out media. Add 1x Fixative and incubate for 15 to 20 minutes.
e. Gently aspirate out 1X fixative and add PBS + 1% saponin. Spin down plates and gently aspirate out PBS + 1% saponin.
f. Repeat one more wash step by adding PBS + 1% saponin and spinning down tissue culture plates. Gently aspriate out PBS + 1% saponin.
g. Add 100-200 mL of PBS + 1% Saponin and add 10 ml of Rabbit anti active caspase 3.
h. Incubate for 30 to 60 minutes and then wash tissue culture plates 2X as described in step **f** above. After the final wash add 100 to 200 mL of PBS + 1% saponin and 10 mL of FITC or PE labeled Goat anti Rabbit. Incubate for 30 to 60 minutes.

i. After final incubation step, gently aspirate out the supernatant. Fill the wells with PBS+1% saponin. Spin down the plate and gently aspirate out the supernatant. Repeat this step one more time. After the final wash add PBS + 2% BSA and observe cells under a fluorescence microscope using a FITC or PE filter or add 1X fixative to store the cells. We recommend analyzing the fixed samples within 24 hours.

X. Immunohistochemistry Protocol for Floating Sections

1. Materials Required but not Supplied.

1. PBS
2. BSA
3. Triton X-100
4. Normal goat serum
5. Slides cover slips and mounting medium

2. Fixation

1. Fix tissues slices with 1X fixative (see section VI of main instruction booklet for preparation of 1X fixative) overnight at 4°C. Alternatively tissue slices maybe cut fresh with out fixation and snap frozen in liquid nitrogen.
2. Make cryosections or Vibratome sections of fixed or snap frozen tissue up to a thickness of 50mm.
3. Wash the fixed cut tissue sections 3 times for 5 minutes each wash in PBS + .1%Triton buffer.
4. Primary antibody preparation; Further dilute the 1X of Rabbit anti active Caspase 3 antibody from **Section VI Experimental Setup and Preparation** 1:10 – 1:20 with PBS + 2% BSA. It is recommended to add 1-2% normal goat serum to the antibody to reduce non-specific binding.
5. Flood the tissue sections with the diluted antibody and incubate in a humidified chamber for 1 to 2 hours at room temperature.
   Note: 1X Rabbit anti active caspase 3 antibody maybe diluted in PBS+ 1%BSA + .1% Triton X-100. Each investigator should titrate out the Rabbit anti active caspase 3 antibody in order to reduce background.
6. Wash the tissue sections 3 times, 10 minutes per wash, in PBS + 1% Triton X-100.
7. Secondary antibody preparation: Further dilute the 1X working dilution of Goat anti Rabbit FITC or Goat anti Rabbit PE labeled antibody from **Section VI Experimental Setup and Preparation** 1:10 – 1:20 with PBS + 2% BSA. Flood the tissue sections with the diluted antibody and incubate in a humidified chamber for 1 to 2 hours at room temperature in the dark.
   Note: Dilute the Goat anti Rabbit FITC or PE labeled antibody in PBS + .1% Triton X-100, 1% BSA. You may add 1% normal goat serum to the diluent in order to reduce background signal. Each investigator should titrate out the Goat anti Rabbit FITC or PE labeled antibody in order to reduce background.
8. Wash the tissue sections 3 times in PBS + .1% Triton X-100.
9. Mount sections on slides using conventional mounting medium. Anti fade mounting media may be used. Sections maybe countered stained prior to mounting.

Publications Cited in Manual

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