

FLICA® 660 Caspase-1 Assay Kit



Catalog #9122, 25-50 Tests

**FOR RESEARCH USE ONLY. NOT FOR
USE IN DIAGNOSTIC PROCEDURES.**

1. INTRODUCTION

FLICA® is a powerful method to assess caspase activity. FLICA probes are cell permeant noncytotoxic Fluorescent Labeled Inhibitors of CASpases that covalently bind with active caspase enzymes^{1,2}. ImmunoChemistry Technologies (ICT) has developed a far-red excitation and emission spectra FLICA 660 probe for the detection of cells bearing active caspase-1.

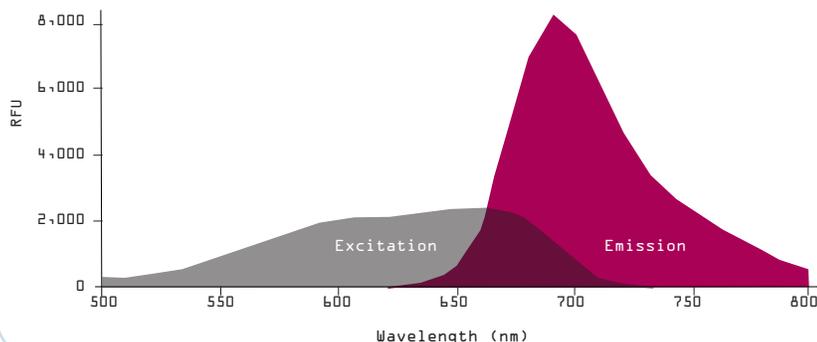
As a part of the pathway to process inflammatory precursors, activation of caspase-1 represents one of many roles caspases have in cellular function and differentiation outside of apoptosis. Exposure of inflammatory effector cells like monocytes and macrophages to pathogen-associated molecular patterns (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) proteins^{3,4}. 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), proteolytically converts the proforms of interleukin 1 β (IL-1 β) and IL-18 in monocytes and macrophages⁵.

ICT's FLICA 660 caspase-1 inhibitor probe contains the preferred binding sequence for caspase-1, Tyr-Val-Ala-Asp (YVAD)⁶. This preferred caspase-1 binding sequence is labeled at the amino terminus end with a far-red fluorescent 660 dye and linked at the carboxyl end to a 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.

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. Unbound FLICA will diffuse out of the cell during the wash step. 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.

FIGURE 1: FLICA 660 EXCITATION AND EMISSION SPECTRA

FLICA 660 was reconstituted in DMSO, diluted in diH₂O, and analyzed on a Molecular Devices Gemini XPS 96-well fluorescence plate reader. The excitation spectrum (grey) was generated using an emission of 760 nm. The emission spectrum (red) was generated using an excitation of 600 nm.



2. KIT CONTENTS

- 1 vial of FLICA 660-YVAD-FMK caspase-1 inhibitor reagent #6323
- 1 bottle of 10X Cellular Wash Buffer (15 mL) #6164
- 1 bottle of Fixative (6 mL) #636

3. STORAGE

Store the unopened kit and each unopened component at 2-8°C until the expiration date. Once reconstituted with DMSO, use FLICA 660 immediately, or store at $\leq -20^{\circ}\text{C}$ for 6 months protected from light and thawed no more than twice during that time.

4. SAFETY DATA SHEETS (SDS)

Safety data sheets are available online at www.immunochemistry.com or by calling 1-800-829-3194 or 952-888-8788.

5. RECOMMENDED MATERIALS

- DMSO, 50 μL per vial to reconstitute FLICA 660
- diH₂O, 135 mL to dilute 10X Cellular Wash Buffer
- Phosphate buffered saline (PBS) pH 7.4, up to 100 mL, 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 labeled

- Reagents to induce caspase-1 activity to create a positive control
- Hemocytometer
- Centrifuge at 200 x g
- 15 mL polypropylene centrifuge tubes (1/sample)
- Hoechst 33342 (catalog #639) or DAPI (catalog #6244) for optional nuclear staining

6. DETECTION EQUIPMENT

FLICA 660 excites at 660 nm and emits at 685-690 nm (Figure 1). 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).

7. 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.

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, or a different representative cell type with known caspase-1 activity
 - d. Negative control: if a different cell type was used to represent caspase-1 positive cells (c), include a negative control using the same cell line as the positive control grown in a normal cell culture environment
3. Flow cytometry controls: additional controls should be established for instrument compensation and gating. Use the control cell line:

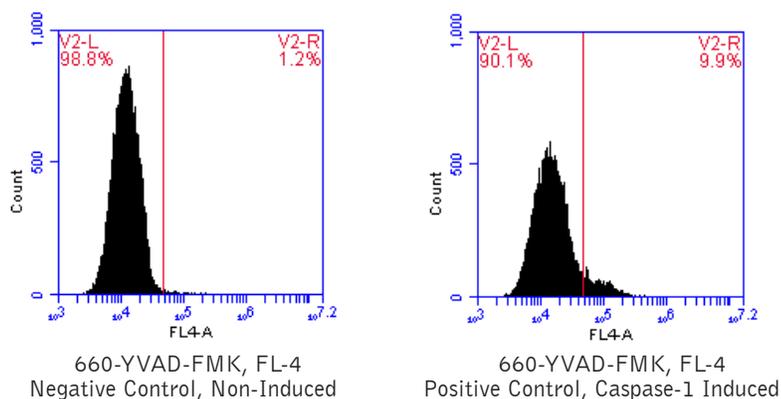
- a. Unlabeled cells induced to activate caspase-1
 - b. Unlabeled cells not induced to activate caspase-1
 - c. Cells labeled with FLICA 660 and induced to activate caspase-1
 - d. Cells labeled with FLICA 660 not induced to activate caspase-1
 - e. Cells stained only with the secondary dye (if applicable) and induced to activate caspase-1
 - f. Cells stained only with the secondary dye (if applicable) not induced to activate caspase-1
 - 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.

8. 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 10 μ M Nigericin for 24 hours has been shown to produce a robust caspase-1 induction response in Jurkat cells.

FIGURE 2: SINGLE COLOR ANALYSIS VIA FLOW CYTOMETRY

THP-1 cells were treated with a negative control (left) or PMA and LPS to induce caspase-1 activity (right), for 51 hours, washed, trypsinized, washed, then stained with ICT's far-red FLICA caspase-1 inhibitor probe, 660-YVAD-FMK (kit #9122), for 1 hour. Cells were washed twice and read on an Accuri C6 flow cytometer. Treatment with the negative control induced caspase-1 activity in only 1.2% of the cell population (left), whereas treatment with PMA and LPS induced caspase-1 activity in 9.9% of the experimental cells (right). This is a ratio of 8:1. Data courtesy of Mrs. Tracy Murphy, ICT, 213:52.



9. 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.

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 $\leq -20^\circ\text{C}$ 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. The working solution is used at approximately 1:30–1:60 in suspension cell samples at $2\text{--}5 \times 10^5$ cells/mL. This calculates to 5-10 μL of FLICA 660 working solution per 300 μL cell sample.
3. The optimal cell concentrations and volumes will vary based on the experimental conditions and method of analysis. Flow cytometry typically requires a lower cell concentration and 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.

10. PREPARATION OF 1X CELLULAR WASH BUFFER

ICT's Cellular Wash Buffer (catalog #6164) 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 dH_2O to create the 1X wash solution. For example, add 15 mL 10X Cellular Wash Buffer to 135 mL dH_2O for a total of 150 mL.
- 1X Cellular Wash Buffer may be stored at $2\text{--}8^\circ\text{C}$ and used within 1 week or frozen and used within 6 months.

11. FIXATIVE

ICT's Fixative (catalog #636) is a formaldehyde solution designed to cross-link 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.

ICT's 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.

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

12. STAINING PROTOCOL

1. Expose cells to the experimental condition and prepare control cell populations (Sections 7 and 8). If analyzing with a flow cytometer, be sure to include all gating and compensation controls.
2. Initial cell concentration should be between $2\text{--}5 \times 10^5$ cells/mL but should not exceed 10^6 cells/mL; cells cultivated in excess of this concentration may begin to naturally enter apoptosis and trigger pan-caspase activity. For analysis by fluorescence microscopy (Section 13), concentrate cells by centrifugation to $2\text{--}5 \times 10^6$ 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 2×10^6 cells/mL is required to obtain 5-20 cells per image field. Flow cytometry (Section 14) has lower cell density requirements, and thus, a concentration as low as 1×10^5 cells/mL is sufficient for flow analysis.
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 the 30-60X FLICA 660 working solution (Section 9). 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 . 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 15–20 minutes to ensure an even distribution of FLICA 660.
6. If cells are to be analyzed with a microscope, cells may be counter-stained with a nuclear stain such as Hoechst 33342 (catalog #639) or DAPI (catalog #6244).
7. Add 2 mL 1X Cellular Wash Buffer (Section 10) and gently mix.
8. Centrifuge at $200 \times g$ for 5 minutes at RT.
9. Carefully remove and discard supernatants. Gently vortex the pellets to disrupt clumping. Resuspend in 1 mL 1X Cellular Wash Buffer and gently mix.

10. Centrifuge cells at 200 x g for 5 minutes at RT.
11. 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.

13. MICROSCOPY ANALYSIS

Follow Section 12, Steps 1-11.

12. Resuspend cells in 300-500 μ L 1X Cellular Wash Buffer (Section 10) and place on ice. At this point, the cells may be stained with other dyes, fixed for future viewing (Step 13) or observed immediately (Step 14).
13. 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 onto a microscope slide and allow to dry.
 - d. Briefly rinse cells with PBS.
 - e. Cover cells with mounting media and coverslip.
 - f. Store slides at 2-8°C for up to 16 hours.
14. To view cells immediately, place 1 drop of cell suspension onto a microscope slide and cover with a coverslip.
15. 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 (Section 6). Cells bearing active caspase-1 enzymes that are covalently bound to FLICA 660-YVAD-FMK will show elevated levels of fluorescence > 680 nm. 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 (catalog #639) can be detected using a UV-filter with excitation at 365 nm and emission at 480 nm; DAPI nuclear stain (catalog #6244) exhibits an optimal dsDNA-bound excitation of 358 nm and an emission maximum of 461 nm.

14. FLOW CYTOMETRY ANALYSIS

Follow Section 12, Steps 1-11, but omit the optional nuclear staining steps.

12. Resuspend cells in 300 μ L 1X Cellular Wash Buffer (Section 10) and place on ice.
13. Run the unstained control. If possible, adjust the voltages to place the unstained sample in the first decade of the FL dot plots.
14. For single-color analyses, use a 633 nm (peak emission) 15 mW helium-neon ion laser or comparable > 640 nm laser illumination source. Measure FLICA 660 emission on the FL4 channel or with emission filters compatible with light emission between 680-690 nm.

15. Generate a histogram with the log FL4 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 FL4 X-axis, whereas caspase-positive (FLICA 660+) cells will appear as a shoulder on the right side or as a separate peak on the right side of the negative peak histogram (Figure 2).
16. For dual-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).
17. Run the dual-color experimental samples and analyze.

15. ACKNOWLEDGEMENT

ImmunoChemistry Technologies, LLC gratefully acknowledges the significant contributions made by one of its founders, Brian W. Lee, Ph.D in the development of this product, including the creation and illustration of its strategy and protocol.

16. REFERENCES

1. Amstad, P. A. et al. Detection of caspase activation in situ by fluorochrome-labeled caspase inhibitors. *Biotechniques* 31, 608-610, 612, 614, passim (2001).
2. Bedner, E., Smolewski, P., Amstad, P. & Darzynkiewicz, Z. Activation of caspases measured in situ by binding of fluorochrome-labeled inhibitors of caspases (FLICA): correlation with DNA fragmentation. *Exp Cell Res* 259, 308-313, doi:10.1006/excr.2000.4955 (2000).
3. Martinon, F., Gaide, O., Petrilli, V., Mayor, A. & Tschopp, J. NALP inflammasomes: a central role in innate immunity. *Semin Immunopathol* 29, 213-229, doi:10.1007/s00281-007-0079-y (2007).
4. Netea, M. G. et al. Differential requirement for the activation of the inflammasome for processing and release of IL-1 β in monocytes and macrophages. *Blood* 113, 2324-2335, doi:10.1182/blood-2008-03-146720 (2009).
5. Thornberry, N. A. et al. A novel heterodimeric cysteine protease is required for interleukin-1 β processing in monocytes. *Nature* 356, 768-774, doi:10.1038/356768a0 (1992).
6. Chapman, K. T. Synthesis of a potent, reversible inhibitor of interleukin-1 β converting enzyme. *Bioorganic & Medicinal Chemistry Letters* 2, 613-618, doi:10.1016/S0960-894X(01)81209-9 (1992).

Thank you for using FLICA! If you have any questions, or would like to share your data, please contact us at 1-800-829-3194 or 952-888-8788, or send an email to help@immunochemistry.com.



ImmunoChemistry Technologies, LLC
9401 James Avenue South #155
Bloomington, MN 55431 USA

toll-free: 1-800-829-3194
local: 952-888-8788
fax: 952-888-8988
www.immunochemistry.com