FLICA® 660 Caspase-3/7 Assay

Catalog #9125

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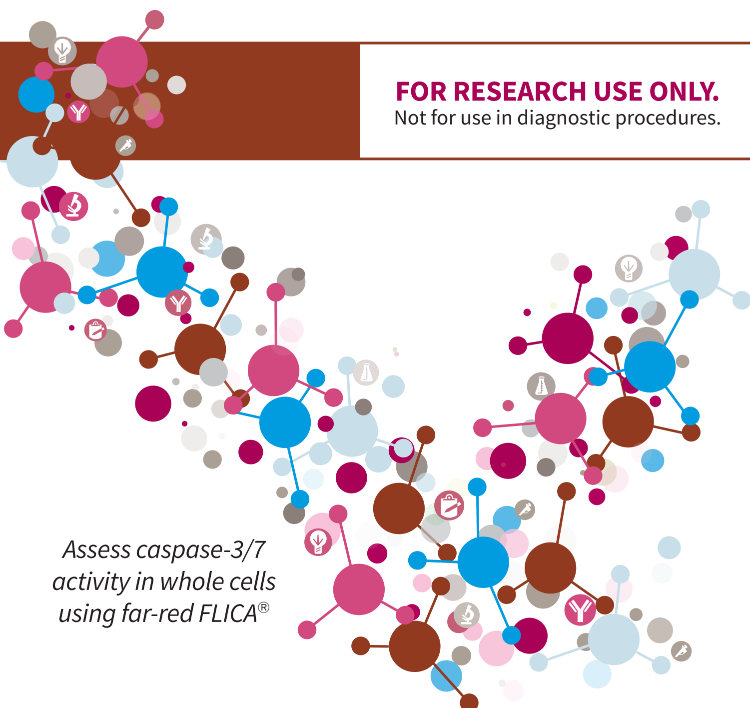


Assess caspase-3/7 activity in whole cells using far-red FLICA®

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 to 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 caspases 3 and 7.

Apoptosis is an evolutionarily conserved process of programmed cell death. It is centered on a cascade of proteolytic enzymes called caspases that are triggered in response to pro-apoptotic signals. Like the majority of other proteases, caspases are synthesized as pro-form precursors that undergo proteolytic maturation, either autocatalytically or in a cascade by enzymes with similar specificity[3]. Active caspase enzymes consist of two large (~20 kD) and two small (~10 kD) subunits that non-covalently associate to form a two heterodimer, tetrameric active caspase[4]. Once activated, caspases cleave protein substrates leading to the eventual disassembly of the cell. Caspases have been identified in organisms ranging from *C. elegans* to humans. Mammalian caspases play distinct roles in both apoptosis and inflammation.

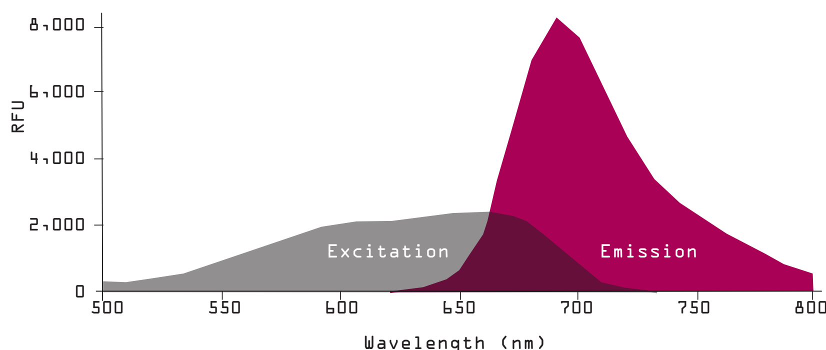
Mammalian caspase enzymes have been classified as initiator, executioner, and inflammatory caspases[5]. Once activated by initiator caspases (such as caspases -8, -9, and -10), executioner caspases -3 and/or -7 cleave specific sets of substrates that lead to apoptosis. Extrinsic activation of apoptosis, such as with FasL, TNF-a, or TRAIL binding to their associated death receptors, triggers the caspase-8 and -10-mediated cascade characteristic of the extrinsic apoptotic pathway, in which caspase-3 plays a dominant role[6]. In intrinsic apoptosis activation, DNA damage or inhibition of DNA repair leads to cytochrome c release from mitochondria, triggering formation of the apoptosome and activation of caspase-9. Once active, caspase-9 cleaves pro-form precusors of effector caspases, such as -3 and -7, leading to the eventual disassembly of the cell.

ICT's FLICA 660 caspase-3/7 inhibitor probe contains the preferred binding sequence, Asp-Glu-Val-Asp (DEVD) for executioner caspases -3 and -7. This preferred caspase-3 and -7 binding sequence, DEVD, 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-DEVD-FMK, optimally excites at 660 nm and emits between 685-690 nm (Figure 1). 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, as well as 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-3 and/or -7 enzyme, it will covalently bind to FLICA 660-DEVD-FMK and retain the far-red fluorescent signal within the cell. Unbound FLICA will diffuse out of the cell during the wash steps. Apoptotic cells will retain a higher concentration of FLICA and fluoresce brighter than non-apoptotic cells. There is no interference from pro-caspases or inactive forms of the enzyme. If the treatment is causing cell death via apoptosis, apoptotic cells will have an elevated level of caspase-3/7 activity relative to non-apoptotic or negative control cells and fluoresce with FLICA. After labeling with FLICA, cells can be counter-stained with other reagents and fixed or frozen.

FIGURE 1: FLICA 660 EXCITATION AND EMISSION SPECTRA

FLICA 660 was reconstituted in DMSO, diluted in diH_2O, 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-DEVD-FMK caspase-3/7 inhibitor reagent #6344
- 1 bottle of 10X Apoptosis Wash Buffer (15 mL) #635
- 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 ≤-20°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
- DiH_2O, 135 mL to dilute 10X Apoptosis 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 apoptosis or trigger caspase activity, such as staurosporine (catalog #6212) or camptothecin (catalog #6210), for creation of 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 optima between 685-690 nm. Due to the long wavelength emission properties of FLICA 660 (>680 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

Apoptosis assessment with FLICA can be completed within a few hours. However, since FLICA is used to label living cells, adequate time must be allotted for the cultivation of cell samples and the experimental treatment or apoptosis induction process. The optimal cell concentrations and sample volumes will vary based on the experimental conditions and method of analysis. As 660-DEVD-FMK preferentially detects the presence of the catalytically active form of caspases -3 and -7, plan the experiment so that FLICA will be diluted and added at the time when caspases are expected to be activated in the cells

1. Culture cells to a density optimal for the specific experiment or apoptosis induction protocol. Cell density should not exceed 10^6 cells/mL. Cells cultivated in excess of this concentration may begin to naturally enter apoptosis. Carefully monitor the density of adherent cell monolayers to avoid excessive levels of confluency.
2. Create experimental and control cell populations:
   a. Treated experimental population(s): cells exposed to the experimental condition or treatment(s)
   b. Negative control: non-treated cells grown in a normal cell culture environment
   c. Positive control: cells induced to undergo apoptosis using a known apoptosis induction protocol.
3. Flow cytometry controls: The following controls should be established for instrument compensation and gating.
   a. Unlabeled cells induced to activate caspases
   b. Unlabeled cells not induced to activate caspases
   c. Cells labeled with FLICA 660 and induced to activate caspases
   d. Cells labeled with FLICA 660 not induced to activate caspases
   e. Cells stained only with a secondary dye (if applicable) and induced to activate caspases
   f. Cells stained only with a secondary dye (if applicable) not induced to activate caspases
   g. Cells stained with both FLICA 660 and a secondary dye (if applicable) and induced to activate caspases
   h. Cells stained with both FLICA 660 and a secondary dye (if applicable) not induced to activate caspases
4. Calculate how much FLICA is needed (Section 9). Initial experiments may be necessary to assess the optimal concentration of 660-DEVD-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. APOPTOSIS INDUCTION

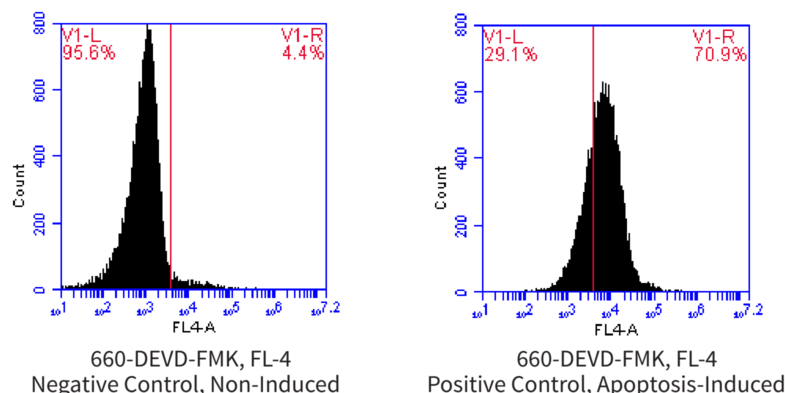
Prior to commencing the experiment, determine a reproducible method for obtaining a positive control by triggering caspase -3 and -7 activity. This process varies significantly with each cell type. For example, apoptosis via caspases -3 and -7 may be induced with 2-4 µg/ml camptothecin or 1-2 µM staurosporine for >4 hours.

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 FLICA 660 to the samples immediately after diluting it with the aque-

FIGURE 2: SINGLE COLOR ANALYSIS VIA FLOW CYTOMETRY

Jurkat cells were treated with a negative control (left) or staurosporine, an apoptosis-inducing agent (right), for 4 hours, then stained with ICT's far-red FLICA caspase-3/7 inhibitor probe, 660-DEVD-FMK (catalog #9125), for 1 hour. Cells were washed twice and read on an Accuri C6 flow cytometer. Treatment with the negative control induced caspase activity in only 4.4% of the cell population (left), whereas treatment with staurosporine induced caspase activity in 70.9% of the experimental cells (right). This is a ratio of 16:1. Data courtesy of Mrs. Tracy Murphy, ICT, 13F38, 022013.



660-DEVD-FMK, FL-4
Negative Control, Non-Induced

660-DEVD-FMK, FL-4
Positive Control, Apoptosis-Induced

ous 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 ≤-20°C for 6 months protected from light and thawed no more than twice during that time.
2. Immediately prior to addition to the samples and controls, dilute FLICA 1:5 by adding 200 µL PBS 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 2-5 x 10^5 cells/mL. Respectively, 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 APOPTOSIS WASH BUFFER

ICT's Apoptosis Wash Buffer (catalog #635) 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 Apoptosis 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 Apoptosis Wash Buffer.

1. 10X Apoptosis 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 Apoptosis Wash Buffer 1:10 in diH_2O to create the 1X wash solution. For example, add 15 mL 10X Apoptosis Wash Buffer to 135 mL diH_2O for a total of 150 mL.
    - 1X Apoptosis Wash Buffer may be stored at 2-8°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. For example, to use Fixative at 1:10, add 100 µL Fixative to 900 µL cells. 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 for 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 2-5 x 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. The optimal cell concentrations for staining will vary based on the experimental conditions and method of analysis. For analysis by fluorescence microscopy (Section 13), concentrate cells by centrifugation to 2-5 x 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 x 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 x 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 of 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. 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 reagent.
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 20 minutes to ensure an even distribution of FLICA 660.
6. If cells are to be analyzed with a microscope, they may be counter-stained with a nuclear stain such as Hoechst 33342 (catalog #639) or DAPI (catalog #6244).
7. Add 2 mL 1X Apoptosis Wash Buffer (Section 10) and gently mix.
8. Centrifuge at 200 x g for 5-10 minutes at RT.
9. Carefully remove and discard supernatants. Gently vortex the pellets to disrupt clumping. Resuspend in 1 mL 1X Apoptosis Wash Buffer and gently mix.
10. Centrifuge cells at 200 x g for 5-10 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 µL 1X Apoptosis 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.

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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, protected from light.

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 enzymes that are covalently bound to FLICA 660-DEVD-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 µL 1X Apoptosis 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. REFERENCES

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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.

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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.

Immunochemistry Technologies, LLC
9401 James Ave. S. #155, Bloomington, MN 55431 USA 800-829-3194
immunochemistry.com

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