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ProductInformation

Caspase 1 Fluorescein (FLICA) Assay

Catalog Number **CS0280**
Storage Temperature 2-8 °C

Technical Bulletin

Product Description
Caspases are detected by immunoprecipitation, immunoblotting with caspase specific antibodies, or by fluorescence employing fluorochrome substrates, which fluoresce upon cleavage by the caspases. The FAM-FLICA Caspase Assays detect active caspases present in living cells utilizing the Fluorochrome Inhibitor of Caspases (FLICA) methodology. When added to the cells, FLICA penetrates the cell membrane and covalently binds to the active intracellular caspases.[1] The FLICA inhibitor was also found to be non-cytotoxic to the cells at the concentrations used in the assay.

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. This results in the cleavage of protein substrates, leading eventually to the disassembly of the cell.[2] Caspases have been identified in organisms ranging from *C. elegans* to humans. In apoptosis, mammalian 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 kDa) and two small (~10 kDa) subunits that form two heterodimers that associate in a tetramer. 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.[3] Caspase enzymes specifically recognize a 4 amino acid sequence on the target substrate, which necessarily includes an aspartic acid residue. This residue is the target for the cleavage reaction, which occurs at the carbonyl end of the aspartic acid residue.[4]

Caspase-1, known also as Interleukin 1β Converting Enzyme (ICE) or IL-1β convertase (IL1BC), is a unique cysteine protease whose inhibition in human blood monocytes blocks production of mature IL1β.

Experiments with ICE-deficient mice have shown that these mice developed normally, appeared healthy, and were fertile. However, they were highly resistant to the lethal effects of endotoxin. With high-dose lipopolysaccharide that killed all wildtype mice within 30 hours, all ICE-deficient mice survived the first 48 hours and 70% of them survived after 7 days. These studies indicate that caspase-1 and ICE inhibitors are potential therapeutic targets for the treatment of inflammatory diseases such as septic shock and inflammatory bowel diseases.[5-8] Constitutive expression of caspase-1 immunoreactivity was found in nerve cells in the arcuate nucleus and in nerve fibers throughout the brain. The distribution pattern of caspase-1 immunoreactive structures is consistent with a role to produce mature IL-1β in regions where IL-1β mediates fever and sleep.[9]

Caspase 1 FLICA Assay contains a carboxyfluorescein (FAM) derivative of tyrosylvalylalanylaspartic acid fluoromethyl ketone (YVAD-FMK). It is an inhibitor with a preference for caspase 1 activity. Because the FAM-YVAD-FMK in the cell becomes covalently bound to the enzyme, it remains in the cell, while unbound reagent is washed away. The green fluorescent signal is a measure of active caspase 1 that was present in the cell at the time the reagent was added. Cells that contain the bound FLICA can be analyzed by multiwell fluorometer plate reader, fluorescence microscopy, flow cytometry or immunoblot protocols.

Other FLICA substrates have been shown to have a preference for specific caspases, such as:
- FAM-VAD-FMK, poly-caspases,
- FAM-VDVAD-FMK, caspase 2,
- FAM-LEHD-FMK, caspase 9;
- FAM-AEVD-FMK, caspase 10;
- FAM-LEED-FMK, caspase 13.

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Reagents

- FAM-YVAD-FMK FLICA Reagent, 4 vials, Catalog Number F8678 - lyophilized, brown powder. Reagent supplied sufficient for 100 assays.
- Wash Buffer, Concentrate 10X, 60 mL, Catalog Number W3764
- Fixative, 6 mL, Catalog Number F8553 - contains formaldehyde, methanol and PBS.
- Propidium iodide, 1 mL, Catalog Number P2746 - 250 μg/ml in water
- Hoechst Stain 33342, 1 mL, Catalog Number H2413 – solution 200 μg/ml in distilled water.

Reagents and equipment required but not provided

- 15 mL polystyrene centrifuge tubes
- Microcentrifuge polypropylene tubes
- Slides and coverslips
- Hemocytometer
- Centrifuge at <400 x g
- Incubator at 37 °C, CO_2
- Calibrated adjustable precision pipettes for volumes between 5 μL and 1,000 μL.
- 150 mL or 600 mL graduated cylinder
- Deionized or distilled water
- Phosphate buffered saline (PBS), pH 7.4
- Dimethyl sulfoxide (DMSO)
- Trypsin-EDTA solution, Catalog Number T3924
- Fluorescence multiwell plate reader with the excitation filter at 488 nm, emission filter at 520 nm, and black round or flat bottom 96-well plates.
- Fluorescence microscope with the following filters:
  FLICA - excitation 490 nm, emission >520 nm;
  PI - excitation at 490 nm, emission at 635 nm;
  Hoechst - a UV-filter with excitation at 365 nm, emission at 480 nm.
- Flow cytometer equipped with a 15 mW, 488 nm argon excitation laser, with the following filters:
  FLICA - excitation 490 nm, emission >520 nm;
  PI - excitation at 490 nm and emission at 635 nm

Precautions and Disclaimer

The kit is for R&D use only, not for drug, household or other uses. Please consult the Material Safety Data Sheet for information regarding hazards and safe handling practices.

Preparation Instructions

Sample Preparation

- The caspase detection assays utilize cells of a chosen cell line, induced to undergo apoptosis plus a non-induced cell control.
- Optimal cell concentration will vary depending on the cell line used and experimental conditions.
- In general, cell density should not exceed 10^6 cells/mL.
- Cells cultivated in excess of 10^6 cells/mL may spontaneously enter apoptosis state due to nutrient deprivation or the accumulation of cell degradation products in the media.
- Prepare a non-induced negative control cell population at the same density as the induced population for every labeling condition.
- Prepare the following 6 cell populations
  1. FLICA labeled non-induced cell population
  2. FLICA labeled induced cell population
  3. FLICA+Hoechst labeled non-induced cell population
  4. FLICA+ Hoechst labeled induced cell population
  5. Hoechst labeled non-induced cell population
  6. Hoechst labeled induced cell population

Induction of Apoptosis

- Use apoptosis induction protocol established in your laboratory.
- Induce apoptosis as you normally would, then label the cells with FLICA.
- If you do not have an established induction protocol, do one of the following:
  1. treat Jurkat cells with 2 μg/mL camptothecin for 3 hours.
  2. treat Jurkat cells with 1 μM staurosporine for 3 hours.
  3. treat HL-60 cells with 4 μg/mL camptothecin for 4 hours.
  4. treat HL-60 cells with 1 μM staurosporine for 4 hours.

Reagent Preparation

150X FLICA Stock Solution

1. The 100-assay kit contains 4 vials of highly concentrated lyophilized FLICA powder.
2. Protect the FLICA reagent from light at all times.
3. To reconstitute, add 50 µL DMSO /vial.
4. Swirl or tilt the vial, allowing the DMSO to travel around the base of the amber vial until powder is completely dissolved. At room temperature, the reagent should dissolve in a few minutes.
5. Pool together the content of the 4 vials (optional).
6. Label as **150X FLICA Stock Solution.**
7. If not used immediately, the 150X concentrate may be frozen in aliquots at –20 °C, protected from light, for up to 6 months.
8. During that time, the 150X FLICA aliquots may be thawed and used twice.

30X FLICA Working Solution
1. Dilute 150X FLICA Stock Solution 1:5 in phosphate buffered saline, pH 7.4, just before it is needed.
2. Each sample to be tested requires 10 μL of 30X FLICA Working Solution, or 2 μL of the 150X FLICA Stock Solution.
3. To prepare 1 vial 30X FLICA Working Solution:
   50 μL (150X stock in DMSO) + 200 μL PBS, pH 7.4
4. Mix by inverting or vortexing the vial at RT.
5. The 30X FLICA Working Solution must be used the same day that it is prepared.

30X FLICA Working Solution from frozen 150X FLICA Stock Solution
1. Thaw the aliquot of 150X FLICA Stock Solution (protect from light).
2. Once thawed, dilute the aliquot as follows:
   10 μL (150X FLICA Stock Solution) + 40 μL PBS, pH 7.4
3. Invert or vortex the vial at RT to mix.
4. Use immediately.

Working Wash Buffer
1. Gently warm the Wash Buffer Concentrate 10X to completely dissolve any salt crystals that may have come out of solution.
2. Add the entire bottle (60 mL) to 540 mL of deionized water H_2O – total 600 mL of Working Wash Buffer, or if you do not plan to use the entire bottle, prepare 1:10 dilutions as needed.
3. Stir the solution for 5 minutes at RT until all crystals are dissolved
4. Store Working Wash Buffer at 2-8 °C, covered, for up to 14 days.

Propidium iodide
1. Propidium iodide (PI) is used to distinguish between apoptotic and necrotic cells, either caspase-negative or caspase-positive.
2. PI provided ready-to-use at 250 μg/mL.
3. PI stains necrotic, and membrane-compromised late apoptotic cells.
4. PI stained cells may be viewed through a fluorescence microscope using excitation 488 - 492 nm and emission maximum 635 nm, or analyzed using a flow cytometer.

Hoechst stain
1. Hoechst stain can be used to label and identify the nuclei of apoptotic cells after labeling with the FLICA reagent.
2. Hoechst stain provided ready-to-use at 200 μg/mL.
3. It is revealed under a microscope using a UV-filter with excitation at 365 nm and emission at 480 nm.

Fixative
1. If the cell populations cannot be evaluated immediately upon completion of the FLICA staining, cells may be fixed and analyzed up to 24 hours later on a microscope or flow cytometer.
2. The fixative is a formaldehyde solution designed to cross-link cell components and will not interfere with the carboxyfluorescein labeling once the FLICA reaction has been completed.
3. After labeling with FLICA, add the fixative into the cell solution at a 1:9 ratio.
   For example, add 100 μL fixative to 900 μL cells.
4. Fixed cells may be stored on ice or at 2-8 °C for up to 24 hours.

Notes:
a. Do not use ethanol-based or methanol-based fixatives to preserve the cells - they will inactivate the FLICA label.
b. Do not add the fixative until the staining and final wash steps have been completed.

Storage/Stability
- The unopened kit and all components are stable at 2 to 8 ºC for the shelf life of the kit.
- Protect the FLICA reagent from light at all times.
- Once reconstituted, the unused 150X FLICA Stock Solution should be stored in aliquots at –20 °C, protected from light.
- The 150X FLICA Stock Solution is stable for up to 6 months and may be thawed twice during that time.

Refer to the Certificate of Analysis for kit shelf life. To obtain C of A go to www.sigma-aldrich.com.

Procedure

General Protocol for FLICA Cell Staining

1. **Culture** cells to a density optimal for apoptosis induction according to your specific induction protocol.

2. **Prepare 6 populations** of experimental cells (see Sample Preparation section*)*.
   Cell density should not exceed 10^6 cells/mL as discussed previously.

3. **Mix** 290-300 µL 2-6 X 10^6 cells + 10 µL 30X FLICA Working Solution

4. **Incubate 1 hour, at 37 °C** with 5% CO_2

5. **1st Wash** Add 2 mL Working Wash Buffer, gently mix. Centrifuge at less than 400 x g, 5 minutes at RT. Remove supernatant (do not disturb cells) Resuspend cells in 1 mL Working Wash Buffer, gently mix

6. **2nd Wash** Repeat washing procedure above

7. **Cell count**
   Mix the cells by slightly flicking the tubes. Determine the concentration of both the induced and non-induced cell populations. To count cells:
   a. Remove 50 µL from each tube
   b. Add to 450 µL PBS (a 1:10 dilution).
   c. Count the cells using a hemocytometer.
   d. After counting, compare the density of each.
   e. The non-induced population may have more cells than the induced population, as some induced cells may be lost during the apoptotic process.
   f. If there is a dramatic loss in stimulated cell population numbers, adjust the volume of the induced cell suspension to match the cell density of the non-induced suspension.
   g. Centrifuge the remaining cells at <400 x g for 5 minutes at RT.
   h. Carefully remove and discard supernatant.

   Note: More sensitive cell lines may only tolerate centrifugation speeds of <200 x g.

   Follow specific detection protocol

96 Well Fluorescent Multiwell Plate Protocol

1. When induced and non-induced samples contain similar cell concentrations, resuspend both in 400 µL PBS.
2. If stimulated cell concentration has decreased substantially, adjust the PBS suspension volume accordingly.
3. Place 100 µL of the cell suspensions in each of the duplicate 2 wells of a **black** multiwell plate.
   Note: *Do not use clear plates. Avoid bubbles*.
4. Read at 490 nm excitation and 520 nm emission, or select the filter pairing which most closely approximates this range.
5. Fluorescein has an optimal excitation range from 488 - 492 nm, and emission range from 515 - 535 nm.
6. Express the results as a graph (see results).
   Note: The amount of FLICA reagent required may vary depending on cell line and research conditions.

Adherent Cell Staining Protocol for Fluorescence Microscopy

1. Trypsinize cells for easy removal from flask using 0.1 mL Trypsin-EDTA solution per 25 cm^2 flask.
2. Count cells.
3. Seed about 10^4-10^5 cells onto a sterile glass coverslip in a 35 mm petri dish or onto chamber slides.
4. Grow cells in their respective cell culture media formulation for 24 hours at 37 °C (as discussed in Sample Preparation Section).
5. Induce cells to undergo apoptosis and sample at time points according to your specific protocol (as mentioned in Induction of Apoptosis section).
6. Add the 30X FLICA Working Solution to the medium at a 1:30 ratio. For example, add 10 µL 30X FLICA to 290 µL medium.
7. Mix well.
8. Incubate cells for 1 hour at 37 °C under 5% CO_2.
9. Remove the medium.
10. If cells are to be monitored using Hoechst stain:
    a. add 1.5 µL Hoechst stain to 300 µL media (0.5% v/v). Add this media to the cells.
    b. Incubate for 5 minutes at 37 °C under 5% CO_2.
11. Wash cells twice with 2 ml Working Wash Buffer.
12. At this point, cells may be analyzed directly, or fixed and analyzed later.
13. To analyze directly:
    a. mount a coverslip with cells facing down onto a microscope slide containing a drop of Working Wash Buffer.
    b. or, remove the plastic frame of the chamber slide; add a drop of Working Wash Buffer onto the glass slide and cover with a coverslip.

14. To fix the cells for later analysis:
    a. add Fixative to Working Wash Buffer at a 1:9 ratio. For example, add 40 µL Fixative to 360 µL Working Wash Buffer .
    b. Mount a coverslip with cells facing down onto a microscope slide containing a drop of Fixative plus Working Wash Buffer .
    c. or, remove the plastic frame of the chamber slide; add a drop of Fixative plus Working Wash Buffer onto the glass slide and cover with a coverslip.
    d. Keep fixed cells at 2-8 °C protected from light for up to 24 hours.
15. View the green fluorescence of caspase positive cells under a fluorescence microscope using an excitation filter at 490 nm, emission >520 nm.
16. View the Hoechst stained nucleus using a UV-filter with excitation at 365 nm and emission at 480 nm. (If these filters are not available, select a filter combination that best approximates these settings.)

Cell Suspension Staining Protocol for Fluorescence Microscopy

1. Culture cells to a density optimal for apoptosis induction according to your specific induction protocol.
2. Grow cells to a density of at least 5 X 10^5 cells/mL, but not exceeding 10^6 cells/mL.
3. Follow the FLICA staining and washing protocol
4. Resuspend the cell pellet in 300 µL Working Wash Buffer
5. Place cells on ice.
6. At this point, the cells may be stained with propidium iodide (PI) for bicolor analysis
7. PI staining allows us to differentiate between necrotic (dead) cells (PI positive, FAM negative) and apoptotic cells (PI-negative and positive, FAM positive).
8. Add 1.5 µL PI solution (0.5% v/v).
9. To view cells place 1 drop of the cell suspension onto a microscope slide and cover with a coverslip;
10. View cells using a long pass filter with the excitation at 490 nm, emission >520 nm; PI has a maximum emission at 617 nm.
11. If not viewing immediately, cells may be fixed for viewing up to 24 hours later.
12. Add 30 µL Fixative to each 300 µL cell suspension (1:10 ratio).
    a. Incubate cells for 15 minutes at RT in the dark.
    b. Dry cells onto a microscope slide.
    c. Briefly wash the cells with PBS.
    d. Cover cells with mounting media and coverslip.
    e. Store slides at 2-8 °C up to 24 hours.
13. Observe cells under a fluorescence microscope using an excitation 490 nm, emission >520 nm to view green fluorescence.
14. Cells bearing active caspase enzymes covalently coupled to the FLICA reagent appear green.
15. Hoechst stained cells can be seen using a UV-filter with excitation at 365 nm and emission at 480 nm. (If these filters are not available, select a filter combination that best approximates these settings.)

Flow Cytometry Protocol with Single-Color Staining

After labeling with FLICA, cells can be analyzed directly by flow cytometry, or the cells may be fixed first and then analyzed by flow cytometry. For a thorough analysis, 2 types of samples are recommended (both should have induced and non-induced populations):

- unstained cells (induced and non-induced).
- cells stained with FLICA (induced and non-induced).

Results

Note The experiments below show activation of multiple caspases in Jurkat cells, using peptide FAM-VAD-FMK, specific for multiple caspases.

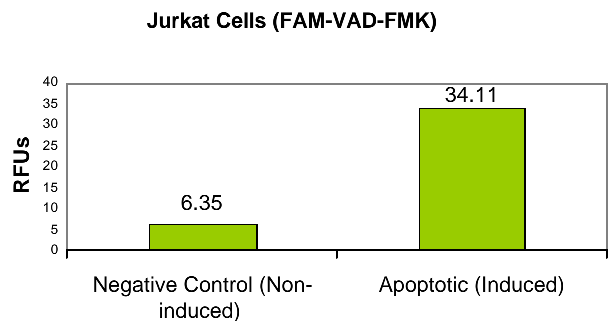
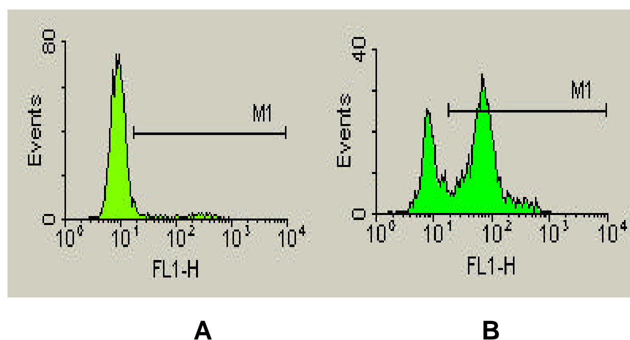


Figure 1. Fluorescent detection of caspases in 96 well plate reader

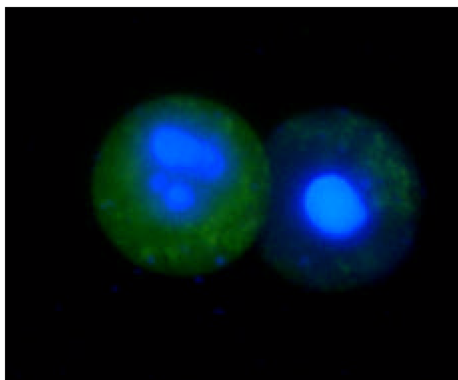
1. Negative control Jurkat cells (DMSO-treated)
2. Apoptosis-induced Jurkat cells (staurosporine-treated)
3. Fluorescence plate reader:
   Excitation – 490 nm
   Emission - 520 nm
4. As the caspases become activated (apoptosis) the green fluorescence increases by over 500%.



A **B**

Figure 2 The flow cytometry histogram
Y axis - number of events, X axis - fluorescein intensity

1. Non-induced Jurkat cells were treated with DMSO (A) or induced with camptothecin (B) for 3 hours
2. Cells were labeled with FAM-VAD-FMK for 1 hour and washed
3. Caspases activity was detected using a BD Facscalibor flow cytometer.
4. 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).



Fluorometric detection of active caspases by fluorescence microscopy and Hoechst staining

1. In the photograph above, cells were induced with staurosporine (1 μM staurosporine for 3 hours) and stained with Hoechst stain
2. Both cells in the picture are undergoing apoptosis (green) and dying (blue Hoechst stain).
3. Cell to the left shows a greater level of caspase activity.

References

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