# Pyroptosis FAM and 660 Caspase-1 Kit

BIO-RAD

## Cat. #ICT9145 - 25-50 Tests

| Component | Storage Conditions | Quantity |
|---|---|---|
| 1 vial FAM-YVAD-FMK Caspase-1 Inhibitor | 2-8°C | 25-50 tests |
| 1 vial Hoechst 33342 (200 µg) | 2-8°C | 1 ml |
| 1 vial Nigericin | -20°C | 0.5 µmoles |
| 1 bottle 10x Cellular Assay Buffer | 2-8°C | 15 ml |
| 1 bottle Fixative | 2-8°C | 6 ml |

## #ICT9146 - 100-200 Tests

| Component | Storage Conditions | Quantity |
|---|---|---|
| 4 vials FAM-YVAD-FMK Caspase-1 Inhibitor | 2-8°C | 100-200 tests |
| 1 vial Hoechst 33342 (200 µg) | 2-8°C | 1 ml |
| 1 vial Nigericin | -20°C | 0.5 µmoles |
| 1 bottle 10x Cellular Assay Buffer | 2-8°C | 60 ml |
| 1 bottle Fixative | 2-8°C | 6 ml |

## #ICT9158- 25 Tests

| Component | Storage Conditions | Quantity |
|---|---|---|
| 1 vial FAM-YVAD-FMK Caspase-1 Inhibitor | 2-8°C | 25 tests |
| 1 vial Hoechst 33342 (200 µg) | 2-8°C | 1 ml |
| 1 vial Nigericin | -20°C | 0.5 µmoles |
| 1 bottle 10x Cellular Assay Buffer | 2-8°C | 15 ml |
| 1 bottle Fixative | 2-8°C | 6 ml |

Each kit is provided in two boxes which should be stored at different temperatures:

- Box one contains Nigericin which should be stored at -20°C. Reconstituted Nigericin is stable for up to one year at -20°C. Aliquot to avoid repeat freezing and thawing
- Box two contains all of the other kit components which should be stored at 2-8°C. Do not freeze
- Once the FAM or 660 substrate is reconstituted with DMSO, use immediately or store at -20°C for up to 6 months

## Background

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 (Martinon F et al. 2007, Netea M et al. 2009). The NLRP3 inflammasome is the most studied of all. Activation of the NLRP3 inflammasome follows exposure to PAMPS (first signal) and ATP (second signal). This leads to oligomerization and assembly of a high molecular weight (~700 kDa) multimeric inflammasome complex, which in turn leads to the conversion of pro-caspase-1 into the catalytically active form. Inflammatory caspases, like caspase-1, play a central role in innate immunity by recognizing foreign danger signals and initiating a two-fold response. Firstly, 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. Secondly, caspase-1 or caspase-11 triggers a form of lytic, programmed cell death known as pyroptosis (Jorgensen I et al. 2015, Schroder K and Tschopp J 2010).

Pyroptosis is a highly inflammatory form of regulated 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.

Bio-Rad's Pyroptosis/Caspase-1 Assay Kit utilizes our popular FLICA technology to detect caspase-1 activation. FLICA probes are cell permeant noncytotoxic Fluorescent Labeled Inhibitors of Caspases that covalently bind with active caspase enzymes (Amstad et al. 2001, Bedner et al. 2000). These kits contain the caspase-1 inhibitor reagent YVAD-FMK, which has the preferred binding sequence for caspase-1, Tyr-Val-Ala-Asp (YVAD) (Chapman KT 1992). This preferred caspase-1 binding sequence is labeled with either FAM, a green fluorescent dye, or 660, a far red fluorescent 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.

FLICA can be added directly to the cell culture medium, incubated, and washed. It 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 YVAD-FMK and retain the 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 counterstained with other reagents and fixed or frozen.

Cells labeled with YVAD-FMK can be counterstained with reagents such as the red live/dead stains propidium iodide and 7-AAD. Nuclear morphology may be concurrently observed using Hoechst 33342 (included in the kit), a blue DNA-binding dye. Cells can be viewed through a fluorescence microscope or flow cytometer.

| Format | Max Ex (nm) | Max Em (nm) | Laser (nm) | ZE5 Filter |
|---|---|---|---|---|
| FAM | 488-492 | 515-535 | 488 | 525/35 |
| 660 | 660 | 685-690 | 640 | 720/60 |

## Experimental Set Up

Staining with caspase-1 positive cells may require cell culture for a period of time to obtain the correct cell concentration and allow experimental treatment to take effect. The recommended staining is 5-10 µl of 30-60x FLISP per 300 µl of cells at a cell concentration of 3-5 x $10^5$ cells/ml, however this may vary depending upon the cells and treatment used.

Include appropriate controls for your experiment, such as:

- Unlabeled treated and untreated cells
- Labeled treated and untreated cells
- Vehicle (DMSO) only treated cells
- A positive control, cells treated with a known caspase-1 inducer may also be required
- A single stained sample with propidium iodide is useful to determine underlying levels of necrosis within your sample
- A single stained sample of FLICA, propidium iodide, and any additional fluorophore may be required to determine compensation levels

## Reagent Preparation

### Nigericin

Add 100 µl of DMSO to form a 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. Immediately prior to addition to the samples and controls, dilute 5 mM Nigericin stock 1:10 in DI water 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 DI. Use Nigericin at 1-20 µM to induce NLRP3 inflammasome and caspase-1 activation in cells. Typical treatment periods range from 3-24 hr at 37°C however you may need to adjust the concentration of Nigericin and treatment period to accommodate the particular cell line and research conditions.

**Danger:** Nigericin is toxic if swallowed, causes skin irritation and serious eye irritation, and may cause respiratory irritation. Avoid contact with skin, eyes, and clothing by wearing a lab coat, gloves, and safety glasses. In case of exposure, immediately flush eyes or skin with water. See SDS for further information.

### FLICA

Add 50 µl DMSO to form the 150-300x stock concentrate of FLICA. Once reconstituted, it may be stored at -20°C for 6 months protected from light and thawed no more than twice during that time. 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 min of dilution into aqueous buffers.

### Wash Buffer

10x Cellular Wash Buffer may form precipitates during cold storage. If this happens, gently warm it until all crystals have dissolved. Do not boil. Dilute 10x Cellular Wash Buffer 1:10 in DI water. For example, add 15 ml 10x Cellular Wash Buffer to 135 ml DI water 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.

## Flow Cytometry Protocol

1. Prepare samples and appropriate controls in tubes or plates in cellular wash buffer. For staining in plates, volumes will need to be adjusted and optimized.
2. Reconstitute FLICA with 50 µl DMSO.
3. Immediately before use, dilute FLICA 1:5 by adding 200 µl PBS.
4. Add 5-10 µl FLICA to each sample (290-295 µl aliquot of cultured cells).
5. Incubate at 37°C protected from light. This can be from 30 min to several hours and should be optimized for the cell line and experimental conditions. Gently resuspend cells approximately every 20 min throughout the staining process.
6. Wash cells with 2 ml wash buffer and spin cells (twice).
7. If desired, label with additional viability dyes such as PI, or an antibody.
8. If desired, fix cells by adding fixative at a 1:5-1:10 ratio.
9. Analyze with a flow cytometer.

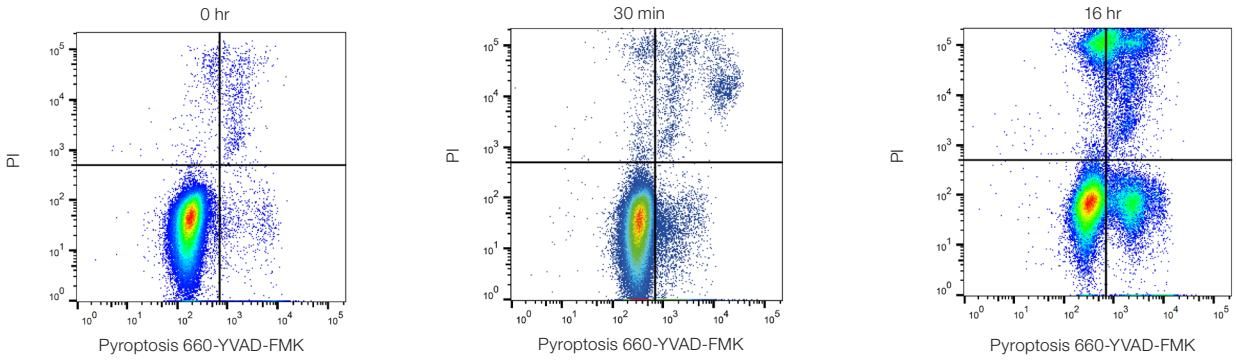


**Fig. 1. Detection of pyroptosis in Jurkat cells.** Jurkat cells were stained with Pyroptosis 660 Caspase-1 Assay, far red (#ICT9158) for 2 hr followed by treatment with 10 µM nigericin as shown. Pyroptosis can be demonstrated by an increase in active caspase-1 detected by an increase in 660 far red fluorescence. Data analyzed on the ZE5 Cell Analyzer.

# Microscopy Protocol for Suspension Cells

1. Prepare samples and appropriate controls resuspended in cellular assay buffer.
2. Reconstitute FLICA with 50 µl DMSO.
3. Immediately before use, dilute FLICA 1:5 by adding 200 µl PBS.
4. Add 5-10 µl FLICA to each sample (290-295 µl aliquot of cultured cells).
5. Incubate at 37°C protected from light. This can be from 30 min to several hours and should be optimized for the cell line and experimental conditions. Gently resuspend cells approximately every 20 min throughout the staining process.
6. Wash cells with 2 ml wash buffer and spin cells (twice).
7. Counterstain with Hoechst 33342 (1.5 µl per 300 µl of cell suspension), other nuclear dyes, or compatible dyes and antibodies if required.
8. Place 15-20 µl of cell suspension onto a microscope slide and cover with a coverslip. Observe on a fluorescent microscope using the correct filters.
9. If desired, fix cells by adding fixative at a 1:5-1:10 ratio and incubate for 15 min in the dark.
10. Place on a microscope slide and allow to dry.
11. Rinse with PBS and mount. Observe on a fluorescent microscope using the correct filters.

# Microscopy Protocol for Adherent Cells

1. Seed $10^{4}$-$10^{5}$ cells onto a sterile coverslip in a 35 mm petri dish, onto chamber slides, or grow in a plate.
2. Reconstitute FLICA with 50 µl DMSO
3. Immediately before use, dilute FLICA 1:5 by adding 200 µl PBS
4. Add FLICA 1:30-1:60 to each sample.
5. Incubate at 37°C protected from light. This can be from 30 min to several hours and should be optimized for the cell line and experimental conditions.
6. Remove the staining solution and incubate for 10 min at 37°C in wash buffer to allow any unbound FLICA to diffuse out of the cells.
7. Carefully remove and replace overlay wash buffer with fresh wash buffer and incubate another 10 min at 37°C.
8. Gently remove overlay wash buffer and replace for a third wash step. Incubate 10 min at 37°C.
9. Remove wash buffer and counterstain with Hoechst 33342 (1.5 ml per 300 ml of wash buffer), other nuclear dyes, or compatible dyes and antibodies if required.
9. Mount the coverslip facing down into a drop of PBS.
10. Observe on a fluorescent microscope using the correct filters.

## References


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