Necrosis vs Apoptosis Assay Kit

Trial Size (50-100 tests) catalog#KF17371
Standard Size (100-200 tests) catalog#KF17372
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1. INTRODUCTION

Assessment of cellular cytotoxicity levels associated with cytolytic activity of T lymphocytes or natural killer cells is an important aspect of immunobiology related research[1]. Early assays developed to assess cytolytic activity utilized the release of ^{51}Cr from membrane compromised target cells, which were passively loaded with this radioactive indicator prior to exposure to the cytotoxic agent or cells[2]. A major draw-back to these ^{51}Cr release assays is their reliance on the use of a radioactive isotope with its associated disposal and safe handling issues. Additionally, chromium uptake methods suff er from variabilities in target cell preloading inconsistencies as well the tendency to spontaneously release the chromium isotope into the media in the absence of any cytotoxicity stimulus[3].

Assessing potential cytotoxicity properties of chemical and biological agents is a mandatory requirement for the safe distribution of pharmaceuticals, vaccines, or additives associated with food product formulations. Early identifi cation of unintended drug, vaccine, or chemical associated cytotoxicity properties is always an early priority of initial FDA approval testing protocols. With cellular cytotoxicity assessment playing a central role in countless research and environmental safety studies, there is an ever present need for simple, straightforward analysis methods like the Necrosis vs Apoptosis Assay kits developed by Immunochemistry Technologies, LLC (ICT).

Neuromics' Necrosis vs Apoptosis Assay kit simultaneously detects both apoptosis associated cytotoxicity events as well as cell death due to necrosis. Apoptotic cells are identifi ed using ICT's Fluorescent Labeled Inhibitor of CAspases (FLICA) reagent probe4-7. Th e FAM-FLICA probe covalently binds to active caspase enzymes, which are up-regulated during apoptosis, thus clearly labeling apoptotic cells for subsequent analysis8,9. Non-apoptotic cells will not contain the active caspase enzymes required for FAM-FLICA to remain covalently bound within the cell structure

Loss of the integrity of the cell membrane, indicative of necrosis or late stage apoptosis, is detected using the vital staining dye, 7-aminoactinomycin D (7-AAD), a red fluorescing live/dead stain. This dye easily penetrates cell membrane-compromised cells, binding tightly to GC rich regions of the DNA10-14. Because 7-AAD alone may not detect cells in the early stages of apoptosis, it is essential to use it in combination with the green-fluorescent FAM-FLICA apoptosis detection reagent15. Combining these two different types of fluorescent cell-status-indicator reagents within a single test can reveal a significant percentage of cells that are 7-AAD-negative (membrane intact live cells) and yet FAMFLICA positive (apoptotic) (Figures 3-6). FAM-FLICA probes optimally excite between 485-495 nm with maximal emission between 515-525 nm. Th e vital staining dye 7-AAD can also be efficiently excited at 485-495 nm, but exhibits an optimal emission well into the red fluorescence range (647 nm). This significant difference in fluorescence emission wavelength between the green FAM (carboxyfluorescein) label on the FAM-FLICA probe and the red 7-AAD vital dye simplifies flow cytometer gating and compensation. The FAM-FLICA probe (apoptosis) is monitored on the FL-1 channel, while 7-AAD (necrosis) is monitored on FL-3. Combining the use of ICT's FAMFLICA apoptosis detection probe with a membrane integrity dye like 7-AAD makes it easy to distinguish between necrosis and apoptosis within a single sample

2. KIT CONTENTS

Trial size kits contain:

- 2 vials of FAM-FLICA poly caspase inhibitor reagent #637
- 1 vial of 7-Aminoactinomycin D (7-AAD) vital dye, 0.26 mg vial #6163
- 1 bottle of 10X Apoptosis Wash Buffer (60 mL) #634
- 1 bottle of Fixative (6 mL) #636

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Standard size kits contain:

• 4 vials of FAM-FLICA poly caspase inhibitor reagent #637
• 2 vials of 7-Aminoactinomycin D (vital dye), 0.26 mg vial #6163
• 2 bottles of 10X Apoptosis Wash Buffer (60 mL) #634
• 1 bottle of Fixative (6 mL) #636

3. STORAGE

Store the unopened kit and each unopened component at 2-8°C until the expiration date. Once reconstituted with DMSO, use FAM-FLICA and 7-AAD immediately, or store at ≤-20°C for 6 months protected from light and thawed no more than twice during that time.

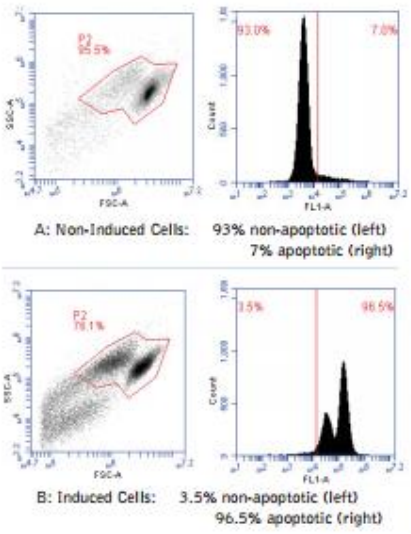
4. SAFETY DATA SHEETS (SDS)
Safety data sheets are available upon request

5. RECOMMENDED MATERIALS
• DMSO, up to 1,000 µL (50 µL per vial to reconstitute FAMFLICA, 260 µL per vial to reconstitute 7-AAD, and more to create controls)
• DiH20, up to 1,080 mL (540 mL per bottle to dilute 10X Apoptosis Wash Buffer)
• Phosphate buffered saline (PBS) pH 7.4, up to 100 mL, to dilute FAM-FLICA and handle cells
• FBS and/or BSA to add to the buffer when handling cells
• Cultured cells treated with the experimental conditions ready to be labeled
• Reagents to induce caspase activity and create controls for FAMFLICA staining (See Figure 1), such as staurosporine (catalog #6212) or camptothecin (catalog #6210)
• 90% ETOH or 3% formaldehyde to create live/dead controls for 7-AAD staining (See Figure 2)
• Hemocytometer
• Centrifuge at < 200 g
• 15 mL polypropylene centrifuge tubes (1 per sample)
• Ice bath

6. DETECTION EQUIPMENT
The assay can be analyzed with a:
• Fluorescence microscope
• Flow cytometer Use fi lter pairings that best approximate these settings:
• FAM-FLICA optimally excites at 488-492 nm and has a peak emission at 515-535 nm (use FL-1 channel).
• 7-AAD optimally excites at 546 nm. It has a peak emission at 647 nm (use FL-3 channel), but displays adequate emission properties when excited within a blue (488-492 nm) light source.

7. EXPERIMENTAL PREPARATION
Staining apoptotic cells with FAM-FLICA and 7-AAD can be completed within a few hours. However, FAMFLICA and 7-AAD are 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 be allotted for the experimental treatment or apoptosis induction, which typically requires a 2-6 hour incubation at 37°C based on the cell line and concentration.

Figure 1: Apoptosis Controls Stained with FAM-FLICA



ICT's green poly caspase inhibitor reagent, FAM-FLICA, was used to assess apoptosis in Jurkat cells. Using FAMFLICA, non-apoptotic cells (unstained, left side of each histogram) can easily be distinguished from apoptotic cells exhibiting green fluorescence (right side of each histogram). The forward and side scatter graphs are also shown. To create a positive control for FAM-FLICA, a population of apoptotic cells is needed (Section 9). If analyzing with a flow cytometer, it will be used to compensate the instrument. In this example, Jurkat cells were grown to 5 x 105 cells/ mL and split into two populations. One population (A, top) was treated with a placebo (DMSO, non-induced) while the other population (B, bottom) was treated with 1 µM staurosporine for 4 hours to induce apoptosis. Cells were stained with FAM-FLICA for 1 hour at 37°C and analyzed using an Accuri C6 fl ow cytometer in FL-1. Only 7% of non-induced cells (A, right) are apoptotic compared with 96.5% of the induced cells (B, right). (ICT 226:17-19.)

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Create cell populations, such as:

a. Cells that were exposed to the experimental treatment.
b. b. A negative control population of cells that received a placebo treatment.

As FAM-FLICA detects the presence of catalytically active forms of caspase enzymes, plan the experiment so that FAMFLICA will be diluted and administered at the time when caspases are expected to be activated in the cells. Culture cells to a density optimal for the specific experimental conditions or apoptosis induction protocol. Cell density should not exceed 106 cells/mL. Cells cultivated in excess of this concentration may begin to naturally enter apoptosis. An initial experiment may be necessary to determine when and how much FAM-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:

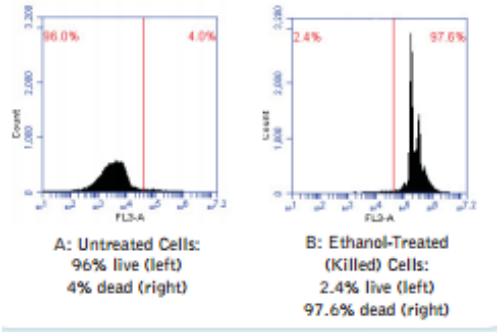
a. Treated experimental population(s): cells exposed to the experimental condition(s).
b. Negative control: non-treated cells grown in a normal cell culture environment.
c. Positive control for FAM-FLICA: cells induced to undergo apoptosis using a known caspase activation protocol (Section 9; Figure 1).
d. Positive control for 7-AAD: cells treated with ethanol or formaldehyde to create a dead cell population (Section 10 and Figure 2).

The induced positive cell population and negative control cell population tubes should come from a common pool of cells and contain similar quantities of cells. Create negative controls by culturing an equal volume of non-induced cells for every labeling condition. For example, if labeling with FAM-FLICA and 7-AAD stain, make 8 populations:

1&2. Unlabeled: induced and non-induced
3&4. FAM-FLICA-labeled: induced and non-induced
5&6. FAM-FLICA-labeled and 7-AAD-labeled: induced and non-induced
7&8. 7-AAD-labeled: induced and non-induced

Figure 2: Live and Dead Controls Stained with 7-AAD

Detection of cell membrane integrity loss, indicative of necrosis or late stage apoptosis, can be done using the red fluorescent live/dead stain, 7-AAD. This vital dye works by penetrating cell membrane-compromised cells and tightly binding to GC rich regions of the DNA. Using 7-AAD, live cells (unstained, left side of each histogram) can easily be distinguished from dead/membrane compromised cells exhibiting red fluorescence (right side of each histogram). To create a positive control for 7-AAD, a population of dead or killed cell is needed (Section 10). If analyzing with a fl ow cytometer, it will be used to compensate the instrument. In this example, Jurkat cells were grown to 5 x 105 cells/mL and split into two populations. One population (A) was left untreated while the other population (B) was treated with 90% ethanol for 60 seconds. Cells that were exposed to ethanol (B) were treated with a 5-fold larger volume of PBS to stop the ethanol surface denaturation process. Cells were pelleted by centrifugation (200 x g for 5 minutes) and resuspended in PBS. Cells were then stained with 7-AAD for 10 minutes on ice, and analyzed using an Accuri C6 fl ow cytometer in FL-3. Only 4% of untreated cells (A) are dead compared with 97.6% of the treated cells (B). Data courtesy of Dr. Kristi Strandberg, ICT 226:30-31.



9. APOPTOSIS INDUCTION (FAM-FLICA CONTROL) Prior to commencing the experiment, determine a reproducible method for obtaining a positive control by triggering caspase activity. This process varies signifi cantly with each cell line. For example, apoptosis may be induced with 2-4 µg/mL camptothecin, or 1-2 µM staurosporine for >4 hours (Figure 1).

10. PREPARATION OF DEAD CELLS (7-AAD CONTROL)

Prior to commencing the experiment, determine a reproducible method for obtaining a population of dead/killed cells to use as a positive control for 7-AAD staining. This can easily be achieved using many different techniques. For example, briefl y expose cells to 90% ethanol for 30-60 seconds at 37°C, or expose cells to 3% formaldehyde for 30 minutes on ice (Figure 2).

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11. PREPARATION OF FAM-FLICA

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

1. Reconstitute each vial of FAM-FLICA with 50 µL DMSO to form the 150X stock. The stock solution should be colorless to light yellow. 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 FAM-FLICA 1:5 by adding 200 µL PBS to each vial to form the 30X FAM-FLICA solution. Use 30X FAM-FLICA within 30 minutes of dilution into aqueous buffers.

• The recommended volume of 30X FAM-FLICA for fl ow cytometry is 5 to 10 µL per 300 µL of cells at 5 x 105 cells/mL. The recommended volume of 30 X FAM-FLICA for microscopy is 10 µL per 300 µL of cells at 5 x 105 cells/mL. These amounts are recommendations, however, the amount needed may vary based on the experimental conditions and the instrument used for analysis. Each investigator should adjust the amount of FAM-FLICA to accommodate the particular cell line and research conditions.

12. PREPARATION OF 1X APOPTOSIS WASH BUFFER

ICT's Apoptosis Wash Buffer (catalog #634, AWB) is an isotonic solution used to wash cells following exposure to FAM-FLICA. It contains mammalian proteins to stabilize cells stained with FAMFLICA, and sodium azide to retard bacterial growth (1X Apoptosis Wash Buffer contains 0.01% w/v sodium azide). Cell culture media containing FBS and other additives may be used to wash cells instead of Apoptosis Wash Buffer.

1. 10X Apoptosis Wash Buffer may form precipitates during cold storage. If this happens, gently warm it until all crystals have dissolved. Do not boil.
2. Dilute 10X Apoptosis Wash Buffer 1:10 in diH2O. For example, add 60 mL 10X Apoptosis Wash Buffer to 540 mL diH2O for a total of 600 mL.

• 1X Apoptosis Wash Buffer may be stored at 2-8°C and used within 1 week or frozen and used within 6 months.

13. PREPARATION OF 7-AAD

Detection of cell membrane integrity loss, indicative of necrosis or late stage apoptosis, is detected using the red fl uorescent live/ dead stain, 7-aminoactinomycin D (7-AAD). This vital dye works by penetrating cell membrane-compromised cells and tightly binding to GC rich regions of the DNA. Because 7-AAD alone may not detect cells in the early stages of apoptosis, it should be combined with the green fl uorescent FAM-FLICA reagent to concurrently label apoptotic cells. 7-AAD is supplied as a lyophilized powder that may be slightly visible as a red sheen inside the vial. Protect from light and use gloves when handling.

1. Reconstitute each vial of 7-AAD with 260 µL DMSO to create a stock concentrate at 1 mg/ mL. Mix by swirling or tilting the vial, allowing the DMSO to travel around the base of the amber vial until the reagent is completely dissolved. At room temperature, the reagent should be dissolved within a few minutes forming a red solution.
2. If storing the stock concentrate for future use, prepare small aliquots (50 µL) to avoid freeze-thaw cycles. The stock concentrate will be stable for 6 months when protected from light and stored at or below -20°C.
3. When ready to stain cells, use 7-AAD at 1:200 dilution. For example, add 2 µL per 400 µL cells.

14. FIXATIVE

ICT's Fixative (catalog #636) is a formaldehyde solution designed to cross-link and aggregate intracellular components. If the stained cell populations cannot be evaluated immediately after labeling with FAM-FLICA, add Fixative at a ratio of 1:10. For example, 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 24 hours, protected from light. ICT's Fixative will not interfere with the carboxyfluorescein (FAM) or 7-AAD labels. Do not use absolute ethanol- or methanol-based fixatives as they will inactivate the FAM-FLICA signal. Do not fi x cells that will be stained later with 7-AAD (stain the cells before using fixative).

• Danger: Fixative is toxic: danger exists of very serious irreversible effects through inhalation, by contact with skin, or if swallowed. Gloves, protective clothing, and eye wear are strongly recommended. When disposing, flush sink with copious amounts of water; see SDS for further information.

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15. STAINING PROTOCOL FOR SUSPENSION CELLS

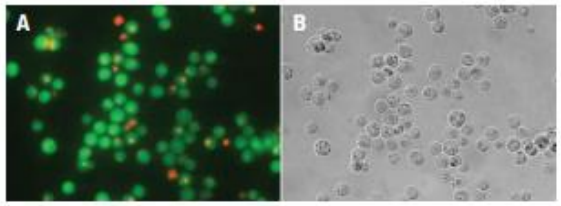
Prepare experimental and control cell populations. Ideally, the cell concentration should be 3-5 x 105 cells/mL. The concentration should not exceed 106 cells/mL, as cells cultivated in excess of this concentration may begin to naturally enter apoptosis. Just prior to staining, cells may need to be concentrated to 2-5 x 106 cells/mL as microscopy analysis methods (Section 17) require high cell concentrations. Start with a larger volume of cells at 3-5 x 105 cells/mL (which is a typical density for cell culture) and then concentrate cells and resuspend to 300 µL per sample when ready for FAM-FLICA staining.

1. Expose cells to the experimental or control condition. If analyzing with a fl ow cytometer, set aside four populations to create instrument control with 7-AAD-positive and 7-AAD-negative cells (Section 10), and FAM-FLICAinduced and FAM-FLICA-non-induced cells (See Figure 5).
2. If analyzing with a fluorescence microscope, concentrate cells to 2-5 x 106 cells/mL just prior to FAM-FLICA staining. Fluorescence microscopy requires an excess of 2 x 106 cells/ mL to obtain 5 - 20 cells per image field. Flow cytometry can analyze samples at 3-5 x 105 cells/mL. Sample fluorescent microscopy results are shown in Figure 3.
3. Transfer 290 µL cells into fresh tubes.
4. Add 10 µL 30X FAM-FLICA solution, forming a final volume of 300 µL. If different cell volumes were used, add 30X FAM-FLICA at a ratio of 1:30. Mix by gently flicking the tubes.
   a. The amount of FAM-FLICA used for fl ow cytometry applications can be diluted 2-fold for some cell lines and experimental conditions, allowing the end user to test a greater number of samples per kit. If staining a lower concentration, add 5 µL of FAM-FLICA to 295 µL of cells. The amount of FAM-FLICA should be optimized for each cell line and experimental condition.
5. 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. As cells may settle on the bottom of the tubes, gently resuspend them by swirling cells every 20 minutes to ensure an even distribution of FAM-FLICA throughout the staining process.
6. Centrifuge at 200 x g for 5 minutes, and then discard the supernatant.
7. Add 2 mL 1X Apoptosis Wash Buffer and gently mix. 8. Centrifuge at 200 x g for 5 minutes at RT.
9. Carefully remove and discard supernatants. Gently vortex the pellets to disrupt clumping. Resuspend in 1 mL 1X Apoptosis Wash Buffer and gently mix. Additional wash steps may be required if high FAM-FLICA background signal is observed.

Figure 3: Microscopy Analysis of Jurkat Cells Stained with FAMFLICA and 7-AAD

Jurkat suspension cells were exposed to 1 µM of staurosporine for 4 hours at 37°C to induce apoptosis. Cells were dually stained with the green fluorescent FAM-FLICA poly caspase probe to detect apoptosis via caspase activity, and the red fluorescent vital dye 7-AAD to detect necrosis.

The image shown below in panel A reveals 4 populations of cells: 1) Live, unstained cells, which do not fluoresce. 2) Early stage apoptotic cells fluoresce green with FAM-FLICA. 3) Dually stained green and red fluorescing cells represent the population of Jurkat cells in mid-to-late stage apoptosis; these cells have active caspase enzymes and compromised cell membranes. 4) Necrotic cells fluoresce red. Panel B shows a corresponding differential interference contrast (DIC) image, which reveals cell morphology. In Figure 5, a similar experiment was performed and cells were analyzed via fl ow cytometry to quantitate each population: 97.1% were apoptotic (Figure 5D, cells in the lower right and upper right quadrants fluoresce green; LR: 46.4% + UR: 50.7%). Microscope images were obtained using an Olympus BH-2 photomicroscope equipped with bright field, DIC, and fluorescence optics. FAM-FLICA and 7-AAD were imaged using a 470-490 nm excitation filter plus >520 nm long pass filter tandem. Data courtesy of Dr. Brian W. Lee (ICT 196:70).



10. Centrifuge cells at 200 x g for 5 minutes at RT.
11. Carefully remove and discard supernatants.
12. Resuspend cells in 400 µL 1X Apoptosis Wash Buffer.
13. Stain with 7-AAD at a final concentration of 5 µg/mL. This can be accomplished by:
    a. Adding the stock solution directly to the cell culture at 1:200; e.g. add 2 µL stock to 400 µL cell suspension.

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b. Or by diluting the stock concentrate 1:10 to form the working solution, and then adding the working solution to the cells at 1:20. For example:
   1. Add 50 µL 7-AAD stock concentrate to 450 µL PBS or sterile media to form the working solution.
   2. Mix by inverting or vortexing the vial at RT.
   3. Store on ice up to 2 hours.
   4. Add the working solution to the cell suspension at approximately 1:20; e.g. add 25 µL diluted 7-AAD working solution into 475 µL cell suspension.

14. Incubate for 10-30 minutes on ice while protected from light.
   b. To analyze using a microscope, refer to Section 17.
   a. To analyze by fl ow cytometer, refer to Section 18.

16. STAINING PROTOCOL FOR ADHERENT CELLS

Adherent cells need to be carefully washed to avoid the loss of any cells that round up and come off the plate surface. Loose cells may be harvested from the plate or slide surface and treated as suspension cells, while those remaining adherent to the surface should be washed as adherent cells. If the adherent cells are trypsinized, the loose cells can be recombined with the trypsinized pool; alternatively, the loose cells can be recombined with the adherent portion when the analysis is performed. If growing adherent cells in a plate, the entire plate may be gently spun as part of the wash process to sediment any loose floating cells. If trypsin is necessary, it is preferable to trypsinize prior to FAM-FLICA staining. Avoid trypsinizing cells prior to labeling with a vital dye, like 7-AAD. Cell membranes exposed to trypsin could be transiently permeant to vital dyes for a variable time depending upon the cell line. Cells may be labeled with FAM-FLICA before or after trypsinization (Figure 6).

1. Culture cells in TC-flasks and expose to the experimental conditions.
2. Collect the cells. Note: Apoptotic cells may detach and begin to float into the media. Save and spin to pellet and include these cells in the analysis. If Trypsin is required to lift the adherent cells:
   a. Rinse the adherent cells with 5-10 mL of PBS to remove any residual trypsin inhibitors present in FBS containing medium.
   b. Trypsinize by adding enough Trypsin/EDTA to cover the cell surface area.
   c. Incubate cells with trypsin at 37°C for several minutes. Check frequently to determine when the cells have lifted.
   d. Neutralize with trypsin inhibitor or cell culture media containing 10-20% FBS.
   e. Pool cells with any pellets created in Step 2.
3. Centrifuge the collected cells at 200 x g for 5 minutes.
4. Remove all but ~100 µL supernatant. Resuspend cells in 300-500 µL cell culture media containing 10-20% FBS. If necessary, count cells and adjust the volume of cell suspension to fi t the experiment. Transfer cells into a 15 mL tube.
5. Add 30X FAM-FLICA at 1:30.
   a. The amount of FAM-FLICA used for flow cytometry applications can be diluted 2-fold for some cell lines and experimental conditions, allowing the end user to test a greater number of samples per kit. If staining a lower concentration, use FAM-FLICA at a dilution between 1:30 and 1:60. The amount of FAM-FLICA should be optimized for each cell line and experimental condition.
6. Incubate 30-60 minutes at 37°C, mixing gently every 10 minutes.
7. Centrifuge at 200 x g for 5 minutes, and then discard the supernatant.
8. Add 2 mL 1X Apoptosis Wash Buffer, and then centrifuge at 200 x g for 5 minutes.
9. Aspirate supernatant and resuspend cells in 2 mL 1X Apoptosis Wash Buffer.
10. Incubate 10 minutes at 37°C to allow any unbound FAMFLICA to diffuse out of the cells.
11. Centrifuge at 200 x g for 5 minutes.
12. Aspirate supernatant and resuspend cells in 400 µL 1X Apoptosis Wash Buffer.
13. Stain with 7-AAD at a fi nal concentration of 5 µg/mL. This can be accomplished by:
   a. Pipetting the stock solution directly into the cell culture at 1:200 (e.g. add 2 µL 7-AAD stock solution to 400 µL cell suspension).
   b. Or by diluting the stock concentrate 1:10 to form the working solution, and then pipetting the working solution into the cells at 1:20. For example:
      1. Add 50 µL 7-AAD stock concentrate into 450 µL PBS or sterile media.
      2. Mix by inverting or vortexing the vial at RT.
      3. Store on ice up to 2 hours.

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4. Add the working solution to the cell suspension at approximately 1:20; e.g. add 25 µL diluted 7-AAD working solution to 475 µL cell suspension.

14. Incubate for 10-30 minutes on ice while protected from light. b. To analyze using a microscope, refer to Section 17. a. To analyze by fl ow cytometer, refer to Section 18.

17. MICROSCOPY ANALYSIS

Follow Section 15 (Steps 1-14), or Section 16 (Steps 1-14).

15. If not viewing immediately, cells may be washed and then fi xed for viewing up to 24 hours later.
    a. Wash sample once with PBS prior to fi xing to remove any unbound 7-AAD from the medium.
    a. Add 30 µL Fixative. If cells were resuspended in a different volume, add Fixative at a ratio of 1:10.
    b. Incubate 15 minutes at RT in the dark.
    c. Place cells onto a microscope slide and allow to dry.
    d. Briefl y rinse cells with PBS.
    e. Cover cells with mounting media and coverslip. f. Store slides at 2-8°C for up to 24 hours.
16. To view cells immediately, place 1 drop of cell suspension onto a microscope slide and cover with a coverslip.
17. Observe cells under a fl uorescence microscope using excitation at 470-490 plus a >520 nm long pass fi lter. Cells bearing active caspase enzymes covalently coupled to FAM-FLICA appear green. Necrotic or late-stage apoptotic cells containing nucleic acid-bound 7-AAD appear red (Figure 3).

18. FLOW CYTOMETRY ANALYSIS

Follow Section 15 (Steps 1-14), or Section 16 (Steps 1-14).

To address compensation issues and set up the fl ow cytometer, prepare 4 instrument control populations: Cells stained only with FAM-FLICA (refer to Section 9 and Figure 1):

1. Induced cells.
2. Non-induced cells. Cells stained with only 7-AAD (refer to Section 10 and Figure 2):
3. Live cells.
4. Killed cells (cell membrane compromised).

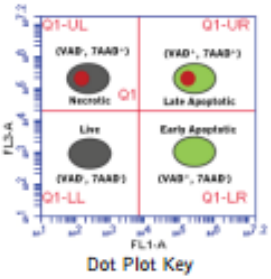
These controls are needed to adjust the instrument PMT's to separate 7-AAD-positive from 7-AAD-negative samples and to compensate for bleed-over of the red 7-AAD signal from FL-3 into FL-1. They will also help to clearly differentiate the FAMFLICA-positive population from the FAM-FLICA-negative population and compensate bleed-over of the green FAM-FLICA signal from FL-1 into FL-3.

While setting up the 7-AAD controls (Section 10), continue working with the test samples and experimental controls as prepared in Section 8.

15. Set up the instrument compensation. a. Read the 7-AAD-positive and 7-AAD-negative controls to compensate bleed-over of the red 7-AAD signal from FL-3 into FL-1. b. Read the FAM-FLICA-positive and FAM-FLICAnegative controls to compensate bleed-over of the green FAM-FLICA signal from FL-1 into FL-3.
16. To read the samples for bicolor analysis:
    a. Measure green FAM-FLICA (FAM = carboxyfl uorescein) on the FL-1 channel.
    b. Measure red fl uorescence (7-AAD) on the FL-3 channel.
    c. Generate a log FL-1 versus log FL-3 dot plot (Figures 5 and 6).
    d. This will reveal 4 populations of cells: (Figure 4)
        1. Live cells (unstained) only emit low level background fl uoresce (lower left).
        2. Cells in early apoptosis fl uoresce green with FAMFLICA (lower right).
        3. Cells in late apoptosis are dually stained with FAMFLICA and 7-AAD; they fl uoresce green (they have active caspases) and red (the cell membrane is compromised; upper right).
        4. Necrotic membrane-compromised cells fluoresce red (upper left)

Figure 4: Quantitate 4 Cell Populations

1. Live, unstained cells do not fluoresce (LL, lower left).
2. Early stage apoptotic cells fluoresce green with FAM-FLICA (LR, lower right).
3. Dually stained green and red fluorescing cells represent cells in mid to late stage apoptosis; these cells have active caspase enzymes and compromised cell membranes (UR, upper right).
4. Necrotic cells fluoresce red (UL, upper left).



Dot Plot Key

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Figure 5: Flow Cytometry Analysis of Jurkat Suspension Cells to Quantify 4 Populations

Jurkat cells were treated with a placebo (non-induced treatment with DMSO; A and B) or treated with 1 µM staurosporine for 4 hours to induce apoptosis via caspase activity (C and D). Cells were then dually stained with ICT's FAMFLICA apoptosis reagent and 7-AAD, a vital dye. Cells were analyzed using an Accuri C6 fl ow cytometer. Compensation was set using cell populations stained individually with either FAM-FLICA or 7-AAD (data not shown). FAM-FLICA was analyzed on FL-1, and 7-AAD was analyzed on FL-3. Forward and side scatter density plots (A, non-induced; C, induced populations) and dual stained density plots (B, noninduced;
D, induced populations) are shown. The density plot key is shown in Figure 4.
Flow cytometry can be used to quantitate 4 populations of cells. Live, unstained cells do not fluoresce (lower left, B and D). Early stage apoptotic cells fluoresce green with FAMFLICA (lower right, B and D). Dually stained green and red fluorescing cells represent the population of Jurkat cells in mid to late stage apoptosis; these cells have active caspase enzymes and compromised cell membranes (upper right, B and D). Necrotic cells fluoresce red (upper left, B and D).
By including FAM-FLICA in the analysis, cells in early apoptosis (lower right, B and D) can be detected, which 7-AAD cannot detect alone. In the non-induced population (B), only 9.8% of cells were apoptotic (LR: 4.5% + UR: 6.3%) compared with 97.1% of the induced population (D; LR: 46.4% + UR: 50.7%). Cells in these quadrants fluoresce green (ICT 226:17-19). In Figure 3, a similar experiment was performed and images were taken using a fluorescence microscope**.**

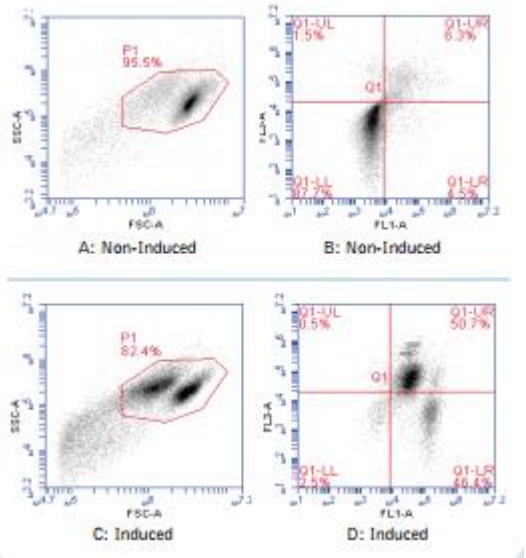
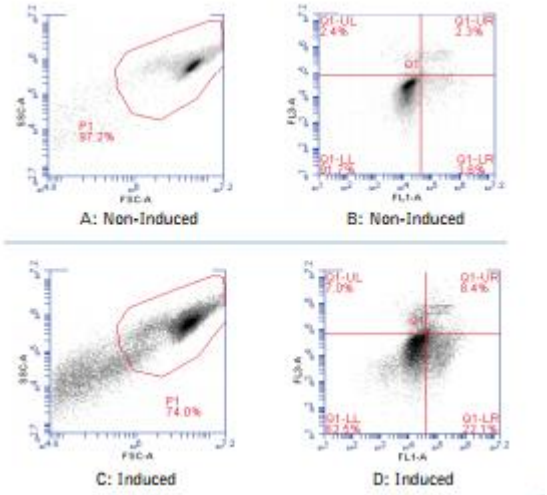


Figure 6: Flow Cytometry Analysis of U-2 OS Adherent Cells to Quantify 4 Populations

U-2 OS cells were treated with a placebo (non-induced, A and B) or treated with 1 µM staurosporine for 4 hours to induce apoptosis via caspase activity (C and D). Cells were lifted off the tissue culture flask by trypsinization and stained with FAM-FLICA. Cells were then stained with 7-AAD and analyzed by flow cytometry using an Accuri C6 fl ow cytometer. Compensation was set using cell populations stained individually with either FAM-FLICA or 7-AAD (data not shown). FAM-FLICA was analyzed on FL-1, and 7-AAD was analyzed on FL-3. Forward and side scatter density plots (A, non-induced; C, induced populations) and dual stained density plots (B, non-induced; D, induced populations) are shown. The density plot key is shown in Figure 4. Flow cytometry can be used to quantitate 4 populations of cells. Live, unstained cells do not fl uoresce (lower left, B and D). Early stage apoptotic cells fl uoresce green with FAM-FLICA (lower right, B and D). Dually stained green and red fl uorescing cells represent the population of U-2 OS cells in mid to late stage apoptosis; these cells have active caspase enzymes and compromised cell membranes (upper right, B and D). Necrotic cells fl uoresce red (upper left, B and D).
By including FAM-FLICA in the analysis, cells in early apoptosis (lower right, B and D) can be detected, which 7-AAD cannot detect alone. In the non-induced population (B), only 5.9% of cells were apoptotic (LR:3.6% + UR: 2.3%) compared with 30.5% of the induced population (D; LR: 22.1% + UR: 8.4%). Cells in these quadrants fluoresce green (ICT 226; 17-19).



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