CHEMICON®
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CaspaTag™ Pan-Caspase *In Situ* Assay Kit, Fluorescein

For 100 Assays

Cat. No. APT400

FOR RESEARCH USE ONLY
NOT FOR USE IN DIAGNOSTIC PROCEDURES

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Background Information

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 the 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 and two small subunits that form two heterodimers, which associate in a tetramer[2-4]. In 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 enzymes specifically recognize a 4 or 5 amino acid sequence on the target substrate, which necessarily includes an aspartic acid residue. This residue is the target for cleavage, which occurs at the carbonyl end of the aspartic acid residue[6]. Caspases can be detected via immunoprecipitation, immunoblotting techniques using caspase specific antibodies, or by employing fluorochrome substrates, which become fluorescent upon cleavage by the caspase.

Test Principle

CHEMICON®'s *In Situ* Caspase Detection Kits use a novel approach to detect active caspases. The methodology is based on Fluorochrome Inhibitors of Caspases (FLICA). The inhibitors are cell permeable and non-cytotoxic. Once inside the cell, the inhibitor binds covalently to the active caspase[7]. This kit uses a carboxyfluorescein-labeled fluoromethyl ketone peptide inhibitor of caspase (FAM-VAD-FMK), which produces a green fluorescence. When added to a population of cells, the FAM-VAD-FMK probe enters each cell and covalently binds to a reactive cysteine residue that resides on the large subunit of the active caspase heterodimer, thereby inhibiting further enzymatic activity. The bound labeled reagent is retained within the cell, while any unbound reagent will diffuse out of the cell and is washed away. The green fluorescent signal is a direct measure of the amount of active caspase present in the cell at the time the reagent was added. Cells that contain the bound labeled reagent can be analyzed by 96-well plate-based fluorometry, fluorescence microscopy, or flow cytometry.

Application

The CHEMICON® *In Situ* FLICA Pan-Caspase Detection Kit is a fluorescent-based assay for detection of active caspases in cells undergoing apoptosis. The kit is for research use only. Not for use in diagnostic or therapeutic procedures.

Kit Components

1. FLICA Reagent (FAM-VAD-FMK): Four lyophilized vials
2. 10X Wash Buffer: 60 mL
3. Fixative: 6 mL
4. Propidium Iodide: 1 mL at 250 µg/mL, ready-to-use
5. Hoechst Stain: 1 mL at 200 µg/mL, ready-to-use

Materials Not Supplied

- Cultured cells with media
- Reagents to induce apoptosis
- 15 mL polystyrene centrifuge tubes
- Amber vials or tubes for storage of 150X concentrate at –20ºC
- 600 mL graduated cylinder
- Microscope slides
- Hemocytometer
- Centrifuge (400 x g)
- 37ºC incubator
- Vortexer
- Adjustable volume pipettor with disposable tips
- Deionized water
- PBS, pH 7.4
- DMSO

Storage

- Store unopened kit materials at 2-8°C up to their expiration date.
- Reconstituted FLICA Reagent (150X) should be frozen at –20ºC for up to 6 months, and may be thawed twice during this time. Aliquot into separate amber tubes if desired. Protect from light at all times.
- Store diluted (1X) wash buffer up to –2-8ºC for 2 weeks.

Precautions

- Use gloves, protective clothing and eyewear when handling the FLICA reagent, Propidium iodide, Hoechst stain and fixative.
- Dispose of all liquid components down the sink and flush with copious amounts of water. Solid components may be tossed in standard trash bins.

Preparation of Reagents

1. **Wash buffer:** If necessary, gently warm the 10X concentrate supplied in the kit to completely dissolve any salt crystals that have come out of solution. Prepare a 1X solution by diluting 1:10 in DI H_2O.
2. **FLICA Reagent (immediately before performing assay):** Reconstitute one vial of lyophilized reagent with 50 µL of DMSO and mix by swirling or tilting the vial until completely dissolved (a few minutes at RT). This 150X stock solution should be used or frozen immediately after preparation. If using immediately, prepare a 1:5 dilution in PBS (pH 7.4), mixing well by vortex, to create a working 30X dilution. ALWAYS protect from light.

Preparation of Samples

- Cells should be cultured to a density optimal for apoptosis induction, not to exceed 10^6 cells/mL.
- Induce apoptosis according to your protocol (camptothecin or staurosporine can be used for treatment for 3-4 hours).
- Concurrently incubate a non-induced negative control cell population at the same density.

- It is recommended that induced and non-induced samples be assayed for each labeling condition. For example, if using both the FLICA reagent and the Hoechst stain, make 8 populations:
    i. Unlabeled, induced and non-induced
    ii. FLICA-labeled, induced and non-induced
    iii. FLICA and Hoechst-labeled, induced and non-induced
    iv. Hoechst-labeled, induced and non-induced

Assay Instructions for Fluorescence Plate Reader

1. Concentrate both induced and non-induced cells to ~1 x 10^7 cells/mL.
2. Transfer 290-300 µL of each cell suspension to sterile tubes.
3. Add 10 µL of freshly prepared 30X FLICA reagent and mix cells by slightly flicking the tubes. *Note: End user should adjust the amount of FLICA reagent used based on cell type and research conditions.*
4. Incubate tubes for 1 hour at 37ºC under 5% CO_2, protecting tubes from light. Swirl tubes once or twice during this time to gently resuspend settled cells.
5. Add 2 mL of 1X wash buffer to each tube and mix.
6. Centrifuge cells at <400 x g for 5 minutes at room temperature.
7. Carefully remove and discard supernatant, and gently vortex cell pellet to disrupt any cell-to-cell clumping.
8. Repeat wash (steps 5-7), this time using 1 ml of 1X wash buffer.
9. Resuspend cell pellet in 1 mL of 1X wash buffer.
10. Remove 50 µL from each tube and dilute 1:10 with 450 µL of PBS. Count cells with a hemocytometer or other method, and compare density of induced and non-induced populations.
11. Centrifuge remaining cells from step 9 at <400 x g for 5 minutes at room temperature; remove and discard supernatant.
12. Resuspend *non-induced cells* in 400 µL PBS.

13. Induced cell populations may have decreased due to loss of cells during apoptosis.

    a. If populations of induced cells (based on count from step 10) were close to that of non-induced, resuspend *induced cells* in 400 µL of PBS.

    b. If there was a dramatic loss in induced cells counted in step 10, resuspend *induced cells* in PBS sufficient to adjust the concentration to that of the non-induced population (<400 µL).

14. Place 100 µL of each cell suspension into each of two wells of a black microtiter plate. Avoid creation of bubbles.
15. Set the fluorescence plate reader to perform an endpoint reading.
16. Read absorbance of each microwell using an excitation wavelength of 490 nm and an emission wavelength of 520 nm.

Sample Data – Fluorescence Plate Reader

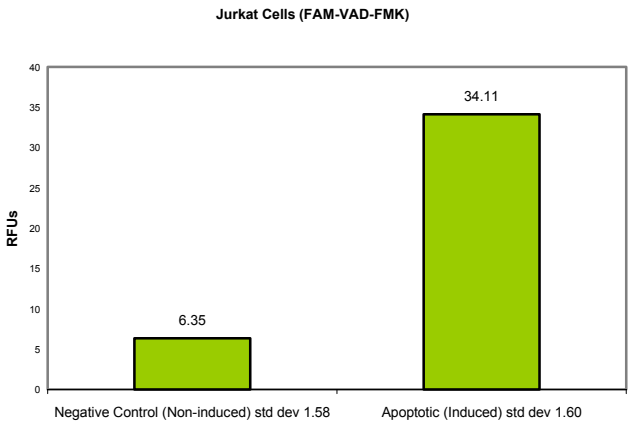

Figure 1. FAM-VAD-FMK fluorometric detection of active caspases in Jurkat cells (SD of 6 wells). Negative control cells were treated with DMSO. Apoptosis was induced by incubation with staurosporine for two hours at 37ºC.

Assay Instructions for Fluorescence Microscopy – Adherent Cells

1. Trypsinize cells.
2. Count cells, and seed 10^4-10^5 cells onto a sterile glass coverslip in a 35 mm petri dish or onto a chamber slide.
3. Grow cells in their respective cell culture media formulation for 24 hours at 37ºC.
4. Induce apoptosis using desired method.
5. Add freshly prepared 30X FLICA reagent solution at a 1:30 dilution in culture medium (10 µL in 290 µL of medium) and mix well. *Note: End user should adjust the amount of FLICA reagent used based on cell type and research conditions.*
6. Incubate for 1 hour at 37ºC under 5% CO_2 and remove medium.
7. Hoechst stain can be used to label the nuclei of dying cells. If this is desired, dilute 1.5 µL Hoechst stain in 300 µL of media (0.5% v/v). Add this media to cells and incubate for 5 minutes at 37ºC under 5% CO_2.
8. Wash cells twice with 2 mL 1X wash buffer.
9. To analyze cells immediately, skip to step 10. However, if desired cells can be fixed at this point for analysis up to 24 hours later:
    a. Dilute Fixative 1:10 with wash buffer.
    b. Mount a cover slip with cells facing down onto a slide containing a drop of diluted fixative. If using chamber slides remove plastic frame, add a drop of diluted fixative onto slide and cover with a cover slip.
    c. Keep fixed cells at 2-8ºC protected from light up to 24 hours. Proceed to step 11.
10. Mount a cover slip with cells facing down onto a slide containing a drop of 1X wash buffer. If using chamber slides remove plastic frame, add a drop of 1X wash buffer onto slide and cover with a cover slip.
11. Observe cells under a fluorescence microscope using a bandpass filter (excitation 490nm, emission 520nm) to view the green fluorescence of caspase-positive cells. Hoechst stain (if used) can be seen using a UV-filter with excitation at 365nm and emission at 480nm.

Assay Instructions for Fluorescence Microscopy – Suspension Cells

1. Transfer 290-300 µL of each cell suspension to sterile tubes.
2. Add 10 µL of freshly prepared 30X FLICA reagent and mix cells by slightly flicking the tubes. *Note: End user should adjust the amount of FLICA reagent used based on cell type and research conditions.*
3. Incubate tubes for 1 hour at 37ºC under 5% CO_2, protecting tubes from light. Swirl tubes once or twice during this time to gently resuspend settled cells.
4. Hoechst stain can be used to label the nuclei of dying cells. If this is desired, add 1.5 µL Hoechst stain (0.5% v/v). Incubate for 5 minutes at 37ºC under 5% CO_2.
5. Add 2 mL of 1X wash buffer to each tube and gently mix.
6. Centrifuge the cells at <400 x g for 5 minutes at room temperature.
7. Carefully remove and discard supernatant, and gently vortex cell pellet to disrupt any cell-to-cell clumping.
8. Repeat wash (steps 5-7), this time using 1 mL of 1X wash buffer.
9. Resuspend cell pellet in 300 µL of 1X wash buffer and place cells on ice.
10. Propidium Iodide can be used to exclude dead cells from the analysis. If desired, add 1.5 µL of PI solution (0.5% v/v). Cells may then be viewed using a long pass filter with excitation at 490nm, emission >520nm. PI has a maximum emission at 637nm.
11. To analyze cells immediately, skip to step 12. However, if desired cells can be fixed at this point for analysis up to 24 hours later:
    a. Add 30 µL of fixative to each tube.
    b. Incubate cells for 15 minutes at room temperature in the dark.
    c. Dry cells onto a microscope slide.
    d. Briefly wash cells with PBS.
    e. Cover cells with mounting media and cover slip.
    f. Keep fixed cells at 2-8ºC protected from light up to 24 hours. Proceed to step 13.

12. Place one drop of cell suspension onto a microscope slide and cover with a cover slip.

13. Observe cells under a fluorescence microscope using a bandpass filter (excitation 490nm, emission 520nm) to view the green fluorescence of caspase-positive cells. Hoechst stain (if used) can be seen using a UV-filter with excitation at 365nm and emission at 480nm.

Sample Data – Fluorescence Microscopy

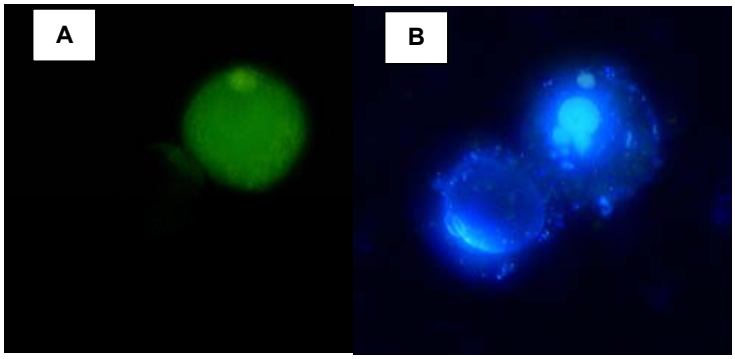

Figure 2. Suspension cells were incubated with 1 µM staurosporine for 3 hours at 37ºC to induce apoptosis. Cells were labeled with FAM-VAD-FMK and subsequently with Hoechst stain. Caspase activity in photo A was detected in one cell using a band pass filter. The second cell is not visible and therefore not apoptotic. Nuclear staining in photo B was revealed using a UV filter.

Assay Instructions for Flow Cytometry

Note: Flow Cytometry analysis can be done with single color (FLICA alone) or dual color staining (FLICA and Propidium Iodide). It is recommended that induced and non-induced samples be run for each labeling condition (unlabeled, FLICA-labeled, PI-labeled, and FLICA/PI-labeled).

1. Transfer 290-300 µL of each cell suspension (~10^6 cells) to sterile tubes.

2. Add 10 µL of freshly prepared 30X FLICA reagent and mix cells by slightly flicking the tubes. *Note: End user should adjust the amount of FLICA reagent used based on cell type and research conditions.*

3. Incubate tubes for 1 hour at 37ºC under 5% CO_2, protecting tubes from light. Swirl tubes once or twice during this time to gently resuspend settled cells.
4. Add 2 mL of 1X wash buffer to each tube and gently mix.
5. Centrifuge the cells at <400 x g for 5 minutes at room temperature.
6. Carefully remove and discard supernatant, and gently vortex cell pellet to disrupt any cell-to-cell clumping.
7. Repeat wash (steps 4-6), this time using 1 ml of 1X wash buffer.
8. Resuspend cell pellet in 400 µL of 1X wash buffer.
9. If using bi-color analysis, add 2 µL of PI solution to one cell suspension. Set aside a second suspension without PI. Skip to step 12.
10. For immediate single-color analysis, put samples on ice and skip to step 11. For single-color analysis up to 24 hours later, add 40 µL fixative and mix. Keep fixed cells at 2-8ºC protected from light. NOTE: Cells to be analyzed with PI cannot be fixed.
11. For single-color analysis, use a 15mW argon ion laser at 488nm. Measure fluorescein on the FL1 channel. Generate a log FL1 (X-axis) versus number of cells (Y-axis) histogram. On the histogram, there will appear two cell populations represented by two peaks. The majority of the caspase negative (-) cells will normally occur within the first log decade of the FL1 (X) axis (first peak), whereas the caspase-positive (+) cell population will appear as a separate peak or as a shoulder of the first peak showing increased fluorescence intensity. Position the vertical cursor in the gap between the two peaks. Events falling to the right of the vertical cursor should be counted as caspase positive (+).
12. For bicolor analysis, measure fluorescein on the FL1 channel and red fluorescence (PI) on the FL2 channel. Generate a log FL1 (X-axis) versus log FL2 (Y-axis) dot plot. Put in quadrant cursors. The 4 quadrant areas contain the following cell populations (Figure 5): (i) quadrant 1, PI positive fluorescein negative cells; (ii) quadrant 2, fluorescein positive PI positive cells; (iii) quadrant 3, fluorescein negative PI negative cells; (iv) quadrant 4, fluorescein positive PI negative cells. The cell population in quadrant 4 consists of living caspase positive (+) cells.

Sample Data – Flow Cytometry

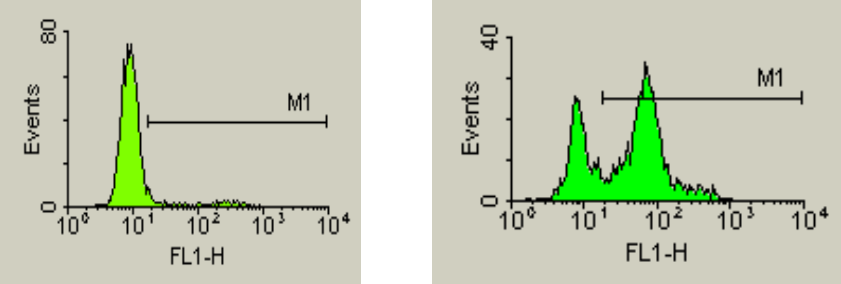

Figure 3. Jurkat cells were treated with DMSO, non-induced cells (A) or camptothecin, induced cells, for 3 hours (B). Cells were labeled with FAM-VAD-FMK for 1 hour, washed, and analyzed. Caspase activity was detected using a BD flow cytometer.

The frequency histogram of the number of events (Y axis) versus fluorescein intensity (X axis) shows 2 peaks: caspase-negative cells occur to the left of the M1 region (unlabeled cells); caspase-positive cells lay within the M1 region (cells were labeled with FLICA).

References

1. Slee, E. A. et al. (1999). *Cell Death and Differ.* **6:** 1067-1074.
2. Walker, N. P. et al. (1994). *Cell* **78:** 343-352.
3. Wilson, K. P. et al. (1994). *Nature* **370:** 270-275.
4. Rotonda, J. et al. (1996). *Nature Struct. Biol.* **3(7):** 619-625.
5. Kumar, S. (1999). *Cell Death and Differ.* **6:** 1060-1066.
6. Thornberry, N. A. et al. (1997). *J. Biol. Chem.* **272(29):** 17907-17911.
7. Ekert, P. G. et al. (1999). *Cell Death and Differ.* **6:** 1081-1086.

Warranty

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