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660 Caspase-1 Assay Kit Data Sheet

Catalog Number: KF17362

Applications: Detects caspase-1 activity.

Storage:

- Store unopened components at 2-8°C until the expiration date
- Once reconstituted with DMSO, use FLICA 660 immediately, or store at ≤-20° for 6 months protected from light. Thaw no more than twice.

Contents

- 1 vial of FLICA 660-YVAD_FMK caspase-1 inhibitor reagent
- 1 bottle of 10x Cellular Wash Buffer (15 mL)
- 1 bottle of Fixative (6 mL)

Recommended Materials

- DMSO, 50 µl per vial to reconstitute FLICA 660.
- DiH_20, 135 mL to dilute 10X Cellular Wash Buffer
- Phosphate buffered saline (PBS) pH 7.4, up to 100 µL per vial to dilute FLICA 660 and handle cells
- FBS and/or BSA to add to the buffer when handling cells
- Cultured cells treated with the experimental conditions ready to be analyzed
- Reagents to induce caspase-1 activity to create a positive control, such as Nigericin
- Hemocytometer
- Centrifuge at <200 x g
- 15 mL polypropylene centrifuge tubes (1 per sample)
- Hoechst 33342 or DAPI for optional nuclear staining

Introduction

FLICA® is a powerful method to assess caspase activity in vitro. FLICA probes are cell permeant noncytotoxic Fluorescent Labeled Inhibitors of CAspases that covalently bind with active caspase enzymes1,2. We have developed a far-red excitation and emission spectra FLICA 660 probe for the detection of cells bearing active caspase-1.

As a part of the pathway to process inflammatory precursors, activation of caspase-1 represents one of many roles caspases have in cellular function and differentiation outside of apoptosis. Exposure of inflammatory effector cells like monocytes and macrophages to pathogen-associated molecular patterns (PAMPS), such as viral or bacterial DNA or RNA and bacterial cell wall components like LPS, will typically trigger conformational changes in NACHT leucine-rich repeat protein family (NLRP) proteins3,4. This leads to oligomerization and assembly of a high molecular weight (~700 kDa) multimeric inflammasome complex, which leads to the conversion of pro-caspase-1 into the catalytically active form. Caspase-1, or interleukin-converting enzyme (ICE), proteolyticly converts the proforms of interleukin 1ß (IL-1ß) and IL-18 in monocytes and macrophages5 . Please note that macrophages and monocytes have been shown to rapidly secrete caspase-1 upon activation6,7.

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. Our FLICA 660 caspase-1 inhibitor probe contains the preferred binding sequence for caspase-1, Tyr-Val-Ala-Asp (YVAD)8 . It should be noted that the YVAD binding sequence is also recognized by caspases 4 and 5. The YVAD binding sequence is labeled at the amino terminus end with a far-red fluorescent 660 dye and linked at the carboxyl end to a

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fluoromethyl ketone (FMK) reactive entity. The resulting cell permeant fluorescent molecule, 660-YVAD-FMK, optimally excites at 660 nm and emits between 685-690 nm. A conventional red HeNe laser with a 633 nm excitation provides excellent excitation efficiency, enabling cells labeled with FLICA 660 to be analyzed with most flow cytometers and fluorescence microscopes equipped with electronic grey scale image capabilities.

Caspases, like most other crucial cell survival enzymes, are somewhat permissive in the target amino acid sequence they will recognize and cleave. Although FLICA reagents contain the different amino acid target sequences preferred by each caspase, they can also recognize other active caspases when they are present. We encourage validation of caspase activity by an orthogonal technique.

To use FLICA, add it directly to the cell media, incubate, and wash. FLICA is cell-permeant and will efficiently diffuse in and out of all cells. If there is an active caspase-1 enzyme inside the cell, it will covalently bind with FLICA 660-YVAD-FMK and retain the far-red fluorescent signal within the cell. The caspase does not cleave the covalently-bound FLICA, and becomes inhibited from further enzymatic activity. Unbound FLICA will diffuse out of the cell during the wash steps. Therefore, positive cells will retain a higher concentration of FLICA and fluoresce brighter than negative cells. There is no interference from pro-caspases or inactive forms of the enzyme. After labeling with FLICA, cells can be counter-stained with other reagents and fixed or frozen. FLICA is for research use only. Not for use in diagnostic procedures

Detection Equipment

FLICA 660 excites at 660 nm and emits at 685-690 nm. Use filter pairings that best approximate these settings.

- Fluorescence microscope - Use band or long pass filter set pairings that best approximate excitation at 660 nm and emission at 685-690 nm. Due to the long wavelength emission properties of FLICA 660 (>650 nm), use a fluorescence microscope with electronic grey scale image capture capabilities.
- Flow cytometer - Use a standard 633 nm excitation laser and 675/25 emission filter set, or similar (often FL-4).

Experimental Preparation & Controls

Staining caspase-1-positive cells with FLICA can be completed within a few hours. However, since FLICA is used to label living cells, adequate time needs to be allotted for the acquisition of functionally appropriate primary cells and expansion of a cell line known to produce caspase-1. The optimal cell concentrations and sample volumes will vary based on the experimental conditions and method of analysis.

The recommended volume of 30-60X FLICA is 5-10 µL per 300 µL of cells at 2-5 x 105 cells/mL (although fluorescence microscopy applications may require a greater cell density). However, the optimal cell concentrations and volumes will vary based on the experimental conditions and method of analysis.

1. Culture cells to a density optimal for the specific experiment or caspase-1 induction protocol. Carefully monitor the density of adherent cell monolayers to avoid excessive levels of confluency. Depending upon the cell line and type, cultivated cells which have reached a confluent monolayer may become spontaneously apoptotic and trigger multicaspase activity. As 660-YVAD-FMK preferentially detects the presence of the catalytically active form of caspase-1, it should not be added to the cells until caspase-1 is expected to be activated.
2. Create experimental and control cell populations:
    a. Treated experimental population(s): cells exposed to the experimental condition or treatment(s)
    b. Negative control: cells grown in a normal cell culture environment c. Positive control: cells induced for caspase-1 activity using a known caspase-1 induction protocol
3. Flow cytometry controls: additional controls should be established for instrument compensation and gating. Use the control cell line:
    a. Unlabeled cells induced to activate caspase-1
    b. Unlabeled cells not induced to activate caspase-1
    c. Cells labeled with FLICA 660 and induced to activate caspase-1
    d. Cells labeled with FLICA 660 not induced to activate caspase-1
    e. Cells stained only with the secondary dye (if applicable) and induced to activate caspase-1
    f. Cells stained only with the secondary dye (if applicable) not induced to activate caspase-1

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   g. Cells stained with both FLICA 660 and the secondary dye (if applicable) and induced to activate caspase-1
   h. Cells stained with both FLICA 660 and the secondary dye (if applicable) not induced to activate caspase-1
4. Calculate how much FLICA is needed (Section 9). Initial experiments may be necessary to assess the optimal concentration of 660-YVAD-FMK and incubation period to adequately label the samples. FLICA should not be reconstituted and diluted until the cells are ready to be labeled.

Caspase-1 Induction
The optimal caspase-1 induction protocol can vary significantly among 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. Caspase-1 activation may be induced in Jurkat cells using Nigericin in cell culture media. For example, exposure to 5-20 μM Nigericin for 24 hours has been shown to produce a robust caspase-1 induction response in Jurkat cells.

Preparation of FLICA 660
FLICA 660 is supplied as a lyophilized powder that is dried onto the base of the amber glass vial. To minimize hydrolysis of the reactive FMK group, FLICA 660 should not be prepared until the samples are ready to be stained. Add it to the samples immediately after diluting it with the aqueous PBS solution. Protect from light and use gloves when handling.

Flow cytometry typically requires less FLICA 660 reagent than fluorescence microscopy. For analysis by flow cytometry or applications where a lower staining concentration is needed, use the FLICA 660 working solution at 1:60. For analysis by fluorescence microscopy or applications where a higher staining concentration is needed, use the FLICA 660 working solution at 1:30.
1. Reconstitute each vial of FLICA 660 with 50 μL DMSO to form the stock concentrate. The stock concentrate should appear as a clear, blue-green solution. Once reconstituted in DMSO, the stock concentrate may be stored at ≤-20°C for 6 months protected from light and thawed no more than twice during that time.
2. Immediately prior to addition to samples and controls, dilute the FLICA 660 stock concentrate 1:5 by adding 200 μL PBS to each vial to form the 30-60X FLICA working solution. Add the working solution to the samples and controls within 15 minutes of preparation to minimize hydrolysis of the FMK reactive group

Preparation Of 1x Cellular Wash Buffer
Cellular Wash Buffer is an isotonic solution used to wash cells following exposure to FLICA. It contains mammalian proteins to stabilize cells stained with FLICA and sodium azide to retard bacterial growth (1X Cellular Wash Buffer contains 0.01% w/v sodium azide). Cell culture media containing FBS and other additives may be used to wash cells instead of Cellular Wash Buffer.
1. 10X Cellular Wash Buffer may form precipitates during cold storage. If this happens, gently warm it until all crystals have dissolved. Do not boil.
2. Dilute 10X Cellular Wash Buffer 1:10 in diH2O to create the 1X wash solution. For example, add 15 mL 10X Cellular Wash Buffer to 135 mL diH2O for a total of 150 mL.

• 1X Cellular Wash Buffer may be stored at 2-8°C and used within 1 week or frozen and used within 6 months.

Fixative
Fixative is a formaldehyde solution designed to crosslink and aggregate intracellular components. If the stained cell populations cannot be evaluated immediately after labeling with FLICA 660, add Fixative at a ratio of 1:5-1:10 and incubate at least 15 minutes. Never add Fixative until all the staining and final wash steps have been completed. Fixed cells may be stored on ice or at 4°C up to 16 hours, protected from light.

Fixative will not chemically interact with the FLICA 660 label. If using absolute ethanol or methanol-based fixatives, caution is recommended as they have been shown to inhibit the fluorescence output of other fluorescent labels, like carboxyfluorescein, and may affect the fluorescence potential of the FLICA 660 label.

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Danger: Fixative contains formaldehyde <10% and methanol <5% and is harmful. Avoid contact with skin, eyes, and clothes by wearing lab coat, gloves, and safety glasses. In case of exposure, immediately flush eyes or skin with water. See SDS for further information.

Staining Protocol

1. Expose cells to the experimental condition and prepare control cell populations (Sections Experimental Preparation & Controls and Caspase-1 Induction) If analyzing with a flow cytometer, be sure to include all gating and compensation controls.
2. Initial cell concentration should be 3-5 x 105 cells/mL but should not exceed 106 cells/mL; cells cultivated in excess of this concentration may begin to naturally enter apoptosis and trigger multicaspase activity. For analysis by fluorescence microscopy (Section Microscopy Analysis), concentrate cells by centrifugation to 2-5 x 106 cells/mL just prior to staining with FLICA 660. Fluorescence microscopy requires a higher concentration of cells to provide an adequate cell density within the field of vision at higher magnifications. For example, an excess of 2 x 106 cells/mL is required to obtain 5-20 cells per image field. Flow cytometry (Section Flow Cytometry Analysis) has lower cell density requirements, and can efficiently analyze samples at 3-5 x 105 cells/mL.
3. Transfer 290-295 µL cells into fresh tubes. Different sample volumes may be used, however this changes the amount of FLICA 660 needed for optimal staining and alters the number of tests per vial.
4. Add 5-10 µL of the 30-60X FLICA 660 working solution (Section Preparation of FLICA 660). The concentration of FLICA 660 should be optimized for each cell line, experimental condition, and method of analysis. Microscopy analysis may require more reagent than flow cytometry. Flow cytometry analysis may provide the sensitivity to detect FLICA 660 when used at 1:60. For example, to stain cells at 1:30, add 10 µL FLICA working solution to 290 µL cells, forming a final volume of 300 µL. To stain cells at 1:60, add 5 µL FLICA working solution to 295 µL cells, forming a final volume of 300 µL. Mix the cell suspension to disperse the FLICA 660 probe.
5. Incubate cells at 37°C protected from light. The incubation period may range from 15 minutes to several hours, depending upon the cell line and experimental conditions. For best results, resuspend the cells every 10–20 minutes to ensure an even distribution of FLICA 660.
6. Add 2 mL 1X Cellular Wash Buffer (Section Preparation of 1X Cellular Wash Buffer) and gently mix.
7. Centrifuge at 200 x g for 5-10 minutes at RT.
8. Carefully remove and discard supernatants. Gently vortex the pellets to disrupt clumping. Resuspend in 1 mL 1X Cellular Wash Buffer and gently mix.
9. Centrifuge cells at 200 x g for 5-10 minutes at RT.
10. Carefully remove and discard supernatants. Gently vortex pellets to disrupt clumping. If analyzing by fluorescence microscopy, repeat wash process a third time. If using a flow cytometer, two wash steps are generally sufficient.
11. Gently vortex pellets to disrupt clumping.
    a. If analyzing with a fluorescence microscope, go to Section Microscopy Analysis
    b. If using a flow cytometer, go to Section Flow Cytometry Analysis

Microscopy Analysis

Follow Staining Protocol

1. Resuspend cells in 300-500 µL 1X Cellular Wash Buffer and place on ice. At this point, the cells may be stained with other dyes, fixed for future viewing (Step 2) or observed immediately (Step 3).
2. If not viewing immediately, cells may be fixed for viewing up to 16 hours later.
    a. Add Fixative at a v/v ratio of 1:5-1:10.
    b. Incubate 15 minutes at RT in the dark.
    c. Place cells on a microscope slide and allow to dry.
    d. Briefly rinse cells with PBS.
    e. Cover with mounting media and coverslip.
    f. Store slides at 2-8°C for up to 16 hours.
3. To view cells immediately, place 1 drop of cell suspension onto a microscope slide and cover with a coverslip.
4. Observe cells under a fluorescence microscope equipped with excitation band pass filter optics capable of efficiently transmitting 660 nm excitation light and a long pass emission filter >680 nm to view far-red fluorescence Cells bearing active caspase-1 enzymes that are covalently bound to FLICA 660-YVAD-FMK will show elevated levels of fluorescence >680 nm.

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Because the human eye is not adept at seeing emission wavelength light greater than 650 nm, the use of electronic gray scale imaging equipment is strongly recommended.
If staining with a nuclear counter-stain, Hoechst 33342 dye can be detected using a UV-filter with excitation at 365 nm and emission at 480 nm; DAPI nuclear stain exhibits an optimal dsDNA-bound excitation of 358 nm and an emission maximum of 461 nm.

FLOW CYTOMETRY ANALYSIS
Follow Staining Protocol, but omit the optional nuclear staining steps.

1. Resuspend cells in 300 µL 1X Cellular Wash Buffer (Section 10) and place on ice.
2. Run the unstained control. Generate a FSC vs SSC dot or density plot and gate on the population of interest. Adjust the voltages, if necessary, so that the cell population is easily distinguished.
3. For single-color analyses, use a 633 nm (peak emission) helium-neon ion laser or comparable >640 nm laser illumination source. Measure FLICA 660 emission on the FL-4 channel or with emission filters compatible with light emission between 680-690 nm.
4. Run single-color controls. Generate a histogram with the log FL-4 on the x-axis versus the number of cells on the y-axis. Caspase-negative (FLICA 660-) cells will fall within the lower log fluorescence output decades of the FL-4 x-axis; caspase-positive (FLICA 660+) cells will appear as a shoulder or as a separate peak on the right side of the negative peak histogram (Figure 2). Adjust the voltage on FL-4, if necessary, to ensure fluorescence is on scale and caspase positive and negative populations are distinguished.
5. For bi-color analyses, run each single-color control. Adjust compensation to remove spectral overlap from interfering FL channels.
   Depending on the instrument and the software used, compensation might be set within the instrument hardware before samples are run or within the software after data collection.
   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 FLICA 660 stained sample being read in FL-4 should have the same MFI in FL-3 as an unstained sample).
6. Run the experimental samples and analyze.

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