Pyroptosis/Caspase-1 Assay

Green; Catalog #9145 & #9146

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Not for use in diagnostic procedures.

1. INTRODUCTION

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[1,2]. Of the inflammasomes, the NLRP3 inflammasome is the most studied. Activation of the NLRP3 inflammasome follows exposure to PAMPS (first signal) and ATP (second signal) (Figure 1). 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. Inflammatory caspases, such as caspase-1, or interleukin-converting enzyme, play a central role in innate immunity by recognizing foreign danger signals and initiating a two-fold response. First, caspase-1, proteolytically converts the proforms of the two important pro-inflammatory cytokines, interleukin 1ß (IL-1ß) and interleukin 18 (IL-18), into their active forms, which are secreted. Second, caspase-1 or caspase-11 triggers a form of lytic, programmed cell death known as pyroptosis[3,4].

Pyroptosis is a highly inflammatory form of programmed cell death that occurs most frequently upon infection with intracellular pathogens and is likely to form part of the antimicrobial immune response. This pathway is distinct from apoptotic cell death in that it results in plasma-membrane rupture and the release of pro-inflammatory cytokines; infected cells eventually swell, burst, and die. This, in turn, attracts other immune cells to fight the infection, leading to inflammation of the tissue, and, in a functional response, rapid clearance of bacterial or viral infections.

ICT's Pyroptosis/Caspase-1 Assay Kit utilizes our popular FLICA® technology to detect caspase-1 activation. FLICA probes are cell permeant noncytotoxic **F**luorescent **L**abeled **I**nhibitors of **CA**spases that covalently bind with active caspase enzymes[5,6]. The kit contains the caspase-1 inhibitor probe FAM-YVAD-FMK, which has the preferred binding sequence for caspase-1, Tyr-Val-Ala-Asp (YVAD)[7]. This preferred caspase-1 binding sequence is labeled with a green fluorescent carboxyfluorescein (FAM) dye and linked to a fluoromethyl ketone (FMK) reactive entity. Caspase-1 will not cleave the FLICA inhibitor probe; instead, it forms an irreversible covalent bond with the FMK group on the reagent and becomes inhibited from further enzymatic activity.

To use FLICA, add it directly to the cell culture medium, 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 FAM-YVAD-FMK and retain the green fluorescent signal within the cell. Unbound FLICA will diffuse out of the cell during the subsequent 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 enzymes. After labeling with FLICA, cells can be counter-stained with other reagents and fixed or frozen.

Assess Caspase-1 activity in whole cells in vitro using FAM-YVAD-FMK.

Cells labeled with FAM-YVAD-FMK can be counter-stained with reagents such as the red live/dead stains Propidium Iodide (catalog #638) and 7-AAD (catalog #6163). Nuclear morphology can be concurrently observed using Hoechst 33342 (included in the kit), a blue DNA-binding dye. Cells can be viewed through a fluorescence microscope (Figures 2 and 4), or the fluorescence intensity can be quantified using a fluorescence plate reader (Figure 3), or flow cytometer (Figure 5). FAM-FLICA optimally excites at 488-492 nm and has a peak emission at 515-535 nm.

Nigericin, a potent microbial toxin derived from *Streptomyces hygroscopicus*, acts as a potassium ionophore, inducing a net decrease in intracellular levels of potassium which is crucial for oligomermization of the NLRP3 inflammasome and activation of caspase-1. Nigericin requires signaling through pannexin-1 to induce caspase-1 activation and IL-1ß processing and release. Nigericin is included in the kit as a positive control. It has been shown to generate a robust caspase-1 activation response in various cell lines, including Jurkat and THP-I cells.

Learn more about all of ICT's products at www.immunochemistry.com or call 1-800-829-3194.

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

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2. KIT CONTENTS

#9145 Trial size kit contain:

- 1 vial of FAM-YVAD-FMK caspase-1 inhibitor reagent, #655
- 1 bottle of 10X Cellular Wash Buffer (15 mL), #6164
- 1 bottle of Fixative (6 mL), #636
- 1 vial of Hoechst 33342, 200 µg/mL (1 mL), #639
- 1 vial of Nigericin, 0.5 µmoles, #6698

#9146 Standard size kit contain:

- 4 vials of FAM-YVAD-FMK caspase-1 inhibitor reagent, #655
- 1 bottle of Fixative (6 mL), #636
- 1 bottle of 10X Cellular Wash Buffer (60 mL), #6165
- 1 vial of Hoechst 33342, 200 µg/mL (1 mL), #639
- 1 vial of Nigericin, 0.5 µmoles, #6698

3. STORAGE

Store the unopened Nigericin at -20°C until the expiration date. Store the remaining unopened kit components at 2-8°C until the expiration date. Once reconstituted with DMSO, use FLICA immediately, or aliquot and store at ≤-20°C for 6 months protected from light and thawed no more than twice during that time. Reconstituted Nigericin is stable for up to 1 year at -20°C. Avoid repeated freeze/thaws; aliquot and store frozen.

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
- DiH_20, 135-540 mL to dilute 10X Cellular Wash Buffer
- Phosphate buffered saline (PBS) pH 7.4, up to 100 mL, to dilute FLICA and handle cells
- FBS and/or BSA to add to the buffer when handling cells
- Cultured cells or tissues treated with the experimental conditions ready to be labeled
- Reagents to induce caspase-1 activity to create a positive control; Nigericin (included in the kit) is one option.
- Hemocytometer
- Black 96-well microtiter plate, flat bottom, non-treated, non-sterile (ICT catalog #266). If using a bottom reading instrument, use a plate with black walls and a clear bottom. If culturing cells in the plate, use a sterile black tissue culture plate.
- Centrifuge at 200 x g
- 15 mL polypropylene centrifuge tubes (1 per sample)

6. DETECTION EQUIPMENT

The assay can be analyzed with a:

- Fluorescence microscope
- Fluorescence plate reader
- Flow cytometer

Use filter pairings that best approximate these settings:

- FAM-FLICA excites at 488-492 nm and has a peak emission at 515-535 nm.
- Hoechst 33342 can be seen using a UV-filter with excitation at 365 nm and emission at 480 nm (Section 13).

7. EXPERIMENTAL PREPARATION

Staining caspase-1 positive cells with FAM-YVAD-FMK can be completed within a few hours. However, since it is used to label living cells, adequate time needs to be allotted for the acquisition of functionally appropriate 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.

In addition, once the proper number of cells has been cultivated, time must be allotted for the experimental treatment or caspase-1 induction process which may take from several hours to multiple days, depending on the methods that are used.

a. Cells that were exposed to the experimental condition or treatment
b. A placebo population of cells that received a blank treatment instead of the experimental treatment

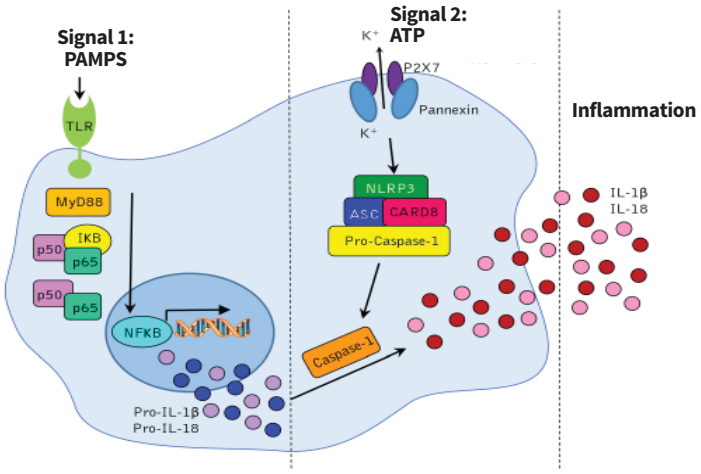
As FAM-YVAD-FMK preferentially detects the presence of catalytically active forms of caspase-1, plan the experiment so that FLICA will be diluted and administered at the time when caspase-1 is expected to be activated in the cells. If this is not known, FLICA can be added so that it is present throughout the induction treatment period for up to 24 hours. Protect samples from light during that time.

The recommended volume of 30-60X FLICA is 5-10 µL per 300 µL of cells at 3-5 x 10^5 cells/mL, but the amount may vary based on the experimental conditions and the instrument used for analysis. Each investigator should adjust the amount of FLICA to accommodate the particular cell line and research conditions.

Culture cells to a density optimal for the specific experiment or caspase-1 induction protocol. Carefully monitor the density of adherent cell

Figure 1: Activation of the NLRP3 inflammasome

Oligomerization of the NLRP3 inflammasome is triggered by two signals. The first signal begins with the recognition of PAMPS (pathogen associated molecular patterns) by Toll-like receptors (TLRs), such as TLR4, which, through an interaction with the adapter protein MyD88, triggers activation of the transcription factor NF-κB. Once activated, NF-κB is translocated to the nucleus, where it leads to the synthesis of the inactive pro-inflammatory cytokine pro-IL-1ß. Another potent pro-inflammatory cytokine precursor, pro-IL-18, is constitutively expressed; however, its expression is increased after cellular activation. The second signal is triggered by an ionic perturbation in the cell, such as an efflux of K^+, caused by the ATP-dependent activation of the purinergic P2X receptor, which subsequently results in the assembly of the NLRP3 inflammasome, caspase-1 activation, and IL-1ß and IL-18 secretion.



monolayers to avoid excess 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. Cell density should not exceed 10^6 cells/mL in suspension cultures. Cells cultivated in excess of this concentration may naturally begin to enter apoptosis. An initial experiment may be necessary to determine when and how much FLICA to use as the resulting positive signal is a direct measurement of caspase activity occurring during the incubation period.

8. CONTROLS

Create experimental samples and control cell populations:

a. Treated experimental population(s): cells exposed to the experimental condition(s).

b. Negative control: non-treated cells grown in a normal culture environment.

c. Positive control: cells induced for caspase-1 activity using a known caspase-1 induction protocol.

A common pool of cells should be used to generate the positive and negative control populations for FLICA, as they should contain similar quantities of cells. For example, when labeling with FLICA, make 4 populations:

1&2. Unlabeled: induced and non-induced

3&4. FLICA-labeled: induced and non-induced

9. CASPASE-1 INDUCTION

The optimal caspase-1 induction protocol will 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-I 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; see next section for additional details.

10. PREPARATION OF NIGERICIN

Nigericin, a potent microbial toxin derived from *S. hygroscopicus,* acts as a potassium ionophore, inducing a net decrease in intracellular levels of potassium which is crucial for oligomerization of the NLRP3 inflammasome and activation of caspase-1 (Figure 1). Nigericin requires signaling through pannexin-1 to induce caspase-1 activation and IL-1ß processing and release. Nigericin is included in this kit as a positive control. It has been shown to generate a robust caspase-1 activation response in various cells lines, including Jurkat and THP-I cells.

Nigericin is supplied lyophilized at 0.5 µmoles per vial. It may be slightly visible as an iridescent sheen or white powder inside the vial. Protect from light and use gloves when handling.

1. Reconstitute each vial of Nigericin with 100 µL DMSO to form the 5 mM stock concentrate. Once reconstituted, it may be aliquoted and stored at ≤-20°C for 1 year protected from light and thawed no more than twice during that time.
2. Immediately prior to addition to the samples and controls, dilute 5 mM Nigericin stock 1:10 in diH_2O to form a 500 µM working solution for use in treating samples. For example, dilute 1:10 by adding 20 µL stock concentrate to 180 µL diH_2O.
3. Use Nigericin at 1-20 µM to induce NLRP3 inflammasome and caspase-1 activation in cells. For example, to use at 10 µM, dilute 500 µM working solution 1:50 in samples; e.g., spike 294 µL cell suspension/overlay medium with 6 µL of 500 µM working solution. Typical treatment periods range from 3-24 hours at 37°C. Each investigator should adjust the concentration of Nigericin and treatment period to accommodate the particular cell line and research conditions. See Figure 5 for time-course study in Jurkat cells treated with 10 µM Nigericin for a period of 1-24 hours at 37°C.

- **Danger:** Nigericin is toxic if swallowed, causes skin irritation, causes serious eye irritation, and may cause respiratory irritation. 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.

11. PREPARATION OF FLICA

FLICA is supplied as a lyophilized powder that is dried onto the base of the amber glass vial. It may be slightly visible as an iridescent sheen inside the vial. Protect from light and use gloves when handling. Because the 30-60X FLICA solution must be used immediately, prepare it just before staining.

1. Reconstitute each vial of FLICA with 50 µL DMSO to form the 150-300X stock concentrate. The stock solution should be clear to slightly yellow or orange in color. Once reconstituted, it 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 each vial to form the 30-60X FLICA solution. Use 30-60X FLICA within 30 minutes of dilution into aqueous buffers.

12. PREPARATION OF 1X CELLULAR WASH BUFFER

ICT's Cellular Wash Buffer (catalog #6164 and #6165, CWB) 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 diH_2O. For example, add 15 mL 10X Cellular Wash Buffer to 135 mL diH_2O 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.

13. HOECHST 33342

Hoechst 33342 is a cell-permeant nuclear stain that emits blue fluorescence when bound to double stranded DNA. It is used to stain the nuclei of living or fixed cells, to distinguish condensed pyknotic nuclei in apoptotic cells, and for cell cycle studies.

Hoechst 33342 is provided ready-to-use at 200 µg/mL. Hoechst 33342 can be used with FLICA to label the nuclei of live, dying, and apoptotic cells. To use, add to samples at 0.5% v/v, and incubate 5 minutes at 37°C. For example, if the cell suspension or overlay medium volume is at 300 µL, add 1.5 µL Hoechst 33342.

When bound to nucleic acids, it has a maximum absorbance at 350 nm and a maximum emission at 480 nm. It is revealed under a microscope using a UV-filter with excitation at 365 nm and emission at 480 nm.

- Hoechst 33342 contains a low concentration of Bis benzimide H 33342 trihydrochloride which is below the threshold for reporting on the safety data sheet (SDS). It is a suspected mutagen at high concentrations. Prolonged skin contact may cause redness and irritation. Because of the small quantity of product, the health hazard is small. See SDS for further information.

14. FIXATIVE

ICT's Fixative 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, add Fixative at a ratio of 1:5-1:10. 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 interfere with the carboxyfluorescein (FAM) label. Do not use absolute ethanol or methanol-based fixatives, as they may inactivate the FAM-FLICA 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.

15. CELL STAINING PROTOCOL

Prepare experimental and control cell populations. Ideally, the cell concentration should be 3-5 x 10^5 cells/mL. The concentration should not exceed 10^6 cells/mL, as cells cultivated in excess of this concentration may begin to naturally enter apoptosis. Just prior to staining with FLICA, cells may need to be concentrated to 2-5 x 10^6 cells/mL as both microscopy and plate reader analysis methods (Sections 16 and 17) require high cell concentrations. Start with a larger volume of cells at 3-5 x 10^5 cells/mL (which is a typical density for cell culture) and then concentrate cells and resuspend to 300 µL per sample when ready for FLICA staining.

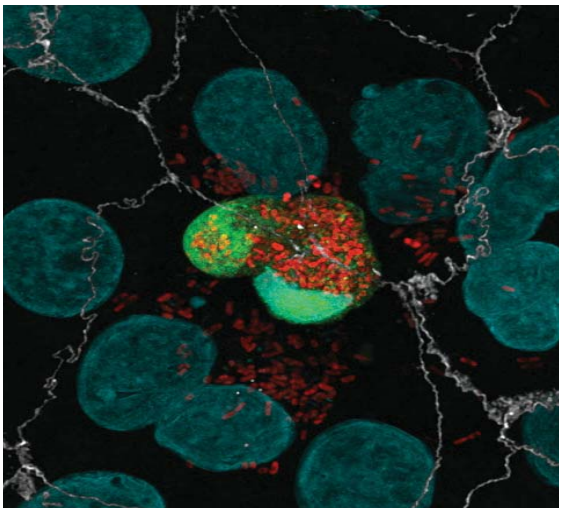
When working with adherent cells, care is recommended during handling to avoid the loss of any cells that round up and come off the culture surface. In microscopy or plate reader applications where trypsinization is not required, adherent cells can be stained and washed directly on the chamber slide, well, coverslip, or culture surface. To avoid losing cells that are no longer adherent during washing, spin down all overlay media and wash buffer and recombine washed cell pellets with the adherent samples prior to analysis. If suspension cells are required, such as in flow cytometry applications, cells may be trypsinized to create suspensions, which may be labeled with FLICA before or after trypsinization. Avoid trypsinizing cells prior to labeling with a live/dead DNA dye, like 7-AAD. Cell membranes exposed to trypsin could be transiently permeable to live/dead dyes for a variable time depending upon the cell line.

1. Expose cells to the experimental and control conditions.
   a. If analyzing with a flow cytometer, set aside two populations to create instrument controls with FLICA. Positive and negative control cells induced and not induced to activate caspase-1 (Section 8).
   b. If analyzing with a fluorescence microscope or plate reader, concentrate cells to 2-5 x 10^6 cells/mL just prior to FLICA staining. Fluorescence microscopy requires an excess of 2 x 10^6 cells/mL to obtain 5-20 cells per image field. Flow cytometry can efficiently analyze samples at 3-5 x 10^5 cells/mL.
2. Transfer 290-295 µL cells into fresh tubes. If staining adherent cells, FLICA can be added directly to the overlay medium.
3. Add 5-10 µL 30-60X FAM-FLICA working solution, forming a final volume of 300 µL. If different cell volumes were used, add FLICA at a ratio of 1:30-1:60. Mix by gently flicking the tubes. The concentration of FLICA should be optimized for each cell line and experimental condition. However, 1:60 is generally sufficient for flow cytometry applications, and 1:30 is recommended for fluorescence microscopy or plate reader applications.
4. Incubate cells at 37°C protected from light. The incubation period may range from 30 minutes to several hours and should be optimized for each cell line and experimental condition. In suspension cell cultures, cells may settle on the bottom of the tubes; gently resuspend by swirling cells every 20 minutes to ensure an even distribution of FLICA.
5. Wash cells. For suspension cells, go to Step 6. For adherent cells, go to Step 7.
6. Wash suspension cells:
   a. Add 2 mL 1X Cellular Wash Buffer and gently mix.
   b. Centrifuge at 200 x g for 10 minutes at RT.
   c. Carefully aspirate supernatant.
   d. Resuspend samples in 1-2 mL wash buffer and gently mix.
   e. Centrifuge a second time at 200 x g for 10 minutes at RT.
   f. Carefully aspirate supernatant.
   g. For flow cytometry analysis, two wash steps are generally sufficient. For microscopy or fluorescence plate reader analysis, repeat wash procedure a third time (resuspend samples, gently pellet by centrifugation, and carefully remove supernatants). Go to Step 8.
7. Wash adherent cells:

   To avoid losing cells that are no longer adherent during washing, spin down all overlay media and wash buffer and recombine washed cell pellets with the adherent samples prior to analysis.

Figure 2: Fluorescence microscopy of epithelial cells undergoing inflammatory cell death

C2BBe1 human colorectal adenocarcinoma cells were grown in polarized monolayers and infected with wild type *Salmonella* constitutively expressing mCherry (red cells, below). After 9 hours, live cells were incubated with ICT's active caspase-1 reagent, FAM-YVAD-FMK (green cell, below) for 1 hour in growth medium, washed, and fixed. The confocal image below reveals an extruding cell that is infected; many red mCherry-labeled *Salmonella* are visible. The infected cell is undergoing a form of inflammatory cell death known as pyroptosis, as evidenced by the positive staining for active caspase-1 visible as increased green fluorescence compared to background levels of fluorescence in the surrounding caspase-1 negative cells. Data courtesy Knodler, Leigh A., et al. Dissemination of invasive *Salmonella* via bacterial-induced extrusion of mucosal epithelia. PNAS. 107:41, 17733-17738 (2010).



a. Carefully remove overlay medium containing FAM-FLICA and replace with 1X Cellular Wash Buffer.
b. Incubate 10 minutes at 37°C to allow any unbound FAM-FLICA to diffuse out of cells.
c. Carefully remove and replace wash buffer with fresh wash buffer and incubate another 10 minutes at 37°C.
d. Gently remove overlay buffer and replace for a third wash step. Incubate 10 minutes at 37°C.
e. Gently remove overlay buffer. Go to Step 8.

8. If using a microscope, cells may be counter-stained with ancillary dyes like the nuclear stain Hoechst 33342 or other compatible fluorescent dye. Do not stain with Hoechst if using a plate reader (Section 17) or flow cytometer (Section 18).

- Resuspend cells or replace overlay buffer, add Hoechst 33342 at 0.5% v/v, and incubate 5 minutes at 37°C. For example, if the cell suspension or overlay medium volume is at 300 µL, add 1.5 µL Hoechst 33342.
- Live/dead cell stains should not be used after trypsinization. Cell membranes exposed to trypsin could be transiently permeable to live/dead dyes for a variable time depending upon the cell line. To identify dead cells with a live/dead stain, perform the FLICA and ancillary dye staining and wash steps prior to using trypsin. To avoid false positives, include another wash step to remove excess live/dead dye prior to trypsinization.
- If using any compatible ancillary dyes, follow the manufacturer's specific instructions for staining samples.

9. Read cells within 4 hours or fix.

- If using a fluorescence microscope, go to Section 16.
- If using a plate reader, go to Section 17.
- If using a flow cytometer, go to Section 18.

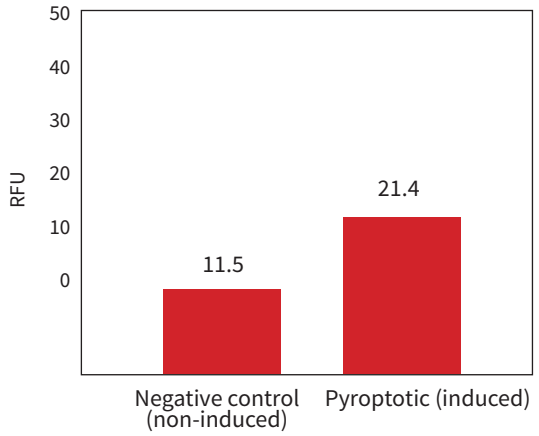
16. MICROSCOPY ANALYSIS

Follow Section 15, Steps 1-9.

1. Resuspend cells or replace overlay medium with 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 with a fluorescence microscope using a bandpass filter (excitation 490 nm, emission >520 nm) to view green fluorescence. Cells bearing active caspase-1 enzymes covalently coupled to FLICA appear green. Hoechst 33342 can be seen using a UV-filter with excitation at 365 nm and emission at 480 nm.

Figure 3: Quantification of caspase-1 activity using a fluorescence plate reader

THP-I cells were plated in 12-well tissue culture plates and treated with either a negative control (non-induced) or 5 ng/mL Phorbol myristate acetate (PMA) (pyroptotic, induced) to cause differentiation into macrophages. After 48 hours, PMA-containing culture medium was removed, wells were rinsed to remove any non-adherent cells, and fresh culture medium containing 10 ng/mL Lipopolysaccharide (LPS) was added to the induced sample wells. After a 2 hour exposure period, LPS-containing medium was removed, cells were rinsed with PBS, and trypsinzed to remove adherent cells. Trypsinized cells were transferred to tubes containing FBS to inactivate trypsin and cells were gently pelleted by centrifugation. Supernatants were carefully removed and samples were resuspended in PBS. Samples were then stained with FAM-YVAD-FMK for 1 hour at 37°C. Following 1 hour staining period, samples were washed three times and read on a Molecular Devices Gemini XPS 96-well fluorescence plate reader set at 488 nm excitation and 530 nm emission using a 515 nm cut-off filter. In the pyroptotic (induced) population, the relative fluorescence units (RFU) of the green fluorescent signal was nearly two times greater than the RFU of the negative (non-induced) population. Data courtesy of Mrs. Tracy Murphy, ICT (207:13-14).



17. FLUORESCENCE PLATE READER ANALYSIS

Follow Section 15, but omit optional nuclear staining with Hoechst 33342.

1. Resuspend cells in 100-300 µL 1X Cellular Wash Buffer and place on ice.
2. Determine the concentration and compare the cell density of each sample. The non-induced population may have more cells than the induced population, as some pyroptotic cells in the induced samples may be lost during the wash steps. Adjust the volume of the cell suspensions to equalize the cell density. When ready to read, cells should be >3 x 10^6 cells/mL. Adherent cells should be cultured to ~90% confluency.
3. If using suspension cells, pipette 100 µL stained and washed cells per well into a black microtiter plate. Do not use clear plates. If using a bottom-reading instrument, use a plate with black walls and a clear bottom. Analyze at least 2 aliquots per sample. Avoid bubbles.
4. Perform an endpoint read. Set the excitation wavelength at 488 nm and the emission wavelength to 530 nm; if possible, use a 515 nm cut-off filter. FAM-FLICA is excited at 488-492 nm and the emission optima is at 515-535 nm.

18. FLOW CYTOMETRY ANALYSIS

Follow Section 15, but omit optional nuclear staining with Hoechst 33342.

1. Resuspend cells in 300 µL 1X Cellular Wash Buffer and place on ice.
2. Cells may be fixed for analysis up to 16 hours later. Add Fixative at a v/v ratio of 1:5-1:10. Store samples at 2-8°C and protected from light.
3. Run the unstained control. If possible, adjust voltages to place the unstained sample in the first decade of the FL dot plots. This is not possible on some instruments, such as an Accuri C6 flow cytometer.
4. For single-color analysis, a 488 nm blue argon laser or comparable can be used with the emission filter pairing that best approximates 530/30 (often FL-1/FITC channel).
5. Generate a histogram with the log FL-1 on the X-axis versus the number of cells on the Y-axis. Caspase negative (FAM-FLICA -) cells will fall within the lower log fluorescence output decades of the FL-1 X-axis, whereas caspase-positive (FAM-FLICA +) cells will appear as a shoulder or as a separate peak on the right side of the negative peak histogram.
6. For dual-color analyses, run each single color control. Adjust compensation to remove spectral overlap from interfering FL channels. For example, if reading FAM-FLICA in FL-1 and 7-AAD, a red live/dead stain, in FL-3:
   a. Subtract a percentage of the fluorescence in the FAM-FLICA channel from the 7-AAD channel (e.g. FL-3 - %FL-1).
   b. Subtract a percentage of the fluorescence in the 7-AAD channel from the fluorescence in the FAM-FLICA channel (e.g. FL-1 - %FL-3).

   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 FAM-FLICA stained sample being read in FL-1 should have the same MFI in FL-3 as an unstained sample).
7. Run the dual-color experimental samples and analyze.

19. 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.

Figure 4: Microscopy analysis of caspase-1 activity in THP-I cells

THP-I cells were treated with either a negative control (Non-Induced, A), or PMA at 5 ng/mL to induce differentiation into macrophages (Induced, B). After 48 hours, PMA was removed from the induced population and replaced with fresh medium containing LPS at 10 ng/mL to induce caspase-1 activation. After 2 hours, cells were stained FAM-YVAD-FMK for 1 hour, washed, and examined under an Olympus IX-70 inverted photomicroscope equipped with phase contrast and fluorescence optics. In the treated sample, many cells appear bright green, indicating an increased level of caspase-1 activity (B, Induced, right). In the non-induced sample, few green cells are visible, indicating a low level of caspase-1 activity (A, Non-Induced, left). Data courtesy of Dr. Brian Lee, ICT (207:11).

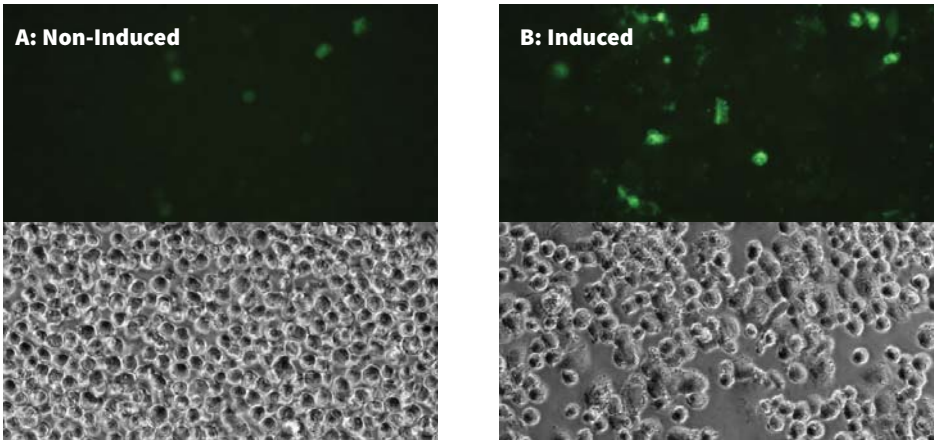


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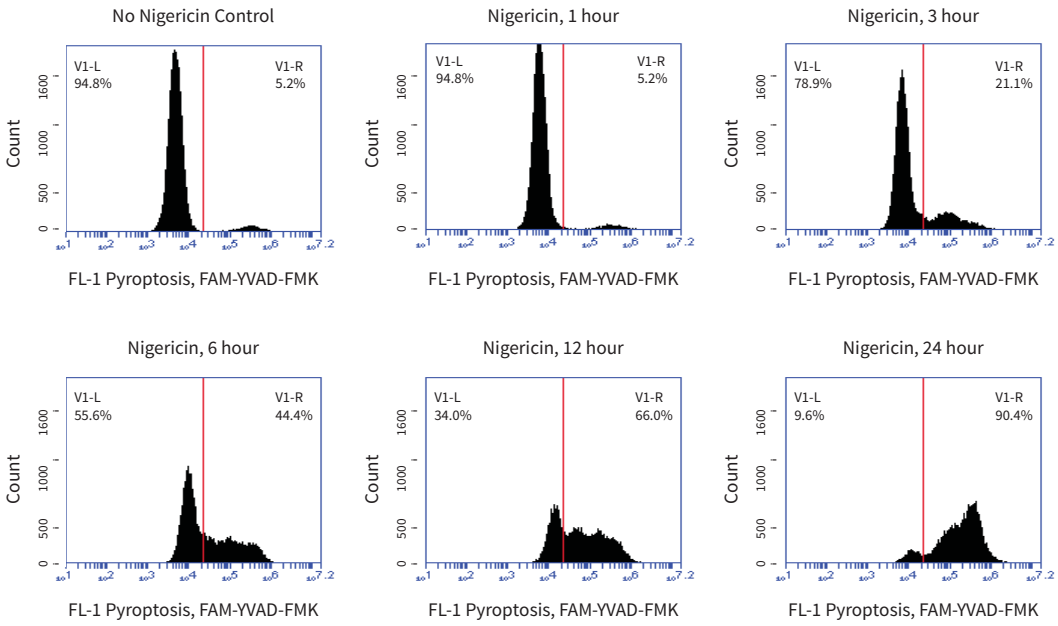
Figure 5: Time-course response in Jurkat cells to nigericin-induced caspase-1 activation

ICT's caspase-1 inhibitor reagent, FAM-YVAD-FMK (kit catalog #9146) was used to monitor the caspase-1 induction response in Jurkat cells treated with Nigericin for various periods of time. A common cell pool was spiked with FAM-YVAD-FMK and divided into separate treatment groups. Starting with 24 hour samples and working backwards, 10 µM Nigericin was added to cells and the samples were incubated at 37°C throughout the induction process. Following their respective treatment exposure periods, the cells were washed and analyzed on an Accuri C6 flow cytometer. The amount of caspase-1 activity detected directly correlated to the duration of the exposure period; the longer the cells were exposed to Nigericin, the larger the proportion of caspase-1 positive cells found in the sample. Data courtesy of Mrs. Tracy Murphy, ICT (220:78).



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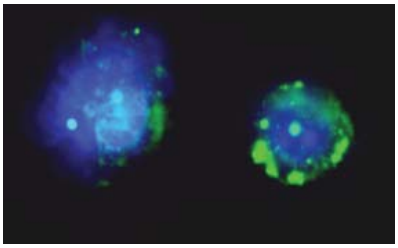

Above: Apoptotic neuroblastoma cells fluoresce green after staining with FAM-FLICA Poly Caspase Assay (#92). Hoechst 33342 (blue in image) nucleic acid stain is included in the kit as well as Propidium Iodide live/dead stain.

At left: SR-FLICA Poly Caspase Assay Kit, standard size (#917)

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