Caspase Assay Kit

Catalog No.	Caspase	Inhibitor Reagent
abx299016	Poly-Caspase	FAM-VAD-FMK
abx299019	Caspase 1, 4 and 5	FAM-YVAD-FMK
abx299026	Caspase 2	FAM-VDVAD-FMK
abx299017	Caspase 3/7	FAM-DEVD-FMK
abx299018	Caspase 6	FAM-VEID-FMK
abx299020	Caspase 8	FAM-LETD-FMK
abx299021	Caspase 9	FAM-LEHD-FMK
abx299027	Caspase 10	FAM-AEVD-FMK

Size: 25 tests / 100 tests

Storage: Store at 4 °C. Avoid prolonged exposure to light. After reconstitution with DMSO, use the Caspase Inhibitor Reagent immediately, or store in the dark at -20 °C for up to 6 months, with no more than 2 freeze/thaw cycles.

Application: For analyzing active caspases in apoptotic cells.

Introduction

Caspases are proteolytic enzymes that play key roles in apoptosis and inflammation in mammalian organisms. In apoptosis, effector caspases (3, 6 and 7) are involved in proteolytic cleavages leading to cell disassembly. Initiator caspases (8, 9 and 10) regulate apoptosis upstream, while caspase 1 is associated with pyroptosis and inflammasome activity. Activated caspase enzymes cleave proteins by recognizing a 3 or 4 amino acid sequence that must include an aspartic acid (D) residue in the P1 position, which is the target for the cleavage reaction at the carbonyl end.

The Caspase Inhibitor Reagent consists of carboxyfluorescein (FAM) and a fluoromethyl ketone (FMK) linked by three or four amino acids (see table above) which are specific for different activated caspases. Caspase enzymes cannot cleave this reagent – instead an irreversible covalent bond is formed between FMK and the enzyme active site, inhibiting further enzyme activity, and simultaneously labeled with the FAM green fluorescent probe, which optimally excites at 488-492 nm and has a peak emission at 515-535 nm. Cells can then be fixed or frozen, or counterstained with Propidium Iodide and 7-AAD (not included) to distinguish apoptosis from necrosis. Nuclear staining can be carried out with Hoechst 33342. Samples should not be paraffin-embedded after labeling as this quenches the FAM dye.

Kit Components (25 tests)

1. Caspase Inhibitor Reagent: 1 vial
2. Fixative Solution: 6 ml
3. Propidium Iodide (0.25 mg/ml): 1 ml
4. Hoechst 33342 (0.2 mg/ml): 1 ml
5. Apoptosis Wash Buffer (10X): 15 ml

Kit Components (100 tests)

1. Caspase Inhibitor Reagent: 4 vials
2. Fixative Solution: 6 ml
3. Propidium Iodide (0.25 mg/ml): 1 ml
4. Hoechst 33342 (0.2 mg/ml): 1 ml
5. Wash Buffer (10X): 60 ml

Materials Required But Not Provided

1. Fluorescence microscope, fluorescence plate reader, and/or flow cytometer
2. Black 96-well plate (if using plate reader)
3. Centrifuge and centrifuge tubes
4. Hemocytometer
5. Pipettes and pipette tips
6. Vortexer
7. Deonized water
8. PBS (pH 7.4)
9. FBS or BSA
10. Reagents to induce caspase activity and create controls (e.g. staurosporine, camptothecin, nigericin)
11. DMSO
12. 90% ethanol or 3% formaldehyde

Protocol

A. Experimental Design and Preparation

Cell staining can be completed within a few hours; however, this assay uses living cells, which require periodic maintenance and cultivation several days in advance. In addition, once the required number of cells has been cultivated, time must be allotted for the experimental treatment or caspase activation process. Create separate cell populations, for example: (1) treated cell population(s): cells that were exposed to the experimental treatment(s); (2) negative control: a negative control population of cells grown in a normal cell culture environment or received a placebo treatment; and (3) positive control: cells induced to activate caspases using a known caspase activation protocol.

Since this assay detects the presence of catalytically active forms of caspase enzymes, plan the experiment so that Caspase Inhibitor Reagent will be diluted and administered when caspases are expected to be activated in the cells. It is recommended to use 10 µl of 30X Caspase Inhibitor Reagent per 300 µl of cells at 3-5 × 10^5 cells/ml, but the amount may vary based on the experimental conditions and the instrument used for analysis. The end user will need to adjust the amount of Caspase Inhibitor Reagent to accommodate the cell line used and the conditions employed.

Culture cells to a density optimal for the specific experimental conditions or apoptosis induction protocol. Cell density should not exceed 10^6 cells/ml. Cells cultivated more than this concentration may begin to naturally enter apoptosis. An initial experiment may be necessary to determine when and how much Caspase Inhibitor Reagent to use as the resulting positive signal is a direct measurement of caspase activity occurring during the incubation period.

B. Sample Preparation

1. Controls

Create experimental samples and control cell populations as per the Experimental Procedure above. The induced positive cell population and negative control cell population tubes should come from a common pool of cells and contain similar quantities of cells. Create negative controls by culturing an equal volume of non-induced cells for every labeling condition. If analyzing cells with a flow cytometer, create Propidium Iodide (PI) instrument controls using formaldehyde or ethanol to compensate for bleed-over of the PI signal into FL-1.

2. Apoptosis Induction

Prior to carrying out the assay, determine a reproducible method for obtaining a positive control by triggering caspase activity. This process varies significantly between different cell lines. For example, apoptosis may be induced with 2-4 µg/ml camptothecin or 1-2 µM staurosporine for > 4 hours.

3. Pyroptosis Induction (Caspase 1 only)

The optimal pyroptosis/caspase 1 induction protocol will vary significantly between cell lines. Determine a reproducible method for obtaining a caspase 1 positive control prior to commencing the experiment. For example, caspase 1 activation may be induced in THP-1 cells using 5-10 ng/ml phorbol myristate acetate (PMA) in cell culture media for 12-24 hours (until cells become adherent), followed by exposure to 100 ng/ml lipopolysaccharide (LPS) and 5 mM adenosine triphosphate (ATP) for 24 hours. Alternatively, pyroptosis may be induced in cells using Nigericin in cell culture media at 1-20 µM for 3-24 hours at 37 °C. The end user will need to adjust the concentration of Nigericin and treatment period to accommodate the cell line used and the conditions employed.

4. Propidium Iodide Controls

Propidium iodide (PI) is used to distinguish between live and dead cells by staining necrotic, dead, and membrane-compromised cells red. Propidium iodide instrument controls for flow cytometry can be set up as follows:

1. Label 2 centrifuge tubes, one as PI-Negative (Live) and the other as PI-Positive (Dead).
2. Add 1-5 × 10^5 non-induced healthy cells to each tube.

3. Centrifuge at 200 × g for 5-10 minutes at room temperature to pellet the cells. Remove the supernatants.
4. To create the PI-Negative (Live) control, resuspend cells in the PI-Negative (Live) tube 300 µl of 1X Apoptosis Wash Buffer or PBS containing 1% BSA to maintain the integrity of the cell membrane.
5. To create the PI-Negative (Dead) control, either:
    a. Resuspend cells in 300 µl of 3% formaldehyde (in 1X Apoptosis Wash Buffer or 97% PBS). Allow to stand for 30 minutes on ice.; or
    b. Resuspend cells in 300 µl of 90% ethanol formaldehyde (in 1X Apoptosis Wash Buffer or 10% PBS). Gently vortex for 30 seconds.
6. Add 1 ml of 1X Apoptosis Wash Buffer or PBS containing 1% BSA.
7. Centrifuge at 200 × g for 5-10 minutes. Remove the supernatants.
8. Resuspend in 500 µl 1X Apoptosis Wash Buffer or PBS containing 1% BSA.
9. If ethanol was added: Add 500 µl of non-induced healthy cells (use roughly the same number of cells originally added) to the ethanol-killed cells and mix, to create a sample with distinct positive and negative peaks.
10. Add Propidium Iodide to a final concentration of 0.5% v/v (e.g. add 2.5 µl of Propidium Iodide to 500 µl of sample).
11. Read immediately on the flow cytometer and compensate bleed-over of the red PI signal from FL-2 or FL-3 into FL-1. It may be easier to compensate PI spillover into FL-1 when read in FL-3. View Propidium Iodide (PI) under a long pass filter with the excitation at 488-492 nm, emission > 610 nm; nuclei-bound PI has a maximum emission at 617 nm.

C. Reagent Information and Preparation

1. Caspase Inhibitor Reagent

The Caspase Inhibitor Reagent should only be prepared immediately before use.

1. Reconstitute a vial of Caspase Inhibitor Reagent with 50 µl DMSO to prepare a 150X Caspase Inhibitor Reagent stock solution. Once reconstituted, the stock solution can be stored in the dark at -20 °C for up to 6 months, with no more than 2 freeze/thaw cycles.
2. Immediately prior to adding samples and controls, add 200 µl PBS to the vial to prepare the 30X Caspase Inhibitor Reagent solution. Use within 30 minutes of preparation.

2. Apoptosis Wash Buffer

The Apoptosis Wash Buffer is used to wash cells following exposure to the Caspase Inhibitor Reagent. It contains mammalian proteins to stabilize cells after staining, and 0.01% sodium azide to inhibit bacterial growth. Cell culture media containing FBS and other additives can be used to wash cells instead of Apoptosis Wash Buffer if desired.

If a precipitate is observed in the 10X Apoptosis Wash Buffer, gently warm until all precipitates have dissolved, do not boil. Dilute 1/10 in deionized water (e.g. add 15 ml of 10X Apoptosis Wash Buffer to 135 ml of deionized water to prepare 150 ml of 1X Apoptosis Wash Buffer). 1X Apoptosis Wash Buffer can be stored at 4 °C for up to 1 week or stored at -20 °C for up to 6 months.

3. Hoechst 33342

Hoechst 33342 is a cell-permeant nuclear stain that emits blue fluorescence when bound to double-stranded DNA. It is used to stain the nuclei of living or fixed cells, to distinguish condensed pyknotic nuclei in apoptotic cells, and for cell cycle studies. When bound to nucleic acids, it has a maximum absorbance at 350 nm and a maximum emission at 480 nm. It is revealed under a microscope using a UV-filter with excitation at 365 nm and emission at 480 nm. It is

provided as a ready-to-use solution (concentration 0.2 mg/ml). Use personal protective equipment (such as lab coats, disposable gloves, lab goggles) when handling this reagent.

4. Fixative Solution

The Fixative Solution is a formaldehyde solution designed to cross-link and aggregate intracellular components. If the stained cell populations cannot be evaluated immediately after labeling with the Caspase Inhibitor Reagent, add Fixative Solution at 1/5 – 1/10 (e.g. add 100 µl Fixative Solution to 900 µl cells, to add Fixative Solution at 1/10). Do not add Fixative Solution until all the staining and final wash steps have been completed. Fixed cells may be stored on ice or at 4 °C for up to 16 hours, avoid exposure to light. The Fixative Solution will not interfere with the FAM dye. Do not use absolute ethanol or methanol-based fixatives as they will inactivate the FAM dye. Do not fix cells that will be stained later with Propidium Iodide.

D. Assay Procedure for Suspension Cells

Create experimental samples and control cell populations as per the Experimental Procedure in Section A. Cells may need to be concentrated to 2-5 × 10^5 cells/ml immediately prior to adding the Caspase Inhibitor Reagent as both microscopy and plate reader analysis methods require high cell concentrations. Start with a larger volume of cells at 3-5 × 10^5 cells/ml (which is a typical density for cell culture) and then concentrate cells and resuspend to 300 µl per sample when ready for staining.

1. Expose cells to the experimental or control condition. If analyzing with a flow cytometer, set aside four populations to create instrument controls with PI-Positive and PI-Negative cells, and Caspase Inhibitor Reagent-induced and Caspase Inhibitor Reagent-non-induced cells.
2. If analyzing with a fluorescence microscope or plate reader, concentrate cells to 2-5 × 10^5 cells/ml just prior to adding the Caspase Inhibitor Reagent. Fluorescence microscopy requires an excess of 2 × 10^6 cells/mL to obtain 5-20 cells per image field. Flow cytometry can analyze samples at 3-5 × 10^5 cells/ml.
3. Transfer 290 µl cells into fresh tubes. Add 10 µl of 30X Caspase Inhibitor Reagent solution to each tube for a final volume of 300 µl. If different cell volumes were used, add 30X Caspase Inhibitor Reagent at 1/30. Mix gently. The amount of Caspase Inhibitor Reagent should be optimized for each cell line and experimental condition.
4. Incubate cells at 37°C in the dark. The incubation period may range from 30 minutes to several hours and should be optimized for each cell line and experimental condition. As cells may settle on the bottom of the tubes, gently resuspend by swirling cells every 10-20 minutes to ensure an even distribution of Caspase Inhibitor Reagent.
5. If cells are to be analyzed with a fluorescence microscope, cells may optionally be counter-stained with Hoechst 33342. Add Hoechst 33342 to a final concentration of 0.5% v/v (e.g. add 1.5 µl Hoechst 33342 to 300 µl cell suspension) and incubate for 5 minutes at 37 °C.
6. Add 2 ml of 1X Apoptosis Wash Buffer and gently mix.
7. Centrifuge at 200 × g for 5-10 minutes at room temperature. Carefully remove and discard supernatants. Gently vortex the pellets to disrupt clumping. Resuspend in 1 ml of 1X Apoptosis Wash Buffer and gently mix.
8. Centrifuge at 200 × g for 5-10 minutes at room temperature. Carefully remove and discard supernatants.
9. If cells are to be analyzed with a fluorescence microscope or fluorescence plate reader, repeat the wash process a third time. If cells are to be analyzed a flow cytometer, two wash steps are generally sufficient.
10. Gently vortex pellets to disrupt clumping. Analyze cells using a fluorescence microscope (Section F), fluorescence plate reader (section G), or flow cytometer (Section H).

E. Assay Procedure for Adherent Cells

Adherent cells need to be handled carefully to avoid the loss of any cells that round up and come off the culture surface. In microscopy or plate reader applications where trypsinization is not required, adherent cells can be stained and washed directly on the chamber slide, well, or culture surface. To avoid losing cells that are no longer adherent during washing, spin down all overlay media and wash buffer and recombine washed cell pellets with adherent samples prior to analysis. Cells may be trypsinized to create suspensions, which may be labeled with Caspase Inhibitor Reagent before or after trypsinization. Avoid trypsinizing cells prior to labeling with a live/dead stain, like PI or 7-AAD. Cell membranes exposed to trypsin could be transiently permeable to live/dead dyes for a variable time depending upon the cell line.

1. Culture cells in T25 flasks, culture dishes, or chamber slides and expose to the experimental or control conditions.
2. If staining cells while adherent, go to Step 4. If suspension cells are required for the final analysis: Remove overlay media. Spin to pellet any loose cells. Trypsinize adherent cells, or alternatively Caspase Inhibitor Reagent can be added first, followed by washing and trypsinization steps. Neutralize with trypsin inhibitor, as found in cell culture media with 20% FBS. Add 2-5 ml media.
3. Prepare trypsinized cells for staining: Centrifuge at 200 × g for 5-10 minutes at room temperature. Remove all but ~100 µl supernatant. Resuspend cells in 300-500 µl cell culture media containing 10-20% FBS. If necessary, count cells and adjust the concentration and volume of cell suspension to fit the experiment.
4. Add 30X Caspase Inhibitor Reagent solution to samples at 1/30 for a final Caspase Inhibitor Reagent concentration of 1X. Mix the cell suspension to disperse the Caspase Inhibitor Reagent. If staining adherent cells, add Caspase Inhibitor Reagent directly to the overlay media. The concentration of Caspase Inhibitor Reagent should be optimized for each cell line, experimental condition, and method of analysis.
5. Incubate at 37 °C for 30-60 minutes, mixing gently every 10-20 minutes to disperse the reagent.
6. Wash cells:
    - **Trypsinized adherent (suspension) cells:** Add 2 ml 1X Apoptosis Wash Buffer and gently mix. Centrifuge at 200 × g for 5-10 minutes at room temperature, and carefully remove the supernatant. Resuspend cells in 2 ml 1X Apoptosis Wash Buffer and gently mix. Centrifuge again at 200 × g for 5-10 minutes at room temperature, and carefully remove the supernatant. For flow cytometry analysis, two wash steps are generally sufficient. For microscopy or fluorescence plate reader analysis, repeat wash procedure a third time (resuspend samples, gently pellet by centrifugation, and carefully the remove supernatant).
    - **Adherent cells:** To avoid losing cells that are no longer adherent during washing, spin down all overlay media and wash buffer and recombine washed cell pellets with the adherent samples prior to analysis. Carefully remove overlay media containing Caspase Inhibitor Reagent and replace with 1X Apoptosis Wash Buffer. Incubate at 37 °C for 10 minutes to allow any unbound Caspase Inhibitor Reagent to diffuse out of cells. Carefully remove the wash buffer and replace with fresh 1X Apoptosis Wash Buffer and incubate at 37 °C for 10 minutes. Carefully remove the wash buffer again, replace with fresh 1X Apoptosis Wash Buffer and incubate at 37 °C for 10 minutes. Gently remove the overlay buffer.
7. If cells are to be analyzed with a fluorescence microscope, cells may optionally be counter-stained with Hoechst 33342 or another compatible fluorescent dye. Add Hoechst 33342 to a final concentration of 0.5% v/v (e.g. add 5 µl Hoechst 33342 to 1 ml cell suspension) and incubate for 5 minutes at 37 °C. If using other compatible ancillary dyes, follow the manufacturer's specific instructions for staining samples.
    - Note: Live/dead cell stains should not be used after trypsinization. Cell membranes exposed to trypsin could

be transiently permeable to live/dead dyes for a variable time depending upon the cell line. To identify dead cells with a live/dead stain, perform the Caspase Inhibitor Reagent and ancillary dye staining and wash steps prior to using trypsin. To avoid false positives, include another wash step to remove excess live/dead dye prior to trypsinization.

8. Read cells within 4 hours or fix. Analyze cells using a fluorescence microscope (Section F), fluorescence plate reader (section G), or flow cytometer (Section H).

F. Analysis with a Fluorescence Microscope

Carry out suspension cell staining (Section D) or adherent cell staining (section E).

1. Resuspend cells or replace overlay media in 300-500 µl 1X Apoptosis Wash Buffer and place on ice. At this point, the cells may be stained with Propidium Iodide for bicolor analysis (Step 2), fixed for future viewing (Step 3), or observed immediately (Step 4).
2. To identify dead cells by staining with Propidium Iodide, add Propidium Iodide at a concentration of 0.5% v/v (e.g. add 1.5 µl of Propidium Iodide to 300 µl of cell suspension). Incubate at 37 °C for 5 minutes. Wash cells to remove excess Propidium Iodide from the media. Centrifuge at 200 × g for 5-10 minutes at room temperature, and carefully remove the supernatant. Gently vortex pellets to disrupt clumping. Carefully remove and discard the supernatant. Resuspend cells in 300 µl of 1X Apoptosis Wash Buffer and gently mix. Go to Step 3 or 4.
3. If cells are not viewed immediately, they be fixed for viewing up to 16 hours later. Add Fixative Solution at 1/5 – 1/10 (e.g. add 100 µl Fixative Solution to 900 µl cells, to add Fixative Solution at 1/10). Incubate in the dark at room temperature for 15 minutes. Place cells onto a microscope slide and allow to dry. Briefly wash cells with PBS, then cover cells with mounting media and coverslip. The slides can be stored in the dark at 4 °C for up to 16 hours.
4. To view cells immediately, place 1 drop of cell suspension onto a microscope slide and cover with a coverslip.
5. Observe cells under a fluorescence microscope using a bandpass filter (excitation 490 nm, emission > 520 nm) to view green fluorescence. Cells bearing active caspase enzymes covalently coupled to the Caspase Inhibitor Reagent appear green. Hoechst 33342 can be seen using a UV-filter with excitation at 365 nm and emission at 480 nm. View nucleic-acid-bound Propidium Iodide under a long pass filter with the excitation at 490 nm, emission > 610 nm (nuclei-bound Propidium Iodide has a maximum emission at 617 nm).

G. Analysis with a Flow Cytometer

Carry out suspension cell staining (Section D) or adherent cell staining (section E), but do **not** carry out Hoechst 33342 staining steps (D5 and E7).

1. Resuspend cells or replace overlay media in 300-500 µl 1X Apoptosis Wash Buffer and place on ice.
2. Cells may be fixed for analysis up to 16 hours later. Add Fixative Solution at 1/5 – 1/10 (e.g. add 100 µl Fixative Solution to 900 µl cells, to add Fixative Solution at 1/10). Fixed samples can be stored in the dark at 4 °C for up to 16 hours.
3. Run the unstained control. If possible, adjust voltages to place the unstained sample in the first decade of the FL dot plots.
4. For single-color analysis, use a 488 nm blue argon laser, or comparable. Measure carboxyfluorescein (FAM) on the FL-1 channel.
5. Generate a histogram with the log FL-1 on the X-axis versus the number of cells on the Y-axis. Caspase-negative cells will occur in the lower log fluorescence output decades of the FL-1 (X) axis, whereas caspase-positive cells will appear as a shoulder on the right side (brighter) or separate peak on the right side of the negative peak

histogram.

6. For bicolor analyses, such as Caspase Inhibitor Reagent + PI, prepare two sets of instrument controls: live and killed cells that are only stained with PI; and induced and non-induced cells that are only stained with Caspase Inhibitor Reagent. These controls are needed to adjust the instrument PMT's to separate PI-positive and PI-negative samples and to compensate for bleed-over of the red PI signal from FL-2 or FL-3 into FL-1. They will also help to clearly differentiate the Caspase Inhibitor Reagent-positive population from the Caspase Inhibitor Reagent-negative population and compensate bleed-over of the green Caspase Inhibitor Reagent signal from FL-1 into FL-2 or FL-3
7. Run each set of single color controls. Adjust compensation to remove spectral overlap from interfering FL channels. When the data have been correctly compensated, the median fluorescence intensity (MFI) values in non-primary detectors of any given single-stained control sample should be the same as an unstained control sample (e.g. a Caspase Inhibitor Reagent-stained sample being read in FL-1 should have the same MFI in FL-3 as an unstained sample). For example, if reading Caspase Inhibitor Reagent in FL-1 and PI in FL-3:
    a) Subtract a percentage of the fluorescence in the Caspase Inhibitor Reagent channel from the fluorescent channel used for PI (e.g. FL-3 - %FL-1).
    b) Subtract a percentage of the fluorescence in the channel used for PI from the fluorescence in the Caspase Inhibitor Reagent channel (e.g. FL-1 - %FL-3).
8. When ready to read the samples for bicolor analysis, measure carboxyfluorescein (FAM) on the FL-1 channel and red fluorescence (PI) on the FL-2 or FL-3 channel. Generate a log FL-1 versus log FL-2 or FL-3 dot plot. This will reveal four populations of cells:
    a) Cells in early apoptosis fluoresce green with Caspase Inhibitor Reagent.
    b) Cells in late apoptosis are dually stained with Caspase Inhibitor Reagent and PI; they fluoresce green (they have active caspases) and red (the cell membrane has permeabilized).
    c) Necrotic cells fluoresce red.
    d) Unstained live cells have minimal fluorescence.

H. Analysis with a Fluorescence Plate Reader

Carry out suspension cell staining (Section D) or adherent cell staining (section E), but do **not** carry out Hoechst 33342 staining steps (D5 and E7).

1. Determine the concentration and compare the cell density of each sample. The non-induced population may have more cells than the induced population, as some apoptotic cells in the induced samples may be lost during the wash steps. Adjust the volume of the cell suspensions to equalize the cell density. When ready to read, cells should be > 3 × 10^6 cells/ml. Adherent cells should be cultured to ~90% confluency.
2. If using suspension cells, pipette 100 µl stained and washed cells per well into a black microtiter plate. Do not use clear plates. If using a bottom-reading instrument, use a plate with black walls and a clear bottom. Analyze at least 2 aliquots per sample. Avoid bubbles.
3. Perform an endpoint read. Set the excitation wavelength at 488 nm and the emission wavelength at 530 nm; if possible, use a 515 nm cut-off filter. The Caspase Inhibitor Reagent excites at 488-492 nm and the emits at 515-535 nm.