Apoptosis Detection Kit, Poly-Caspases

For the detection of active poly-caspases in living cells without lysis

Cat. No. KT-053

For Research Use Only. Not for Use in Diagnostic Procedures.

PRODUCT INFORMATION

Apoptosis Detection Kit, Poly-Caspases
Cat. No. KT-053

PRODUCT
The **K-*ASSAY*®** Apoptosis Detection Kit, Poly-Caspases is for the detection of active poly-caspases in living cells without lysis. It can be used to evaluate apoptotic events using three different fluorescence detection methods: 96-well microtiter plate fluorometry and flow cytometry for quantitation and fluorescence microscopy for qualitative analysis.

DESCRIPTION
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 cell.

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 kDa) and two small (~10 kDa) subunits that form two heterodimers which associate in a tetramer. In common with other proteases, caspases are synthesized as precursors that undergo proteolytic maturation, either auto-catalytically or in a cascade by enzymes with similar specificity.

Caspase enzymes specifically recognizes a four amino acid sequence on the target substrate that includes an aspartic acid residue. This residue is the target for the cleavage reaction, which occurs at the carboxyl end of the aspartic acid residue. Caspases can be detected via immunoprecipitation or immunoblotting techniques using caspase-specific antibodies, or by employing fluorochrome substrates, which become fluorescent upon cleavage by the caspase.

PRINCIPLE

The Apoptosis Detection Kit, Poly-Caspases uses a novel approach to detect active caspases. The methodology is based on peptide fluorochrome inhibitor of caspases (FLICA). These inhibitors are cell permeable and non-cytotoxic. 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 active caspase heterodimer, thereby inhibiting further enzymatic activity. Because the FLICA Reagent becomes covalently coupled to the enzyme, it is retained within the cell, while any unbound FLICA Reagent will 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 reagent can be analyzed by 96-well-plate based fluorometry, fluorescence microscopy, or flow cytometry.

This Apoptosis Detection kit (Cat. No. KT-053) uses the FAM-VAD-FMK (carboxyfluorescein (FAM)-labeled valylalanylaspartic acid (VAD) fluoromethyl ketone (FMK)) as a label that is used to measure the amount of active caspases that were present in the cell at the time when the reagent was added. Other fluorescence Apoptosis Detection Kits are also available to analyze specific caspases: FAM-YVAD-FMK for caspase-1 (KT-055); FAM-VDVAD-FMK (KT-057) for caspase-2; FAM-DEVD-FMK (KT-059) for caspase-3 and caspase-7; FAM-VEID-FMK (KT-061) for caspase-6; FAM-LETD-FMK (KT-063) primarily for caspase-8; FAM-LEHD-FMK (KT-065) primarily for caspase-9; FAM-AEVD-FMK (KT-067) for caspase-10; and FAM-LEED-FMK (KT-069) primarily for caspase-13.

Following the suggested protocol below, each sample requires 10 µL of 30X reagent solution (equal to 2 µL of 150X FAM-VAD-FMK Reagent stock). The kit will test 100 samples (KT-053).

The Apoptosis Detection Kit, Poly-Caspases is designed to evaluate apoptotic events using three different fluorescence detection methods: 96-well microtiter plate fluorometry and flow cytometry for quantitation and fluorescence microscopy for qualitative analysis. The reagent has an optimal excitation range of 488-492 nm, and emission range of 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 (PI) or Hoechst Stain.

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

When 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 FAM-VAD-FMK 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 performed 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.

COMPONENTS

- 4 vial FAM-VAD-FMK Reagent, Lyophilized
- 1 vial Wash Buffer, 10X (60 mL)
- 1 vial Fixative (6 mL)
- 1 vial Propidium Iodide (PI), 250 µg/mL (1 mL)
- 1 vial Hoechst Stain, 200 µg/mL (1 mL)

Kit size is 100 tests.

Materials or equipment required but not provided

- 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
- 600 mL graduated cylinder
- Slides
- Hemocytometer
- Clinical centrifuge at <400 X g
- 37°C CO_2 incubator
- Vortex mixer
- Pipette(s) capable of dispensing at 10µL, 50µL, 200µL, 300µL, 1mL
- Deionized H_2O (diH_2O), 540 mL
- Phosphate Buffered Saline (PBS) pH 7.4, up to 100 mL
- Dimethylsulfoxide (DMSO), 200µL
- Ice or 4°C refrigerator to store cells
- Instrumentation (not all are required)
    - 96-well fluorescence plate reader with excitation 488 nm / emission 520 nm filter pairing, and **black** round or flat bottom 96-well microtiter plates.
    - Fluorescence microscope with appropriate filters (excitation 490 nm, emission >520 nm for FAM-VAD-FMK; excitation at 490 nm and emission at 635 nm for PI; and if Hoechst is used, a UV-filter with excitation at 365 nm and emission at 480 nm) and slides.
    - Flow cytometer equipped with a 15 mW, 488 nm argon excitation laser, with appropriate filters (excitation 490 nm, emission >520 nm for FAM-VAD-FMK; excitation at 490 nm and emission at 635 nm for PI).

PROTOCOLS

Preparation of 1X Wash Buffer

The Wash Buffer is supplied as a 10X concentrate which must be diluted to 1X with diH_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. If not using the entire bottle, dilute the 10X Wash Buffer 1:10 in diH_2O. For example, add 10 mL 10X Wash Buffer to 90 mL diH_2O (to make 100 mL).

3. Otherwise, add the entire bottle (60 mL) of 10X Wash Buffer to 540 mL of diH_2O (to make 600 mL).
4. Let the solution stir for 5 minutes or until all crystals have dissolved.
5. If not using the 1X Wash Buffer the same day it was prepared, store it covered at 4°C for up to 14 days.

Warning: The Wash Buffer contains sodium azide, which is toxic if swallowed or absorbed through the skin. Sodium azide reacts with lead and copper sink drains forming explosive compounds. When disposing of excess Wash Buffer, flush sink with copious amounts of water.

Propidium Iodide (PI)

PI is provided at 250 µg/mL and ready to use.
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. The dye excites at 488-492 nm with an emission maximum at 635 nm.

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

Hoechst Stain

Hoechst Stain is provided at 200 µg/mL and ready to use.
Hoechst Stain can be used to label the nuclei of dying cells after labeling with the probe reagent. It is revealed under a microscope using a UV-filter with excitation at 365 nm and emission at 480 nm.

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

Fixative

After labeling, add the Fixative into the cell suspension at a 1:10 ratio. For example, add 100 µL Fixative to 900 µL cell suspension. Fixed cells may be stored on ice or at 4°C up to 24 hours.

If the stained cell populations cannot be evaluated immediately after staining, cells may be fixed and analyzed up to 24 hours later on a microscope or flow cytometer. The Fixative is a formaldehyde solution designed to cross-link cell components and will not interfere with the carboxyfluorescein labeling once the probe reaction has taken place.

- Do not use ethanol-based or methanol-based Fixatives to preserve the cells. They will inactivate the probe label.
- Never add the Fixative until the staining and final wash steps have been completed.

Preparation of 150X FAM-VAD-FMK Stock

The FAM-VAD-FMK Reagent is supplied as a highly concentrated lyophilized powder. Reconstitute in DMSO to prepare a 150X stock, and then dilute 1:5 in PBS to form a 30X working solution. For best results, the 30X working solution should be prepared immediately prior to use. The reconstituted 150X stock can be stored at –20°C protected from light for future use.

1. Reconstitute each vial of lyophilized FAM-VAD-FMK with 50 µL DMSO. This yields a 150X concentrate (kit contains 4 vials).
2. Mix by swirling or tilting the vial, allowing 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. If using later, aliquot and store it at –20°C.
   - **The newly reconstituted 150X FAM-VAD-FMK stock must be used or frozen immediately after it is prepared and protected from light during handling.**

Preparation of 30X FAM-VAD-FMK Solution for Immediate Use

Using the freshly reconstituted 150X FAM-VAD-FMK stock, prepare the 30X working-strength FAM-VAD-FMK solution by diluting the stock 1:5 in PBS at pH 7.4. Following the suggested protocol here, each test sample requires 10 µL of 30X FAM-VAD-FMK solution (or 2 µL of the 150X FAM-VAD-FMK 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.
3. Mix by inverting or vortexing the vial at RT.
   - **The 30X working strength FAM-VAD-FMK solution must be used the same day that it is prepared.**

Storage of 150X FAM-VAD-FMK Stock for Future Use

If all of the 150X FAM-VAD-FMK stock will not be used immediately after reconstitution, the unused portion may be stored at -20°C for up to 6 months. During that time, the 150X FAM-VAD-FMK stock may be thawed up to two times. After the

second thaw, discard any remaining 150X FAM-VAD-FMK 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 "Preparation of 30X FAM-VAD-FMK Solution from a Frozen Aliquot" instructions below.

Preparation of 30X FAM-VAD-FMK Solution from a Frozen Aliquot

The 150X FAM-VAD-FMK stock previously reconstituted and stored at –20°C may be used 2 more times within 6 months.

1. Thaw the 150X FAM-VAD-FMK stock and protect from light.
2. Dilute the 150X stock solution 1:5 in PBS pH 7.4. For example, mix 10 µL of 150X FAM-VAD-FMK stock with 40 µL of PBS.
3. Mix by inverting or vortexing the vial at RT.
4. If the 150X FAM-VAD-FMK stock was frozen immediately after reconstitution and was never thawed, return the remaining stock to the freezer. If the stock was previously thawed once, discard the remaining stock.
5. Proceed to the labeling protocol.

PROCEDURE

Overview of the Protocol

Staining apoptotic cells with the Apoptosis Detection Kit, Poly-Caspases can be completed within a few hours. However, the kit is used to stain 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 induction process (which typically requires a 2-4 hour incubation at 37°C). Therefore, the kit reagents should be prepared at the end of the apoptosis induction process so that the 30X FAM-VAD-FMK solution is used immediately. The following is a brief overview of the 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 control cell population at the same density as the induced population for every staining condition. For example, if staining with FAM-VAD-FMK and Hoechst Stain, prepare 8 populations:

    a) Unlabeled (for both induced and non-induced populations)
    b) Labeled with FAM-VAD-FMK only (for both induced and non-induced populations)
    c) Labeled with Hoechst only (for both induced and non-induced populations)
    d) Labeled with FAM-VAD-FMK and Hoechst (for both induced and non-induced populations)

3) Induce apoptosis following your protocol (Four example protocols are described in "Induction of Apoptosis" below).
4) Prepare 1X Wash Buffer.
5) Prepare 150X FAM-VAD-FMK stock.
6) Prepare 30X FAM-VAD-FMK solution.
7) Stain cells with 30X FAM-VAD-FMK solution, incubate for 1 hour, and wash cells.
8) If desired, stain cells with PI.
9) If desired, stain cells with Hoechst Stain.
10) If desired, fix cells (see "Fixative" section).
11) Analyze data via microtiter plate fluorometry, flow cytometry, and/or fluorescence microscopy.

Induction of Apoptosis

This kit works with your current apoptosis protocols. Induce apoptosis as you normally would, then label the cells with FAM-VAD-FMK. 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 (Kamiya cat. no. AP-002) 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.

96-Well Fluorescence Plate Reader Staining Protocol

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

1. Prepare 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 cultured 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 "Induction of Apoptosis," pg. 5).
3. At the same time, culture an equal volume of non-induced cells as a negative control. Make sure that both tubes of cells contain similar number 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 staining. 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. However, more of the FAM-VAD-FMK Reagent may be needed per sample. Larger volume cell suspensions can be labeled nicely using 25 cm^2 tissue culture flasks (laid flat) as the incubation vessel.
   - **When ready to label with the 30X FAM-VAD-FMK 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 FAM-VAD-FMK solution directly to the 290–300 µL cell suspension.
6. Or, if a different cell volume was used, add the 30X FAM-VAD-FMK solution at a 1:30 ratio. For example, if 2.9 mL of cell suspension was used, add 100 µL of the 30X FAM-VAD-FMK solution (forming a final volume of 3 mL).
   - **Each investigator should adjust the amount of FAM-VAD-FMK Reagent used to accommodate their particular cell line and research conditions.**
7. Mix the cells by lightly 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 once or twice during this incubation time. This will ensure an even distribution of the labeling 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 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. Repeat Steps 11-14
16. 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 17). 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 21).
17. Centrifuge the remaining cells at <400 X g for 5 minutes at RT.
18. Carefully remove and discard supernatant.
19. Resuspend non-stimulated cells in 400 µL PBS
20. If it is not necessary to adjust the cell concentrations (as discussed in Step 16d), resuspend the stimulated cells in 400 µL PBS as well.
21. If it is necessary to adjust the cell concentrations (as discussed in Step 16d), 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 induceded cell population numbers.
22. Place 100 µL/well of the cell suspensions into duplicate wells of a **black** microtiter plate. Do not use clear plates. Avoid bubbles.
23. Measure the fluorescence intensity of fluorescein.

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 at 520 nm. Fluorescein has an optimal excitation range of 488 - 492 nm, and emission range of 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 fluorescence intensities in induced versus non-induced Jurkat cells is shown in Figure 1.

96-Well Fluorescence Plate Reader Sample Data (using FAM-VAD-FMK)

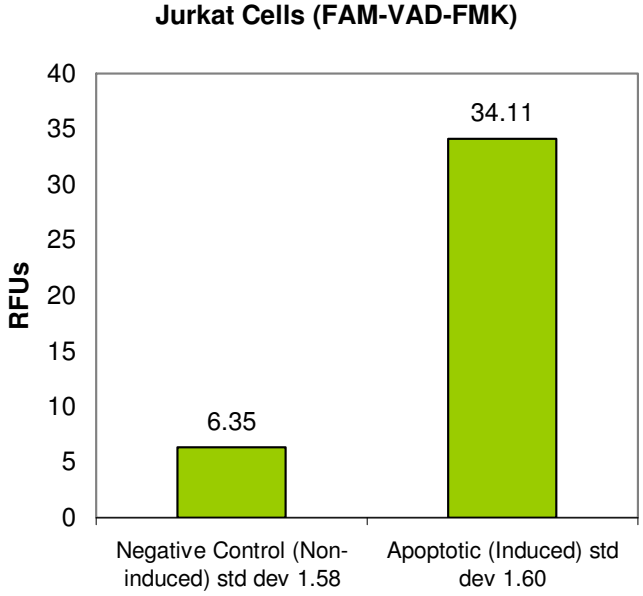


Figure 1. FAM-VAD-FMK fluorometric detection of active caspases in Jurkat cells (mean n=6).

In Figure 1, cells were either treated with DMSO (negative, non-induced cells) or with staurosporine (apoptotic, induced cells) for 2 hours at 37°C. Cells were labeled with FAM-VAD-FMK for 60 minutes at 37°C. Samples were read on a 96-well fluorescence plate reader (Molecular Devices, Gemini XS) set at 490 nm excitation and 520 nm emission using a 495 nm cut-off filter. As caspases became more active, indicating apoptosis, the amount of green fluorescence increased by over 500% in the induced Jurkat cells.

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.
5. Induce cells to undergo apoptosis and take samples at time points according to your specific protocol.
6. Add the 30X FAM-VAD-FMK solution to the medium at a 1:30 ratio. For example, add 10 µL 30X FAM-VAD-FMK to 290–300 µL medium.
   - **Each investigator should adjust the amount of FAM-VAD-FMK 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, dilute 1.5 µL Hoechst Stain with 300 µL medium (0.5% v/v) and add to cells.
    a. Incubate for 5 minutes at 37°C under 5% CO_2.
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, place a drop of 1X Wash Buffer onto the glass slide and mount a coverslip with cells facing down on the slide. 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 µL Fixative to 360 µL 1X Wash Buffer.
    a. Place a drop of diluted Fixative onto a microscope slide and mount a coverslip with cells facing down onto a microscope slide. Or, remove the plastic frame of the chamber slide, add a drop of diluted Fixative onto the glass slide and cover with a coverslip.
    b. Keep fixed cells at 4° C protected from light for up to 24 hours.
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, use 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.)

Fluorescence Microscopy Staining Protocol for Suspension Cells

1. Culture cells to a density optimal for apoptosis induction according to your specific induction protocol.
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.
4. At the same time, culture an equal volume of non-induced cells as a negative control cell population. Make sure that both the negative control and induced positive cell population tubes contain similar numbers of cells.
5. Transfer 290–300 µL of each positive and negative control cell populations into fresh tubes. If desired, larger cell volumes can be used. However, more of the 30X FAM-VAD-FMK 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-VAD-FMK solution, cells should be at least 5 X 10^5 cells/mL. Cell density can be determined by counting cell populations on a hemocytometer.**
6. Add 10 µL of the 30X working dilution FAM-VAD-FMK solution directly to each 290–300 µL cell suspension.
7. Or, if a larger cell volume was used, add the 30X FAM-VAD-FMK solution at a 1:30 ratio. For example, if 2.9 mL of cell suspension was used, add 100 µL of the 30X FAM-VAD-FMK solution (forming a final volume of 3 mL).
   - **Each investigator should adjust the amount of labeling reagent used to accommodate their particular cell line and research conditions.**
8. Mix the cells by gently 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 labeling 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. Repeat Steps 12-15.
18. Resuspend the cell pellets in 300 µL 1X Wash Buffer (higher volumes may be used if a larger staining cell volume was used).
19. Place cells on ice.
20. At this point, the cells may be stained with PI for bicolor analysis (Step 21) and analyzed immediately (Step 22), or fixed for future viewing (Step 23).
21. 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 and emission at >520 nm; PI has an emission peak at 637 nm.
22. To view cells immediately, place 1 drop of the cell suspension onto a microscope slide and cover with a coverslip. Go to Step 24.
23. 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. Place a drop of cell suspension onto a microscope slide and dry cells.
    c. Briefly wash the cells with PBS.
    d. Cover cells with mounting media and coverslip.
    e. Store slides at 4°C up to 24 hours.
24. 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-VAD-FMK 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.)

Examples of FAM-VAD-FMK and Hoechst staining of Jurkat cells are shown in Figures 2, 3, and 4.

Fluorescence Microscopy Sample Data

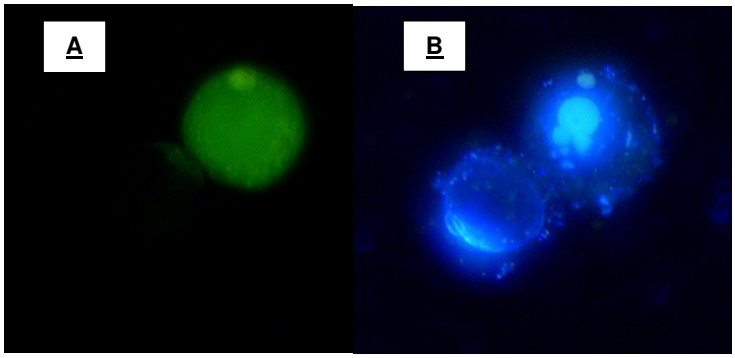
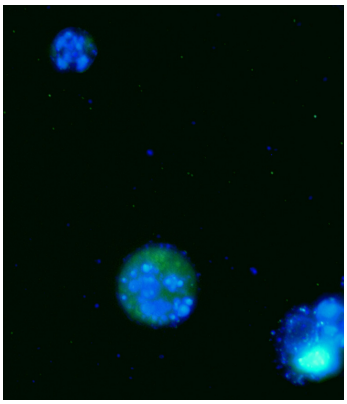


Figure 2. Suspension cells were incubated with 1 µM staurosporine for 3 hours at 37°C to induce apoptosis. Cells were labeled with FAM-VAD-FMK for 60 minutes at 37°C. Cells were washed and then incubated with Hoechst Stain for 5 minutes. Wet-mount slides were prepared and 2 photos were taken of the same cells. (A) Caspase activity was detected using a band pass filter (excitation at 488 nm, emission at 520 nm). (B) Nuclear staining by Hoechst Stain was revealed using a UV filter (excitation at 365 nm, emission at 480 nm).

In Figure 2, Photo A, only one cell appears green. The cell was apoptotic and stained positive for poly-caspase activity with the FAM-VAD-FMK Reagent. The other cell, which is not visible, did not bind to the reagent because it was not apoptotic. The same cells, photographed under a different wavelength for Hoechst Stain, appear blue (Photo B). The cell in the top right of photo B (which appears green in photo A) has a brightly stained nucleus – with its chromosomes condensing in the cell, a sign of dying cells. The cell in the bottom left of photo B (which is not visible in photo A) does not have a brightly stained nucleus, therefore it is not apoptotic nor necrotic.

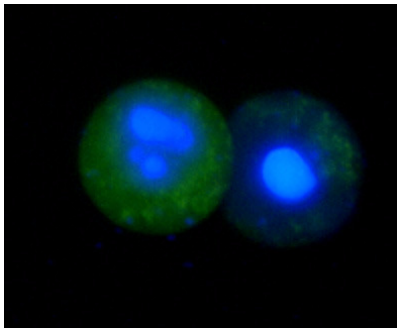
Figure 3. Suspension cells were incubated with 1 µM staurosporine for 3 hours at 37°C to induce apoptosis. Cells were then labeled with FAM-VAD-FMK for 60 minutes at 37°C. Cells were then washed and then incubated with Hoechst Stain for 5 minutes and wet-mount slides prepared. Two photos were taken of the same cells and superimposed. Caspase activity (green) was detected using a band pass filter (excitation at 488 nm, emission at 520 nm). Nuclear staining by Hoechst Stain (blue) was revealed using a UV filter (excitation at 365 nm, emission at 480 nm).



In Figure 3, only one cell of the three cells appears green (middle of the photo). The cell is apoptotic and stained positive for poly-caspase activity with the FAM-VAD-FMK Reagent. It also has many bright blue spots from the Hoechst Stain, indicating that the cell is beginning to die.

All the three cells were stained with Hoechst. The top left cell is somewhat blue throughout the cell. The cells is not dying and is neither necrotic nor apoptotic. The middle cell has many bright blue spots, indicating that chromosome condensation has begun and the cell is beginning to die. The bottom right cell has a brightly stained blue nucleus indicating that it is dying. This cell did not stain green, indicating that it is either necrotic (caspases were not activated and therefore could not bind to FAM-VAD-FMK), or apoptotic but far past the active caspase stage (and therefore did not stain green with FAM-VAD-FMK because the caspases were no longer active).

Figure 4. Cells were prepared as in Figure 3 and photos superimposed. Both cells are apoptotic (green) and dying (blue nuclei). As the left cell is much brighter green than the right cell, the left cell had more active caspases.



Flow Cytometry with Single-Color Staining

Positive control samples can be prepared by inducing cells in suspension culture to undergo apoptosis (see "Induction of Apoptosis" Section, pg. 5, for examples of apoptosis induction protocols). Following this flow cytometry protocol, each sample requires 10 µL of 30X FAM-VAD-FMK solution (equal to 2 µL of 150X FAM-VAD-FMK stock). After labeling with FAM-VAD-FMK, cells can be analyzed immediately 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 positive induced and negative non-induced populations).

a) Unstained (for both positive and negative cell populations)
b) Stained with FAM-VAD-FMK (for both positive and negative cell populations)

1. Culture cells to a density optimal for apoptosis induction according to your specific induction protocol.
2. Induce apoptosis following your protocol.
3. Culture an equal volume of non-induced cells as a negative control. Make sure that both tubes of cells contain similar numbers of cells.
   - **Ideally, cells should be at ~ 1 X 10^6 cells/mL for labeling. If necessary, cells may be induced to undergo apoptosis at lower concentrations, and then concentrated just prior to labeling to 0.5-2 X 10^6 cells/mL by centrifugation for 5 minutes at <400 X 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. However, more of the labeling 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-VAD-FMK solution directly to the 300 µL cell suspension.
6. Or, if a different cell volume was used, add the 30X FAM-VAD-FMK solution at a 1:30 ratio. For example, if 2.9 mL of cell suspension was used, add 100 µL of the 30X FAM-VAD-FMK solution (forming a final volume of 3 mL).
   - **Each investigator should adjust the amount of FAM-VAD-FMK Reagent used to accommodate their particular cell line and research conditions.**
7. Mix the cells by gently 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, gently resuspend them by swirling cells once or twice during this incubation time. This will ensure an even distribution of the FAM-VAD-FMK 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 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. Repeat Steps 11-13
16. Resuspend the cell pellet in 400 µL 1X Wash Buffer.
17. At this point, cells may be fixed (Step 18) or analyzed directly (Step 19).
18. 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 4° C, protected from light for up to 24 hours. Go on to Step 20.
19. Put samples on ice.
20. 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 (+).

21. See Figure 5 below for an example of single color analysis using flow cytometry.

Flow Cytometry Sample Data (using FAM-VAD-FMK)

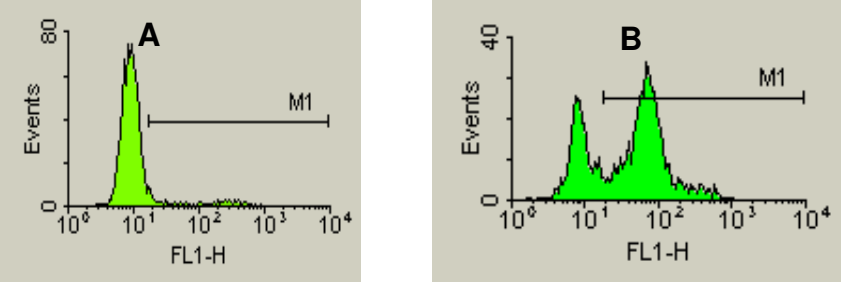


Figure 5. Jurkat cells were treated with A) DMSO (non-induced cells) or B) camptothecin (induced cells) for 3 hours. Cells were labeled with FAM-VAD-FMK for 1 hour, washed, and analyzed using BD Facscalibor flow cytometer.

The frequency histogram of the number of events (Y axis) versus fluorescein intensity (X axis) shows 2 peaks: caspase-negative cells occur to the left of the M1 region; caspase-positive cells lay within the M1 region.

Flow Cytometry with Bicolor Staining

If a bicolor analysis is desired, cells may be stained with PI along with the FAM-VAD-FMK Reagent. Using PI, a flow cytometer can distinguish live cells from necrotic, dead, and membrane-compromised cells in both caspase-negative and caspase-positive populations. Positive control samples can be prepared by inducing cells in suspension culture to undergo apoptosis (see "Induction of Apoptosis" Section, pg. 5, for examples of apoptosis induction protocols). After labeling with FAM-VAD-FMK 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:

a. Unstained (both induced and non-induced populations)
b. Stained with FAM-VAD-FMK only (both induced and non-induced populations)
c. Stained with PI only (both induced and non-induced populations)
d. Stained with FAM-VAD-FMK and PI (both induced and non-induced populations)

1. Culture cells to a density optimal for apoptosis induction according to your specific induction protocol.
2. Induce apoptosis following your protocol.
3. Culture an equal volume of non-induced cells as negative control populations. Make sure that all tubes of cells contain similar numbers of cells.
   - **Ideally, cells should be at ~ 1 X 10^6 cells/mL for labeling. If necessary, cells may be induced to undergo apoptosis at lower concentrations, and then concentrated just prior to labeling to 0.5-2 X 10^6 cells/mL by centrifugation for 5 minutes at <400 X 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. However, more of the FAM-VAD-FMK 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.)
4. Add 10 µL 30X FAM-VAD-FMK solution directly to the 300 µL cell suspension.
6. Or, if a larger cell volume was used, add the 30X FAM-VAD-FMK solution at a 1:30 ratio. For example, if 2.9 mL of cell suspension was used, add 100 µL of the 30X FAM-VAD-FMK solution (forming a final volume of 3 mL).
   - **Each investigator should adjust the amount of FAM-VAD-FMK Reagent used to accommodate their particular cell line and research conditions.**
7. Mix the cells by gently 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 labeling 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 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. Repeat Steps 11-13.
16. Resuspend the cell pellet in 400 µL 1X Wash Buffer.
17. Stain one 400 µL aliquot of FAM-VAD-FMK -treated induced cells with 2 µL PI.
18. Cells that are to be analyzed by the bicolor PI screening protocol cannot be fixed.
19. Set aside a second aliquot of FAM-VAD-FMK –treated, induced cells that does not contain PI.
20. Mix cells.
22. Put samples on ice. Keep bicolor cells at 4°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 (see Fig. 5, pg. 11): (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 live caspase positive (+) cells.

STORAGE

- Store the unopened kit (and unopened components) at 4°C until the expiration date.
- Protect the probe reagent from light at all times.
- Once reconstituted, the 150X FAM-VAD-FMK Reagent 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 4°C up to 14 days.

WARNINGS AND PRECAUTIONS

- PI and Hoechst Stain are potential mutagens.
- Use gloves and protective eye wear while handling the probe reagent, PI, 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.

FOR RESEARCH USE ONLY