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APOPTOSIS DETECTION KITS

Green Fluorescent Caspase Activity Assays

FAM-FLICA™ Reagent Manual

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Caspase-10b	RA15047	Rabbit IgG	H; M	WB
Caspase-12	RA15048	Rabbit IgG	M; R	WB
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Cathepsin B (Human)	GT15046	Goat IgG	H	WB; E
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1. Caspase Kits and Introduction

Name	Catalog #
Polycaspase Assay Kit, green	KF17200
Polycaspase Assay Kit, red	KF17300
Caspase 3 & 7 Assay Kit, green	KF17203
Caspase 3 & 7 Assay Kit, red	KF17301
Caspase 8 Assay Kit, green	KF17105
Caspase 9 Assay Kit, green	KF17206
Caspase 9 Assay Kit, red	KF17302

Introduction

Apoptosis is an evolutionarily conserved form of cell suicide, which follows a specialized cellular process. The central component of this process is a cascade of proteolytic enzymes called caspases. These enzymes participate in a series of reactions that are triggered in response to pro-apoptotic signals and result in the cleavage of protein substrates, causing the disassembly of the cell¹.

Caspases have been identified in organisms ranging from *C. elegans* to humans. The mammalian caspases play distinct roles in apoptosis and inflammation. In apoptosis, caspases are responsible for proteolytic cleavages that lead to cell disassembly (effector caspases), and are involved in upstream regulatory events (initiator caspases). An active caspase consists of two large (~20 kD) and two small (~10 kD) subunits that form two heterodimers which associate in a tetramer²⁻⁴. In common with other proteases, caspases are synthesized as precursors that undergo proteolytic maturation, either autocatalytically or in a cascade by enzymes with similar specificity⁵.

Caspase enzymes specifically recognize a 4 amino acid sequence (on the target substrate) which necessarily includes an aspartic acid residue. This residue is the target for the cleavage reaction, which occurs at the carbonyl end of the aspartic acid residue⁶. Caspases can be detected via immunoprecipitation, immunoblotting techniques using caspase specific antibodies, or by employing fluorochrome substrates which become fluorescent upon cleavage by the caspase.

FLICA Apoptosis Detection Kits use a novel approach to detect active caspases. The methodology is based on a Fluorochrome Inhibitor of Caspases. Once inside the cell, the fluorescent caspase inhibitor binds covalently to the active caspase⁷. These inhibitors are cell permeable and non-cytotoxic. For kits using green fluorescence, a carboxyfluorescein-labeled fluoromethyl ketone peptide inhibitor of caspases is used, FAM-XXX-FMK where XXX refers to the caspase inhibitor. (Neuromics also offers SR-FLICA Apoptosis Detection Kits that use sulforhodamine-labeled inhibitors; see above).

When added to a population of cells, the FAM-XXX-FMK peptide-probe enters each cell and covalently binds to a reactive cysteine residue that resides on the large subunit of the active caspase heterodimer, thereby inhibiting further enzymatic activity. Because the FAM-XXX-FMK reagent becomes covalently coupled to the active enzyme, it is retained within the cell, while any unbound FAM-XXX-FMK diffuse out of the cell and is washed away. The remaining green fluorescent signal is a direct measure of the number of active caspase enzymes that were present in the cell at the time the reagent was added. Cells that contain the bound FAM-FLICA inhibitor can be analyzed by 96-well-plate based fluorometry, fluorescence microscopy, or flow cytometry.

Because the FAM-FLICA reagent FAM-VAD-FMK irreversibly binds to many activated caspases (caspase-1, -3, -4, -5, -6, -7, -8 and -9), it can be used as a generic probe for the detection of most caspases. In contrast the other FAM-FLICA reagents are used to measure specific Caspases (i.e. Caspase 8 or Caspase 9 (see table above)).

Following the suggested protocols listed here, each sample requires 10 μ L of 30X FAM-FLICA solution (equal to 2 μ L of 150X FAM-FLICA stock).

The FAM-FLICA kit was designed to evaluate apoptotic events using 3 different fluorescence detection methods: 96-well microtiter plate fluorometry for quantitation; fluorescence microscopy for qualitative analysis; and flow cytometry for quantitation. The fluorescent reagent has an optimal excitation range from 488-492 nm, and emission range from 515 - 535 nm (the excitation / emission pairs which best approximate this optimal range should be used). Cells labeled with the reagent may be read immediately or preserved for 24 hours using the fixative. Unfixed samples may be analyzed with propidium iodide or Hoechst stain.

Using a fluorescence plate reader (with **black** microtiter plates), apoptosis can be quantitated as the amount of green fluorescence emitted from probes bound to caspases. Cell populations in more advanced stages of apoptosis will have a higher RFU intensity than cell populations in earlier stages.

Viewing cells through a fluorescence microscope, apoptotic cells will fluoresce green, while non-apoptotic cells will appear mostly unstained. As apoptosis progresses, the amount of active caspase enzymes capable of binding the fluorescently labeled inhibitor increases and eventually reaches a maximum level. Therefore, cells in more advanced stages of apoptosis will appear brighter green than cells in earlier stages.

Using a flow cytometer, analysis is done using a 15 mW argon ion laser at 488 nm. Fluorescein is measured on the FL1 channel, and a log FL1 (X-axis) versus number of cells (Y-axis) histogram may be generated. On this histogram, there will appear two cell populations represented by two peaks. The majority of the caspase negative cells will occur within the first log decade of the FL1 (X) axis (first peak), whereas the caspase-positive cell population will appear as a separate peak or as a shoulder of the first peak showing increased fluorescence intensity. Cells with the active caspase (those undergoing apoptosis) will fluoresce green.

2. Contents of the FAM-FLICA Apoptosis Detection Kit:

- FAM-FLICA Reagent, 25-test kits 1 vial; 100-test kits 4 vials.
- 10X Wash Buffer, 60 mL or 15 mL
- Fixative, 6 mL
- Propidium Iodide, 1 mL
- Hoechst Stain, 1 mL

3. Recommended Materials and Equipment (not all are required):

- Cultured cells with media
- Reagents to induce apoptosis
- 15 mL polystyrene centrifuge tube (1 per sample)
- Amber vials or polypropylene tubes for storage of 150X concentrate at -20°C , if aliquoted
- 150 mL or 600 mL graduated cylinder
- Slides and Hemocytometer
- Clinical centrifuge at $<400 \times g$
- 37°C CO_2 incubator
- Vortexer
- Pipette(s) capable of dispensing at $10\mu\text{L}$, $50\mu\text{L}$, $200\mu\text{L}$, $300\mu\text{L}$, 1mL
- dH_2O and Phosphate Buffered Saline (PBS) pH 7.4
- Dimethyl Sulfoxide (DMSO), $50\mu\text{L}$ or $200\mu\text{L}$ needed
- Ice or 4°C refrigerator to store cells

4. Instrumentation (not all are required):

- 96-well fluorescence plate reader with excitation at 488 nm, emission 520 nm filter pairings, and **black** round or flat bottom 96-well microtiter plates.
- Fluorescence microscope with appropriate filters (excitation 490 nm, emission >520 nm for FAM-FLICA; excitation at 490 nm and emission at 635 nm for PI; and if Hoechst is used, a UV-filter with excitation at 365 nm, emission at 480 nm) and slides.
- Flow cytometer with a 15 mW, 488 nm argon excitation laser, with appropriate filters (excitation 490 nm, emission >520 nm for FAM-FLICA; excitation at 490 nm and emission at 635 nm for PI).

5. Storage and Shelf-Life

- Store the unopened kit (and each unopened component) at 2°C to 8°C until the expiration date.
- Protect the FAM-FLICA reagent from light at all times.
- Once reconstituted, the 150X FAM-FLICA stock should be stored at -20°C protected from light. This reagent is stable for up to 6 months and may be thawed twice during that time.
- Once diluted, store the 1X wash buffer at $2 - 8^{\circ}\text{C}$ up to 14 days.

6. Safety Information

- Use gloves while handling the FAM-FLICA reagent, propidium iodide, Hoechst stain, and fixative.
- Dispose of all liquid components down the sink and flush with copious amounts of water. Solid components may be tossed in standard trash bins.

7. Overview of the POLYCASP Protocol

Staining apoptotic cells with the FAM-FLICA kit can usually be completed within a few hours. **Once made, the 30X FAM-FLICA solution must be used immediately.** Because time is needed for the culturing cells and inducing apoptosis, the FAM-FLICA reagents should be prepared at the end of your apoptosis induction process. The following is a quick overview of the FAM-FLICA protocol:

1. Culture cells to a density optimal for apoptosis induction according to your specific induction protocol, but not to exceed 10^6 cells/mL.
2. At the same time, culture a non-induced negative control cell population at the same density as the induced population for every labeling condition.
3. Induce apoptosis following your protocol
4. Prepare 1X wash buffer (Section 9).
5. Prepare 150X FAM-FLICA stock (Section 13).
6. Prepare 30X FAM-FLICA solution (Section 14 or 16).
7. Stain cells with 30X FAM-FLICA solution, incubate for 1 hour, and wash cells (Section 17, 20, 21, 23, or 24).
8. If desired, label cells with propidium iodide (Section 10).
9. If desired, label cells with Hoechst stain (Section 11).
10. If desired, fix cells (Section 12).
11. Analyze data via microtiter plate fluorometry, fluorescence microscopy, or flow cytometry.

8. Induction of Apoptosis

The FAM-FLICA kit works with your current apoptosis protocols - induce apoptosis as you normally would, then label the cells with FAM-FLICA inhibitor.

9. Preparation of 1X Wash Buffer

The wash buffer is supplied as a 10X concentrate which must be diluted to 1X with dH₂O prior to use.

1. If necessary, gently warm the 10X concentrate to completely dissolve any salt crystals that may have come out of solution.
2. For the FAM-FLICA-25 Kit, add the entire bottle (15 mL) of 10X wash buffer to 135 mL of dH₂O (to make 150 mL).
3. Or, for the FAM-FLICA -100 Kit, add the entire bottle (60 mL) of 10X wash buffer to 540 mL of dH₂O (to make 600 mL).
4. Or, if not using the entire bottle, dilute the 10X wash buffer 1:10 in DI H₂O. For example, add 10 mL 10X wash buffer to 90 mL dH₂O (to make 100 mL).
5. Let the solution stir for 5 minutes or until all crystals have dissolved.
6. If not using the 1X wash buffer the same day it was prepared, store it covered at 2° - 8°C for up to 14 days.

Warning: The wash buffer contains sodium azide, which is harmful if swallowed or absorbed through the skin. Sodium azide can react with lead and copper sink drains forming explosive compounds. When disposing of excess wash buffer, flush sink with copious amounts of water.

10. Propidium Iodide

Propidium iodide (PI) may be used to distinguish between live cells and dead cells, either caspase-negative or caspase-positive. PI stains necrotic, dead, and membrane-compromised cells. They may be viewed through a fluorescence microscope, or analyzed on a flow cytometer (Section 21 or 24). The dye excites at 488 - 492 nm and exhibits an emission maximum at 635 nm. PI is provided ready-to-use at 250 µg/mL.

Warning: Propidium iodide is a potential mutagen. Use of gloves, protective clothing, and eyewear are strongly recommended. When disposing, flush sink with copious amounts of water.

11. Hoechst Stain

Hoechst stain can be used to label the nuclei of dying cells after labeling with the FAM-FLICA reagent. It is revealed under a microscope using a UV-filter with excitation at 365 nm and emission at 480 nm (Section 20 or 21). Hoechst stain is provided ready-to-use at 200 µg/mL.

Warning: Hoechst stain is a potential mutagen. Use of gloves, protective clothing, and eyewear are strongly recommended. When disposing, flush sink with copious amounts of water; see MSDS for further information.

12. Fixative

If the stained cell populations cannot be evaluated immediately upon completion of the FAM-FLICA staining protocol, cells may be fixed and analyzed up to 24 hours later on a microscope or flow cytometer. The fixative is a formaldehyde solution and will not interfere with the carboxyfluorescein labeling once the FAM-FLICA reaction has taken place.

After labeling, add the fixative into the cell solution at a 1:10 ratio. For example, add 100 µL fixative to 900 µL cells (Section 20, 21, or 23). Fixed cells may be stored on ice or at 4°C up to 24 hours.

Do not use ethanol-based or methanol-based fixatives to preserve the cells - they will inactivate the fluorescent label.

Never add the fixative until the staining and final wash steps have been completed.

13. Reconstitution of the 150X FAM-FLICA Stock

The FAM-FLICA reagent is supplied as a highly concentrated lyophilized powder. It must first be reconstituted in DMSO, forming a 150X stock concentrate, and then diluted 1:5 in PBS to form a final 30X working solution. For best results, the 30X working solution should be prepared immediately prior to use; however, the reconstituted 150X stock concentrate can be stored at -20°C for future use.

The newly reconstituted 150X FAM-FLICA stock must be used or frozen immediately after it is prepared and protected from light during handling.

1. Reconstitute each vial of lyophilized FAM-FLICA with 50 µL DMSO. This yields a 150X concentrate. (The FAM-FLICA -25 kit contains 1 vial; the FAM-FLICA -100 kit contains 4 vials.)
2. Mix by swirling or tilting the vial, allowing the DMSO to travel around the base of the amber vial until completely dissolved. At room temperature (RT), this reagent should be dissolved within a few minutes.
3. If immediately using this solution, dilute it to 30X (Section 14).
4. Or, if using later, aliquot and store it at -20°C (Section 15).

14. Preparation of 30X FAM-FLICA Solution for Immediate Use

Using the freshly reconstituted 150X FAM-FLICA stock, prepare the 30X working-strength FAM-FLICA solution by diluting the stock 1:5 in PBS at pH 7.4. Following the suggested protocols here, each sample to be tested requires only 10 µL of 30X FAM-FLICA solution (or 2 µL of the 150X FAM-FLICA stock).

1. If you are using the entire vial, add 200 μL PBS pH 7.4 to each vial (each vial contains 50 μL of the 150X stock; this yields 250 μL of a 30X solution).
2. If not using the entire vial, dilute the 150X stock 1:5 in PBS, pH 7.4. For example, add 10 μL of the 150X stock to 40 μL PBS (this yields 50 μL of a 30X solution). Store the unused 150X stock at -20°C (Section 15).
3. Mix by inverting or vortexing the vial at RT.

The 30X working strength FAM-FLICA solution must be used the same day that it is prepared.

15. Storage of 150X FAM-FLICA Stock for Future Use

If not all of the 150X **FAM-FLICA** stock will be used the same time it is reconstituted, the unused portion may be stored at -20°C for 6 months. During that time, the 150X FAM-FLICA stock may be thawed and used twice. After the second thaw, discard any remaining 150X FAM-FLICA stock. If you anticipate using it more than twice, make small aliquots in amber vials or polypropylene tubes and store at -20°C protected from light. When ready to use, follow Section 16 below.

16. Preparation of 30X FAM-FLICA Solution from a Frozen Aliquot

If some of the 150X FAM-FLICA reagent was previously reconstituted and then stored at -20°C , it may be used 2 more times within 6 months.

1. Thaw the 150X FAM-FLICA stock and protect from light.
2. Once the aliquot has become liquid, dilute the 150X stock solution 1:5 in PBS, pH 7.4. For example, mix 10 μL of 150X FAM-FLICA reagent with 40 μL of PBS.
3. Mix by inverting or vortexing the vial at RT.
4. If the 150X FAM-FLICA stock was frozen immediately after reconstitution and was never thawed, return it to the freezer. If the stock was thawed once before, discard it.
5. Proceed to the labeling protocol (Section 17, 20, 21, 23, 24, or 25).

17. 96-Well Fluorescence Plate Reader Staining Protocol

Following this fluorescence plate reader protocol, each sample requires 10 μL of 30X FAM-FLICA solution (equal to 2 μL of 150X FAM-FLICA stock).

1. As discussed in Section 7, culture cells to a density optimal for apoptosis induction according to your specific induction protocol.

Cell density in the cell culture flasks should not exceed 10^6 cells/mL. Cells cultivated in excess of this concentration may begin to naturally enter apoptosis. Optimal cell concentration will vary depending on the cell line used.

2. Induce apoptosis following your protocol (as mentioned in Section 8).
3. At the same time, culture an equal volume of non-induced cells for a negative control cell population. Make sure that both tubes of cells contain similar quantities of cells. Cells can be concentrated just prior to induction to $2 - 6 \times 10^6$ cells/mL. (Cells may be induced at even lower concentrations, but must be concentrated to $\sim 1 \times 10^7$ cells/mL for FAM-FLICA labeling. If necessary, cells can be concentrated by centrifugation for 5 minutes at $<400 \times g$ at RT.)
4. Once induction is completed, transfer 290 – 300 μL of each cell suspension to sterile tubes. Larger cell volumes can also be used as determined by each investigator, however more of the FAM-FLICA reagent may be needed per sample. Larger volume cell suspensions label nicely using 25 cm^2 tissue culture flasks (laid flat) as the incubation vessel.

When ready to label with the 30X FAM-FLICA solution, cells should be at least 5×10^5 cells/100 μL aliquot per microtiter plate well. Density can be determined by counting cell populations on a hemocytometer.

5. Add 10 μL 30X FAM-FLICA solution directly to the 290 – 300 μL cell suspension.
6. Or, if a different cell volume was used, add the 30X FAM-FLICA solution at a 1:30 ratio. For example, if 2.9 mL of cell suspension was used, add 100 μL of the 30X FAM-FLICA solution (forming a final volume of 3 mL).

Each investigator should adjust the amount of FAM-FLICA reagent used to accommodate their particular cell line and research conditions.

7. Mix the cells by slightly flicking the tubes.
8. Incubate cells for 1 hour at 37°C under 5% CO_2 , protecting the tubes from light. As cells may settle on the bottom of the tubes, gently resuspend them by swirling cells once or twice during this incubation time. This will ensure an even distribution of the FAM-FLICA reagent among all cells.
9. Add 2 mL of 1X wash buffer to each tube.
10. Mix the cells.
11. Centrifuge cells at $<400 \times g$ for 5 minutes at room temperature (RT).
12. Carefully remove and discard supernatant.
13. Gently vortex the cell pellet to disrupt any cell-to-cell clumping.
14. Resuspend the cell pellet in 1 mL 1X wash buffer.
15. Centrifuge cells at $<400 \times g$ for 5 minutes at RT.
16. Carefully remove and discard supernatant.
17. Gently vortex the cell pellet to disrupt any cell-to-cell clumping.
18. Resuspend the cell pellet in 1 mL 1X wash buffer.

19. Determine the concentration of both the induced and non-induced cell populations. This can be done while the cells are being pelleted down for the last time (Step 20). To count cells:
 - a. Remove 50 μ L from each tube.
 - b. Add to 450 μ L PBS (forming a 1:10 dilution of each).
 - c. Count the cells using a hemocytometer.
 - d. After counting, compare the density of each. The non-induced population may have more cells than the induced population, as some induced cells may be lost during the apoptotic process. If there is a dramatic loss in stimulated cell population numbers, adjust the volume of the induced cell suspension to match the cell density of the non-induced suspension (Step 24).
20. Centrifuge the remaining cells at $<400 \times g$ for 5 minutes at RT.
21. Carefully remove and discard supernatant.
22. Resuspend non-stimulated cells in 400 μ L PBS
23. If it is not necessary to equilibrate the cell concentrations (as discussed in Step 19d), resuspend the stimulated cells in 400 μ L PBS as well.
24. If it is necessary to equilibrate the cell concentrations (from Step 19d), adjust the suspension volume of the PBS for the induced cells to approximate the cell density of the non-induced population. This adjustment step is optional if your cell treatment does not result in a dramatic loss in stimulated cell population numbers.
25. Place 100 μ L of the cell suspensions into each of 2 wells of a **black** microtiter plate. Do not use clear plates. Avoid bubbles.
26. Measure the fluorescence intensity of fluorescein (Section 18).

18. 96-Well Fluorescence Plate Reader Set Up

1. Set the plate reader to perform an endpoint read.
2. Set the excitation wavelength at 490 nm and the emission wavelength to 520 nm. Fluorescein has an optimal excitation range from 488 - 492 nm, and emission range from 515 - 535 nm. Select the filter pairing which most closely approximates this range; the filter pairing used may differ slightly from these optimal settings.
3. Read the sample.
4. An example of differential FAM-FLICA fluorescence intensities in induced versus non-induced Jurkat cells is shown in Figure 1 using a 96-well fluorescence plate reader.

19. Fluorescence Microscopy Staining Protocol for Adherent Cells

1. Trypsinize cells.
2. Count cells.
3. Seed about 10^4 - 10^5 cells onto a sterile glass coverslip in a 35 mm petri dish or onto chamber slides.
4. Grow cells in their respective cell culture media formulation for 24 hours at 37°C (as discussed in Section 7).

5. Induce cells to undergo apoptosis and sample at time points according to your specific protocol (as mentioned in Section 8).
6. Add the 30X FAM-FLICA solution to the medium at a 1:30 ratio. For example, add 10 μ l 30X FAM-FLICA to 290 – 300 μ l medium.

Each investigator should adjust the amount of FAM-FLICA reagent used to accommodate their particular cell line and research conditions.

7. Mix well.
8. Incubate cells for 1 hour at 37°C under 5% CO₂.
9. Remove the medium.
10. If cells are to be monitored using Hoechst stain, add 1.5 μ L Hoechst stain to 300 μ L media (0.5% v/v). Add this media to the cells.
 - a. Incubate for 5 minutes at 37°C under 5% CO₂.
 - b. Go on to Step 11.
11. Wash cells twice with 2 ml 1X wash buffer.
12. At this point, cells may be analyzed directly (Step 13), or fixed and analyzed later (Step 14).
13. To analyze directly, mount a coverslip with cells facing down onto a microscope slide containing a drop of 1X wash buffer. Or, remove the plastic frame of the chamber slide, add a drop of 1X wash buffer onto the glass slide and cover with a coverslip. Go on to Step 15.
14. To fix the cells and analyze later, add fixative to wash buffer at a 1:10 ratio. For example, add 40 μ L fixative to 360 μ L 1X wash buffer.
 - a. Mount a coverslip with cells facing down onto a microscope slide containing a drop of fixative plus wash buffer. Or, remove the plastic frame of the chamber slide, add a drop of fixative plus wash buffer onto the glass slide and cover with a coverslip.
 - b. Keep fixed cells at 2°C - 8°C protected from light for up to 24 hours. Go on to Step 15.
15. Observe cells under a fluorescence microscope using a bandpass filter (excitation 490 nm, emission >520 nm) to view the green fluorescence of caspase positive cells. If Hoechst stain was also used, it can be seen using a UV-filter with excitation at 365 nm and emission at 480 nm. (If these filters are not available, select a filter combination that best approximates these settings.)

20. Fluorescence Microscopy Staining Protocol for Suspension Cells

1. Culture cells to a density optimal for apoptosis induction according to your specific induction protocol (as discussed in Section 7).
2. Cultivate or concentrate cells to a density of at least 5×10^5 cells/mL.

Cell density in the cell culture flasks should not exceed 10^6 cells/mL. Cells cultivated in excess of this concentration may

begin to naturally enter apoptosis. Optimal cell concentration will vary depending on the cell line used.

3. Induce cells to undergo apoptosis and take samples according to your specific protocol (as mentioned in Section 8).
4. At the same time, culture an equal volume of non-induced cells for a negative control cell population. Make sure that both the negative control and induced positive cell population tubes contain similar quantities of cells.
5. Transfer 290 – 300 μL of each induced and negative control cell populations into fresh tubes. Or, if desired, larger cell volumes can be used, however more of the 30X FAM-FLICA solution may be required. Larger volume cell suspensions label nicely using 25 cm^2 tissue culture flasks (laid flat) as incubator vessels.

When ready to label with the 30X FAM-FLICA solution, cells should be at least 5×10^5 cells/mL. Density can be determined by counting cell populations on a hemocytometer.

6. Add 10 μL of the 30X working dilution FAM-FLICA solution directly to each 290 – 300 μL cell suspension.
7. Or, if a larger cell volume was used, add the 30X FAM-FLICA solution at a 1:30 ratio. For example, if 2.9 mL of cell suspension was used, add 100 μL of the 30X FAM-FLICA solution (forming a final volume of 3 mL).

Each investigator should adjust the amount of FAM-FLICA reagent used to accommodate their particular cell line and research conditions.

8. Mix the cells by slightly flicking the tubes.
9. Incubate cells for 1 hour at 37°C under 5% CO_2 , protecting the tubes from light. As cells may settle on the bottom of the tubes, gently resuspend them by swirling cells once or twice during this incubation time. This will ensure an even distribution of the FAM-FLICA reagent among all cells.
10. If cells are to be monitored using Hoechst stain, add 1.5 μL Hoechst stain (0.5% v/v). Incubate for 5 minutes at 37°C under 5% CO_2 .
11. Add 2 mL of 1X wash buffer to each tube.
12. Gently mix.
13. Centrifuge the cells at <400 X g for 5 minutes at RT.
14. Carefully remove and discard supernatants.
15. Gently vortex the pellets to disrupt any cell-to-cell clumping.
16. Resuspend cells in 1 mL 1X wash buffer.
17. Gently mix.
18. Centrifuge the cells at <400 X g for 5 minutes at RT.
19. Carefully remove and discard supernatants.

20. Gently vortex pellets to disrupt any cell-to-cell clumping.
21. Resuspend the cell pellets in 300 μL 1X wash buffer (higher volumes may be used if a larger staining cell volume was used).
22. Place cells on ice.
23. At this point, the cells may be stained with propidium iodide (PI) for bicolor analysis (Step 24), observed immediately (Step 25), or fixed for future viewing (Step 26).
24. To exclude dead cells from the analysis, 1.5 μL PI solution may be added at this point (0.5% v/v). Cells may then be viewed using a long pass filter with the excitation at 490 nm, emission >520 nm; PI has a maximum emission at 637 nm.
25. To view cells immediately, place 1 drop of the cell suspension onto a microscope slide and cover with a coverslip; go to Step 27.
26. If not viewing immediately, cells may be fixed for viewing up to 24 hours later. If cell pellets were resuspended in 300 μL wash buffer, add 30 μL fixative to each tube. If cells were resuspended in a different volume, add the fixative at a 1:10 ratio into the volume of cell suspension to be fixed. For example, if 3 mL was used, add 300 μL fixative.
 - a. Incubate cells for 15 minutes at RT in the dark.
 - b. Dry cells onto a microscope slide.
 - c. Briefly wash the cells with PBS.
 - d. Cover cells with mounting media and coverslip.
 - e. Store slides at 2° – 8°C up to 24 hours. Go on to Step 27.
27. Observe cells under a fluorescence microscope using a bandpass filter (excitation 490 nm, emission >520 nm) to view green fluorescence. Cells bearing active caspase enzymes covalently coupled to the FAM-FLICA reagent appear green. If Hoechst stain was also used, it can be seen using a UV-filter with excitation at 365 nm and emission at 480 nm. (If these filters are not available, select a filter combination that best approximates these settings.)

21. Flow Cytometry with Single-Color Staining

Positive control samples can be prepared by inducing cells in suspension culture to undergo apoptosis (see Section 8 for examples of apoptosis induction protocols). Following this flow cytometer protocol, each sample requires 10 μL of 30X FAM-FLICA solution (equal to 2 μL of 150X FAM-FLICA stock). After labeling with FAM-FLICA, cells can be analyzed directly by flow cytometry, or the cells may be fixed first and then analyzed by flow cytometry. For a thorough analysis, 2 types of samples are recommended (both should have induced and non-induced populations):

- a. unstained cells (induced and non-induced).
 - b. cells stained with FAM-FLICA (induced and non-induced).
1. As discussed in Section 7, culture cells to a density optimal for apoptosis induction according to your specific induction protocol.
 2. Induce apoptosis following your protocol (as mentioned in Section 8).
 3. Culture an equal volume of non-induced cells for a negative control cell population. Make sure that both tubes of cells contain similar quantities of cells.

Ideally, cells should be at $\sim 1 \times 10^6$ cells/mL for FAM-FLICA labeling. If necessary, cells may be induced to undergo apoptosis at lower concentrations, but then concentrated just prior to labeling to $0.5 - 2 \times 10^6$ cells/mL by centrifugation for 5 minutes at $<400 \times g$ at RT.

4. Once induction is completed, transfer 300 μL of each cell suspension to sterile tubes. (Larger cell volumes can also be used as determined by each investigator, however more of the FAM-FLICA reagent may be needed per sample. Larger volume cell suspensions label nicely using 25 cm^2 tissue culture flasks (laid flat) as the incubation vessel.)
5. Add 10 μL 30X FAM-FLICA solution directly to the 300 μL cell suspension.
6. Or, if a different cell volume was used, add the 30X FAM-FLICA solution at a 1:30 ratio. For example, if 2.9 mL of cell suspension was used, add 100 μL of the 30X FAM-FLICA solution (forming a final volume of 3 mL).

Each investigator should adjust the amount of FAM-FLICA reagent used to accommodate their particular cell line and research conditions.

7. Mix the cells by slightly flicking the tubes.
8. Incubate cells for 1 hour at 37°C under 5% CO_2 , protecting the tubes from light. As cells may settle on the bottom of the tubes, gently resuspend them by swirling cells once or twice during this incubation

time. This will ensure an even distribution of the FAM-FLICA reagent among all cells.

9. Add 2 mL of 1X wash buffer to each tube.
10. Mix the cells.
11. Centrifuge cells at $<400 \times g$ for 5 minutes at room temperature (RT).
12. Carefully remove and discard supernatant.
13. Gently vortex the cell pellet to disrupt any cell-to-cell clumping.
14. Resuspend the cell pellet in 1 mL 1X wash buffer.
15. Centrifuge cells at $<400 \times g$ for 5 minutes at RT.
16. Carefully remove and discard supernatant.
17. Gently vortex the cell pellet to disrupt any cell-to-cell clumping.
18. Resuspend the cell pellet in 400 μL 1X wash buffer.
19. At this point, cells may be fixed (Step 20) or analyzed directly (Step 21).
20. If desired, fix cells by adding 40 μL fixative to the 400 μL cell suspension and mix. Or, if a different volume was used, add the fixative at a 1:10 ratio into the volume of cell suspension to be fixed. Keep fixed cells at $2^\circ\text{C} - 8^\circ\text{C}$ protected from light for up to 24 hours. Go on to Step 22.
21. Put samples on ice.
22. For single-color analysis, use a 15 mW argon ion laser at 488 nm. Measure fluorescein on the FL1 channel. Generate a log FL1 (X-axis) versus number of cells (Y-axis) histogram. On the histogram, there will appear two cell populations represented by two peaks. The majority of the caspase negative (-) cells will normally occur within the first log decade of the FL1 (X) axis (first peak), whereas the caspase-positive (+) cell population will appear as a separate peak or as a shoulder of the first peak showing increased fluorescence intensity. Position the vertical cursor in the gap between the two peaks. Events falling to the right of the vertical cursor should be counted as caspase positive (+).

22. Flow Cytometry with Bicolor Staining

If a bicolor analysis is desired, cells may be stained with PI along with the FAM-FLICA reagent. PI stains necrotic, dead, and membrane-compromised cells. Using PI, a flow cytometer can distinguish among live cells, dead cells, caspase-negative, and caspase-positive cells. Positive control samples can be prepared by inducing cells in suspension culture to undergo apoptosis (see Section 8 for examples of apoptosis induction protocols). After labeling with FAM-FLICA and PI, cells can be analyzed directly by flow cytometry. For a thorough bicolor analysis, 4 types of samples are recommended to set up electronic compensation and quadrant statistics (both types should have induced and non-induced populations):

- a. unstained cells (induced and non-induced).
- b. cells stained with FAM-FLICA only (induced and non-induced).
- c. cells stained with PI only (induced and non-induced).
- d. cells stained with FAM-FLICA and PI (induced and non-induced).

1. As discussed in Section 7, culture cells to a density optimal for apoptosis induction according to your specific induction protocol.
2. Induce apoptosis following your protocol (as mentioned in Section 8).
3. Culture an equal volume of non-induced cells for negative control cell populations. Make sure that all tubes of cells contain similar quantities of cells.

Ideally, cells should be at $\sim 1 \times 10^6$ cells/mL for FAM-FLICA labeling. If necessary, cells may be induced to undergo apoptosis at lower concentrations, but then concentrated just prior to labeling to $0.5 - 2 \times 10^6$ cells/mL by centrifugation for 5 minutes at $<400 \times g$ at RT.

4. Once induction is completed, transfer 300 μ L of each cell suspension to sterile tubes. (Larger cell volumes can also be used as determined by each investigator, however more of the FAM-FLICA reagent may be needed per sample. Larger volume cell suspensions label nicely using 25 cm^2 tissue culture flasks (laid flat) as the incubation vessel.)
5. Add 10 μ L 30X FAM-FLICA solution directly to the 300 μ L cell suspension.
6. Or, if a larger cell volume was used, add the 30X FAM-FLICA solution at a 1:30 ratio. For example, if 2.9 mL of cell suspension was used, add 100 μ L of the 30X FAM-FLICA solution (forming a final volume of 3 mL).

Each investigator should adjust the amount of FAM-FLICA reagent used to accommodate their particular cell line and research conditions.

7. Mix the cells by slightly flicking the tubes.
8. Incubate cells for 1 hour at 37°C under 5% CO₂, protecting the tubes from light. As cells may settle on the bottom of the tubes, gently resuspend them by swirling cells once or twice during this incubation time. This will ensure an even distribution of the FAM-FLICA reagent among all cells.
9. Add 2 mL of 1X wash buffer to each tube.
10. Mix the cells.
11. Centrifuge cells at $<400 \times g$ for 5 minutes at room temperature (RT).
12. Carefully remove and discard supernatant.
13. Gently vortex the cell pellet to disrupt any cell-to-cell clumping.
14. Resuspend the cell pellet in 1 mL 1X wash buffer.
15. Centrifuge cells at $<400 \times g$ for 5 minutes at RT.
16. Carefully remove and discard supernatant.
17. Gently vortex the cell pellet to disrupt any cell-to-cell clumping.
18. Resuspend the cell pellet in 400 μ L 1X wash buffer.
19. Stain one 400 μ L aliquot of FAM-FLICA-treated induced cells with 2 μ L PI.

Cells that are to be analyzed by the bicolor PI screening protocol cannot be fixed.

20. Set aside a second aliquot of FAM-FLICA -treated induced cells that does not contain PI.
21. Mix cells.
22. Put samples on ice. Keep bicolor cells at 2°C - 8°C protected from light for up to 24 hours.
23. For bicolor analysis, measure fluorescein on the FL1 channel and red fluorescence (PI) on the FL2 channel. Generate a log FL1 (X-axis) versus log FL2 (Y-axis) dot plot. Put in quadrant cursors. The 4 quadrant areas contain the following cell populations (Figure 5): (i) quadrant 1, PI positive fluorescein negative cells; (ii) quadrant 2, fluorescein positive PI positive cells; (iii) quadrant 3, fluorescein negative PI negative cells; (iv) quadrant 4, fluorescein positive PI negative cells. The cell population in quadrant 4 consists of living caspase positive (+) cells.

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