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Sulforhodamine FLICA
Apoptosis Detection Kit
Caspase Assay

Poly-Caspases FLICA (SR-VAD-FMK)
Caspase 3 FLICA (SR-DEVD-FMK)

For Research Use Only.

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1. Introduction

Apoptosis, or programmed cell death, is a highly conserved biochemical mechanism that allows cells to die in a controlled and organized manner. This death process is essential for normal cellular differentiation and tissue homeostasis within multicellular organisms[1].

Programmed cell death proceeds in a multi-step process[2]. These stages consist of: an initiation phase, during which time the cell obtains the initial cell death activation signal; a commitment phase, when the cell becomes committed to apoptosis; an amplification phase, involving multiple caspase activation; and a demolition phase, consisting of caspase-mediated destruction of the cellular structure.

Caspase enzymes play a central role as executioners in the apoptotic cell death process[3]. The term caspase was derived from **C**ysteinyl-directed **asp**artate-specific prote**ase** and was created to simplify the naming system as more of these enzymes were discovered[4]. Caspases have an absolute requirement for aspartic acid in the P1 amino acid position of the target substrate sequence[5]. The catalytic domain of the caspase heterodimer, which consists of 2 large (~20KD) and 2 small (~10KD) subunits, targets sequences of 4 amino acids on the substrate molecule[6]. Cleavage occurs at the carbonyl end of the aspartic acid residue[7].

FLICA Apoptosis Detection Kits use a novel approach to detect active caspases. The methodology is based on a Fluorochrome Inhibitor of Caspases (FLICA). Once inside the cell, the FLICA inhibitor binds covalently to the active caspase[8]. These inhibitors are cell permeable and non-cytotoxic. For kits using red fluorescence, a sulforhodamine-labeled fluoromethyl ketone peptide inhibitor of caspases is used. (ICT also offers a line of green FLICA Apoptosis Detection Kits that use carboxyfluorescein-labeled inhibitors; please contact ICT for more details.)

The red FLICA kits contain a fluorescent-labeled inhibitor from ICT, either SR-VAD-FMK (sulforhodaminyl-L-valylalanylaspartyl fluoromethyl ketone), or SR-DEVD-FMK (sulforhodaminyl-L-aspartylglutamylvalylaspartylfluoro-methyl ketone). VAD is an amino acid sequence targeted by all caspases, while DEVD is targeted by caspase-3. When added to a population of cells, the FLICA probe enters each cell and covalently binds to a reactive cysteine residue that resides on the large subunit of the caspase heterodimer, thereby inhibiting further enzymatic activity. Because the FLICA reagent is covalently coupled to the enzyme, it is retained in the cell, while any unbound FLICA reagent will diffuse out of the cell and is washed away. The remaining red fluorescent signal is a direct measure of the number of active caspase enzymes that were present in the cell when the reagent was added. Cells that contain the bound FLICA can be analyzed by 96-well-plate based fluorometry, and fluorescence microscopy.

Because the FLICA reagent SR-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 comparison, when the FLICA reagent SR-DEVD-FMK enters the cell, it primarily binds to caspase-3, therefore it can be used to measure the amount of active caspase-3 that was present in the cell at the time when the SR-DEVD-FMK FLICA reagent was added.

Following the suggested protocols listed here, each sample requires 10 µL of 30X FLICA solution (equal to 2 µL of 150X FLICA stock). The FLICA-25 Kit will test 25 samples; the FLICA-100 Kit will test 100 samples.

The FLICA kit was designed to evaluate apoptotic events using 2 different fluorescence detection methods: fluorescence microscopy for qualitative analysis; and 96-well microtiter plate fluorometry for quantitation. The FLICA reagent excites at 550 nm and has a maximum emission range of 590 – 600 nm (the excitation / emission pairs which best approximate this optimal range should be used). Microscopy samples may also be labeled with Hoechst stain and read using a UV-filter with excitation at 365 nm and emission at 480 nm. Cells labeled with FLICA may be read immediately or preserved for 24 hours using the fixative.

Viewing cells through a fluorescence microscope, apoptotic cells will fluoresce red, while non-apoptotic cells will appear mostly unstained. As apoptosis progresses, the amount of active caspase enzymes capable of binding the FLICA fluorescent inhibitor probe increases and eventually reaches a maximum level. Therefore, cells in more advanced stages of apoptosis will appear brighter red than cells in earlier stages (see Section 22 for sample data).

Using a fluorescence plate reader (with a **black** microtiter plate), apoptosis can be quantitated as the amount of red fluorescence emitted from FLICA probes bound to caspases. Cell populations in more advanced stages of apoptosis will have a higher RFU intensity than cell populations in earlier stages (see Section 19 for sample data).

With the SR-VAD-FMK poly caspase kit, cells with active caspases (those undergoing apoptosis) fluoresce red. Using the SR-DEVD-FMK kit, only cells with active caspase-3 fluoresce red.

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2. Contents of the SR Caspase Detection Kit:

- SR-XXX-FMK FLICA Reagent, lyophilized - the 25 kits contain 1 vial of the reagent; the 100 kits contain 4 vials.
- 10X Wash Buffer, Part# 634 (60 mL) or 635 (15 mL)
- Fixative, Part# 636 (6 mL)
- Hoechst Stain, Part# 639 (1 mL)
- Assay Manual, Part# 800013
- MSDS sheets

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

- Cultured cells with media
- Reagents to induce apoptosis
- 15 mL polypropylene 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
- Hemocytometer
- Clinical centrifuge at <400 X g
- 37°C CO_2 incubator
- Vortexer
- Pipette(s) capable of dispensing at 10µL, 50µL, 200µL, 300µL, 1mL
- dI H_2O, 135 mL or 540 mL needed
- Phosphate Buffered Saline (PBS) pH 7.4, up to 100 mL needed
- Dimethyl Sulfoxide (DMSO), 50µL or 200µL needed
- Ice or 4°C refrigerator to store cells

4. Instrumentation (not all are required):

- 96-well fluorescence plate reader with excitation at 550 nm, emission 595 nm filter pairings. Fluorometer should be capable of reading **black** round or flat bottom 96-well microtiter plates.
- Fluorescence microscope with appropriate filters (excitation 550 nm, emission >580 nm for FLICA; and if Hoechst is used, a UV-filter with excitation at 365 nm, emission at 480 nm) and slides.

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 FLICA reagent from light at all times.
- Once reconstituted, the 150X 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°C up to 14 days.
- Replacement components can be ordered by calling ICT at 1-800-829-3194 or 952-888-8788.

6. Safety Information

- Use gloves while handling the FLICA reagent, 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.
- MSDS sheets are available at www.immunochemistry.com or by calling 1-800-829-3194 or 952-888-8788.

7. Overview of the FLICA Protocol

Staining apoptotic cells with the FLICA kit can usually be completed within a few hours. However, the FLICA kit is used with living cells, which require periodic maintenance and cultivation several days in advance. In addition, once the proper number of cells has been cultivated, time must still be allocated for the induction process (this typically requires a 2-4 hour incubation at 37°C). Therefore, as the 30X FLICA solution must be used immediately, the FLICA reagents should be prepared at the end of your apoptosis induction process. The following is a quick overview of the 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. For example, if labeling with FLICA and Hoechst stain, make 8 populations:
   a. Unlabeled, induced and non-induced populations.
   b. FLICA labeled, induced and non-induced populations.
   c. FLICA and Hoechst labeled, induced and non-induced populations.
   d. Hoechst labeled, induced and non-induced populations.
3. Induce apoptosis following your protocol (4 sample protocols are mentioned in Section 8).
4. Prepare 1X wash buffer (Section 9).
5. Prepare 150X FLICA stock (Section 12).
6. Prepare 30X FLICA solution (Section 13 or 15).
7. Stain cells with 30X FLICA solution, incubate for 1 hour, and wash cells (Section 16, 19, or 20).
8. If desired, label cells with Hoechst stain (Section 10)
9. Analyze data.
10. Fix cells, if desired (Section 11).

8. Induction of Apoptosis

The FLICA kit works with your current apoptosis protocols - induce apoptosis as you normally would, then label the cells with FLICA. Four quick examples of protocols to induce apoptosis in suspension culture are:

1) treating Jurkat cells with 2 μg/ml camptothecin for 3 hours.
2) treating Jurkat cells with 1 μM staurosporine for 3 hours.
3) treating HL-60 cells with 4 μg/ml camptothecin for 4 hours.
4) treating HL-60 cells with 1 μM staurosporine for 4 hours.

9. Preparation of 1X Wash Buffer

The wash buffer is supplied as a 10X concentrate which must be diluted to 1X with DI H_20 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 FLICA-25 Kit, add the entire bottle (15 mL, part# 635) of 10X wash buffer to 135 mL of DI H_2O (to make 150 mL).
3. Or, for the FLICA-100 Kit, add the entire bottle (60 mL, part# 634) of 10X wash buffer to 540 mL of DI H_2O (to make 600 mL).
4. Or, if not using the entire bottle, dilute the 10X wash buffer 1:10 in DI H_2O. For example, add 10 mL 10X wash buffer to 90 mL DI H_2O (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 at 2° - 8°C for up to 14 days. If more buffer is needed, please contact ICT at 1-800-829-3194 or 952-888-8788 for technical assistance or to order a replacement component.

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; see MSDS for further information.

10. Hoechst Stain

Hoechst stain can be used to label the nuclei of dying cells after labeling with the FLICA reagent. It is revealed under a microscope using a UV-filter with excitation at 365 nm and emission at 480 nm (Section 19 or 20). 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.

11. Fixative
If the stained cell populations cannot be evaluated immediately upon completion of the FLICA staining protocol, cells may be fixed and analyzed up to 24 hours later through a microscope. The fixative is a formaldehyde solution designed to cross-link cell components and will not interfere with the SR labeling once the FLICA reaction has taken place.
After labeling with FLICA (and Hoechst if desired), add the fixative into the cell solution at a 1:10 ratio. For example, add 100 µL fixative to 900 µL cells (Section 19 or 20). Fixed cells should 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 SR label. Never add the fixative until the staining and final wash steps have been completed.**

12. Reconstitution of the 150X FLICA Stock
The FLICA reagent is supplied as a highly concentrated lyophilized powder. It must first be reconstituted, forming a 150X stock concentrate, and then diluted 1:5 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 later use.

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

1. Reconstitute each vial of lyophilized FLICA with 50 µL DMSO. This yields a 150X concentrate. (The FLICA-25 kit contains 1 vial; the 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 13).
4. Or, if using later, aliquot and store it at –20°C (Section 14).

13. Preparation of 30X FLICA Solution for Immediate Use
Using the freshly reconstituted 150X FLICA stock, prepare the 30X working-strength FLICA solution by diluting the stock 1:5 in PBS pH 7.4. Using the suggested protocols here, each sample to be tested requires only 10 µL of 30X FLICA solution (or 2 µL of the 150X 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). The FLICA-25 Kit contains 1 vial; the FLICA-100 Kit contains 4 vials.

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 until ready to use (Section 14).
3. Mix by inverting or vortexing the vial at RT.

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

14. Storage of 150X FLICA Stock for Future Use
If not all of the 150X 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 FLICA stock may be thawed and used twice. After the second thaw, discard any remaining 150X 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 15 below.

15. Preparation of 30X FLICA Solution from a Frozen Aliquot
If some of the 150X 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 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 FLICA reagent with 40 µL of PBS.
3. Mix by inverting or vortexing the vial at RT.
4. If the 150X 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. Go on to the labeling protocol (Section 16, 19, or 20).

16. 96-Well Fluorescence Plate Reader Staining Protocol
Following the fluorescence plate reader protocol, each sample requires 10 µL of 30X FLICA solution (equal to 2 µL of 150X 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 X 10^6 cells/mL. (Cells may be induced at even lower concentrations, but must be concentrated to ~ 1 X 10^7 cells/mL for FLICA labeling. If necessary, cells can be concentrated by centrifugation for 5 minutes at <400 X 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 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 FLICA solution, cells should be at least 5 X 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 FLICA solution directly to the 290 – 300 µL cell suspension.
6. Or, if a larger cell volume was used, add the 30X FLICA solution at a 1:30 ratio. For example, if 2.9 mL of cell suspension were used, add 100 µL of the 30X FLICA solution (forming a final volume of 3 mL).

- **Each investigator should adjust the amount of 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 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 X 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 X 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 µL from each tube.
    b. Add to 450 µL 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 X g for 5 minutes at RT.
21. Carefully remove and discard supernatant.
22. Resuspend non-stimulated cells in 400 µL PBS
23. If it is not necessary to equilibrate the cell concentrations (as discussed in Step 19d), resuspend the stimulated cells in 400 µL 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 µL 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 sulforhodamine (excitation 550 nm, emission 595 nm) using a fluorescence plate reader. Your filter pairing may differ slightly from these optimal settings. Select the filter pairing which most closely approximates this range (Section 17).

17. 96-Well Fluorescence Plate Reader Set Up

1. Set the plate reader to perform an endpoint read.
2. Set the excitation wavelength at 550 nm and the emission wavelength to 595 nm. Your filter pairing may differ slightly from these optimal settings. Select the filter pairings that most closely approximate this range.
3. Read the sample.

18. 96-Well Fluorescence Plate Reader Sample Data

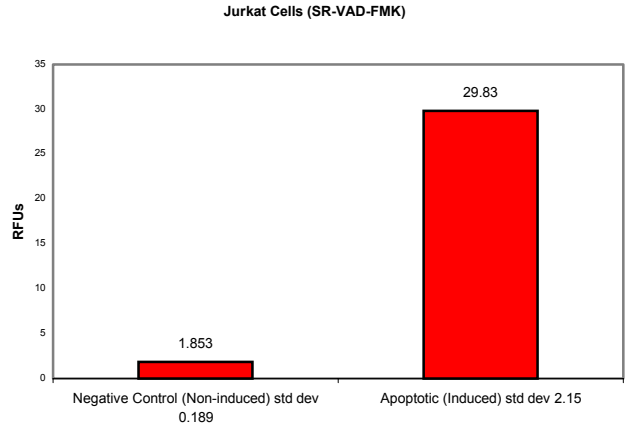

Figure 1. SR-VAD-FMK fluorometric detection of active caspases in Jurkat cells (SD of 4 wells).

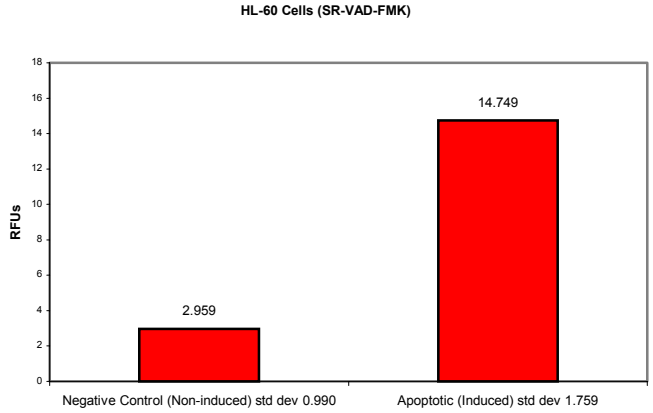

Figure 2: SR-VAD-FMK fluorometric detection of active caspases in HL-60 cells (SD of 18 wells).

In Figures 1 and 2, cells were either treated with DMSO (negative, non-induced cells – bars on the left side of each graph) or with staurosporine (apoptotic, induced cells - bars on the right side of each graph) for 3 hours at 37°C. Cells were labeled with SR-VAD-FMK solution for 60 minutes at 37°C. Samples were read on a 96-well fluorescence plate reader (Molecular Devices, Gemini XS) set at 550 nm excitation and 595 nm emission using a 570 nm cut-off filter. As the caspases became more active, indicating apoptosis, the amount of red fluorescence increased by 1500% in the Jurkat cells and 500% in HL-60 cells.

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 cover slip 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 FLICA solution to the medium at a 1:30 ratio. For example, add 10 µl 30X FLICA to 290 – 300 µl medium.

Each investigator should adjust the amount of 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% CO_2.
9. Remove the medium.
10. If cells are to be monitored using Hoechst stain, add 1.5 µL Hoechst stain to 300 µL media (0.5% v/v). Add this media to the cells.
    a. Incubate for 5 minutes at 37°C under 5% CO_2.
    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 cover slip 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 cover slip. 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 µL fixative to 360 µL 1X wash buffer.
    a. Mount a cover slip 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 cover slip.
    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 550 nm, emission >580 nm) to view the red 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 X 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 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 FLICA solution, cells should be at least 5 X 10^5 cells/mL. Density can be determined by counting cell populations on a hemocytometer.**

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

- **Each investigator should adjust the amount of 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 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 observed immediately, or fixed for future viewing. To view cells immediately, go to Step 24. If cells are to be fixed for later viewing, go to Step 25.
24. To view cells immediately, place 1 drop of the cell suspension onto a microscope slide and cover with a cover slip; go to Step 26.
25. 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 cover slip.
    e. Store slides at 2° – 8°C up to 24 hours.
    f. When ready to read, go on to Step 26.
26. Observe cells under a fluorescence microscope using a bandpass filter (excitation 550 nm, emission >580 nm) to view red fluorescence. 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.) Cells bearing caspase enzymes covalently coupled to the FLICA reagent appear red. SR-VAD-FMK binds to all caspases, while SR-DEVD-FMK binds to caspase-3.

21. Fluorescence Microscopy Sample Data

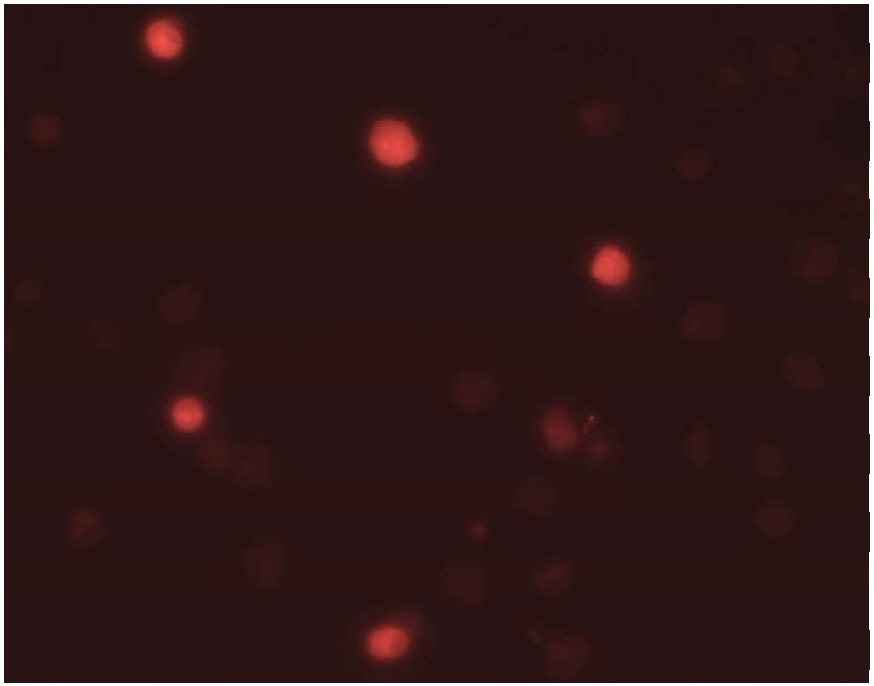

Figure 3: Jurkat cells, grown in suspension, were incubated with 1 μM staurosporine for 3 hours at 37°C to induce apoptosis. Cells were then labeled with SR-VAD-FMK for 60 minutes at 37°C. Slides were prepared and samples were viewed through a fluorescence microscope using a broad band path filter.

In Figure 3, 6 cells were labeled with the SR-VAD-FMK reagent poly caspase kit. 5 cells appear very bright red, indicating a high amount of active caspases. Therefore, these cells were undergoing apoptosis at the time the reagent was added. 1 cell appears faint red in the lower right hand quadrant of the picture. As this cell was labeled with SR-VAD-FMK, but not as brightly as the other cells, it had a lower concentration of active caspases, and was probably just beginning to enter apoptosis at the time the reagent was added. Non-apoptotic cells did not fluoresce.

22. References

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