Product Manual

Carboxyfluorescein Multi-Caspase Activity Kit

Catalog #: BML-AK117

Apoptosis Detection

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Product Manual

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Please read entire booklet before proceeding with the assay.

Carefully note the handling and storage conditions of each kit component.

Please contact Enzo Life Sciences Technical Support if necessary.

SECTION 1

BACKGROUND

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.

The caspases (**C**ysteinyl **A**spartate-**Sp**ecific Prote**ases**) are a family of enzymes that play essential roles in apoptosis (programmed cell death) and inflammation. As the name caspase implies, a conserved cysteine residue functions in catalysis and substrate cleavage occurs on the carboxyl-side of an aspartate residue. All caspases are synthesized as single polypeptide zymogens. Proteolytic activation of the zymogens can occur through autocatalysis, through cleavage by another, active caspase or through cleavage by some serine proteases (e.g. granzyme B, cathepsin-B). The active caspases are tetramers formed from two large (usually ~20 kD) and two small (~10 kD) subunits. Residues from both types of subunit contribute to formation of the substrate binding sites (two per tetramer). Small subunits derive from the C-terminal part of the zymogen polypeptide, by cleavage at one site, or two closely spaced sites, C-terminal to the active-site cysteine. The large subunits are formed from the remainder of the zymogen, usually minus the N-terminal prodomain. Caspase-9 is an exception in that, in vivo, the active, mature enzyme retains the N-terminal prodomain as part of the large subunit (35 or 37 kD)[3,4,5].

FAM-VAD-FMK is a carboxyfluorescein derivative of valylalanylaspartic acid fluoromethyl ketone (VAD-FMK) which is a potent inhibitor of caspase activity. The FAM-VAD-FMK reagent enters the cell and covalently binds to the reactive cysteine (Cys 285) on the large subunit of the caspase heterodimer. Use of the VAD multi-caspase substrate allows the detection of caspases 1-9[6].

The Carboxyfluorescein Multi-Caspase Activity Kit detects active caspase enzymes in living cells. Carboxyfluorescein (FAM) labeled caspase inhibitor probes bind to active caspase enzymes in living cell systems. These inhibitors are both cell-permeable and non-cytotoxic during the time of the labeling procedure. Once inside an apoptotic cell, these probes attach a green fluorescent label to the active site of the caspase enzyme allowing for detection by fluorescence microscopy and 96-well-plate based fluorometry.

Following the suggested protocols listed here, each sample requires 10µl of 30X FAM-VAD-FMK solution (equal to 2µl of 150X FAM-VAD-FMK stock). The FAM-VAD-FMK Kit will test 100 samples.

The FAM-VAD-FMK reagent excites at 488-492nm and has a maximum emission range of 515-535nm. Use the excitation / emission pairs on your microscope or fluorometer which best approximates these optimal ranges. Cells labeled with this reagent may be read immediately or preserved for 24 hours using the fixative included with the kit.

The FAM-VAD-FMK kit was designed to evaluate apoptotic events using 3 different fluorescence detection methods: a fluorescence microscope; and a fluorescence 96-well microtiter plate reader and flow cytometry. Use of the fluorescence microscope provides a qualitative view of the level of apoptosis induced as compared to the use of a fluorometer or flow cytometer which will generate quantitative data.

Using a fluorescence plate reader, the amount of caspase bound FAM-VAD-FMK can be quantitated as the amount of green fluorescence emitted from a fixed number of cells in a black microtiter plate well. When compared to non-stimulated control cells, the amount of green fluorescence will increase as cells enter later stages of apoptosis. Non-apoptotic cells do not contain elevated levels of effector caspases and thus do not bind the green fluorescent FAM-VAD-FMK inhibitor probe (see Section 16 for sample data).

Viewing cells through a fluorescence microscope, non-apoptotic cells will appear mostly unstained. In contrast, apoptotic cells will appear increasingly green as apoptosis progresses to more advanced stages. In these stages, the amount of active caspase enzyme capable of binding the FAM-VAD-FMK fluorescent inhibitor probe reaches maximum levels. This translates into an increased green fluorescence of the affected cells

Using a flow cytometer, analysis is done using a 15 mW argon ion laser at 488nm. 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 axis (first peak), whereas the caspase–positive cell population will appear as a separate peak or as a shoulder of the first peak showing increased fluorescence intensity. Cells with the active caspase will fluoresce green.

SECTION 2

REFERENCES

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2. E.A. Slee, et al. Cell Death and Differ 1999 6:1067.
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6. P.G. Ekert, et al. Cell Death Differ. 1999 6:1086.

PLEASE READ ENTIRE BOOKLET BEFORE PROCEEDING WITH THE ASSAY. CAREFULLY NOTE THE HANDLING AND STORAGE CONDITIONS OF EACH KIT COMPONENT. PLEASE CONTACT Enzo Life SciencesTECHNICAL SERVICES FOR ASSISTANCE IF NECESSARY.

SECTION 3

MATERIALS SUPPLIED

NOTE ON STORAGE AND SHELF LIFE:

Store the unopened kit (and each unopened component) at 2-8°C until the expiration date.

Protect the FAM-VAD-FMK reagent from light at all times.

Once reconstituted, the 150X FAM-VAD-FMK 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 7 days.

- KI-185 FAM-VAD-FMK Reagent, lyophilized
- KI-186 10X Wash Buffer (2 X 30ml)
- KI-187 Fixative (2 X 3ml)
- KI-188 Propidium Iodide (1ml)
- KI-189 Hoechst Stain
- AK-117 Instruction Manual

SECTION 4

OTHER MATERIALS NEEDED

Depending on experimental protocol used, the following materials may be needed.

- Cultured cells with media
- Reagents to induce apoptosis
- 15ml polystyrene centrifuge tube (1 per sample)
- 150ml or 600ml graduated cylinder
- Hemocytometer
- Clinical centrifuge at 400 X g
- Pipette(s) capable of dispensing at 10µl, 300µl, 1ml
- Phosphate Buffered Saline (PBS) pH 7.4
- Dimethyl Sulfoxide-DMSO (50µl or 200µl)
- Vortexer
- Amber vials or polypropylene tubes for storage of 150X concentrate at –20°C
- 37°C CO_2 incubator
- Ice or 4°C refrigerator to store fixed cells
- Slides
- 96-well fluorescence plate reader with excitation at 488nm, emission 520nm filter pairings. Fluorometer should be capable of reading black round or flat bottom 96-well microtiter plates.
- 96-well black microtiter plates
- Fluorescence microscope with appropriate filters (excitation 490nm, emission >520nm) and slides.
- Flow cytometer equipped with a 15 mW, 488nm argon excitation laser, with appropriate filters.

SECTION 5

SAFETY INFORMATION

Handle with care

Use gloves while handling the FAM-VAD-FMK reagent and the 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.

SECTION 6

OVERVIEW OF THE FAM-VAD-FMK PROTOCOL

Staining apoptotic cells with the FAM-VAD-FMK kit can usually be completed within a few hours. However, the FAM-VAD-FMK 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 FAM-VAD-FMK solution must be used immediately, the FAM-VAD-FMK reagents should be prepared at the end of your apoptosis induction process. The following is a quick overview of the FAM-VAD-FMK 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 FAM-VAD-FMK and Hoechst stain, make 8 populations.
   a. Unlabeled, induced and non-induced populations
   b. FAM-VAD-FMK labeled, induced and non-induced populations
   c. FAM-VAD-FMK and Hoechst labeled, induced and non-induced populations.
   d. Hoechst labeled, induced and non-induced populations.
3. Induce apoptosis following your protocol (2 sample protocols are mentioned in Section 7).
4. Prepare 1X wash buffer (Section 9).
5. Prepare 150X FAM-VAD-FMK stock (Section 12).
6. Prepare 30X FAM-VAD-FMK solution (Section 13 or 15).
7. Stain cells with 30X FAM-VAD-FMK solution, incubate for one hour and wash cells.
8. If desired, label cells with propidium iodide (Section 10).
9. If desired, label cells with Hoechst stain (Section 11).
10. Fix cells, if desired (Section 8).
11. Analyze data via microplate fluorometry, fluorescence microscopy, or flow cytometry.

SECTION 7

INDUCTION OF APOPTOSIS

The FAM-VAD-FMK kit works with your current apoptosis protocols. 2 examples of protocols to induce apoptosis in suspension culture are: a) treating Jurkat cells with 2µg/ml camptothecin (or 1 µM staurosporine) for 3 hours; and b) treating HL-60 cells with 4µg/ml camptothecin (or 1 µM staurosporine) for 4 hours.

SECTION 8

FIXATIVE

If the stained cell populations cannot be evaluated immediately upon completion of the FAM-VAD-FMK staining protocol, cells may be fixed and viewed up to 24 hours later. The fixative is a formaldehyde solution designed to cross-link cell components and will not interfere with the carboxyfluorescein labeling. The fixative is supplied ready-to-use. Once the FAM-VAD-FMK reaction has taken place, add the fixative into the cell solution at a 1:10 ratio. For example, add 100µl fixative to 900µl cells (Section 18 or 19). Fixed cells should be stored on ice or at 4°C up to 24 hours.

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

Never add the fixative until the staining and final wash steps have been completed as the fixative will interfere with the staining process.

SECTION 9

PREPARATION OF 1X WASH BUFFER

The wash buffer is supplied as a 10X concentrate which must be diluted to 1X with DI H20 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. Add the entire bottle (60ml) of 10X wash buffer to 540ml of DI H_2O. **Or**, if not using the entire bottle, dilute the 10X wash buffer 1:10 in DI H_2O. For example, add 10ml 10X wash buffer to 90ml DI H_2O.
3. Let solution stir for 5 minutes or until the crystals have dissolved.
4. If not using the 1X wash buffer the same day it was prepared, store it at 2°- 8°C for up to 14 days.

SECTION 10

PROPIDIUM IODIDE

Propidium iodide (PI) may be used to distinguish between live cells and diad 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 488-492nm and exhibits an emission maximum at 635nm. PI is provided ready to use at 250 µg/ml.

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

SECTION 11

HOECHST STAIN

Hoechst stain can be used to label the nuclei of dying cells after labeling with the FAM-VAD-FMK reagent. It is revealed during fluorescence microscopy using a UV-filter with excitation at 365nm and emission at 480nm. 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.

SECTION 12

RECONSTITUTION OF THE 150X FAM-VAD-FMK STOCK

The FAM-VAD-FMK 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 FAM-VAD-FMK stock must be used or frozen immediately after it is prepared and protected from light during handling.

1. Reconstitute each vial of lyophilized FAM-VAD-FMK with 50 µl DMSO. This yields a 150X concentrate. (The FAM-VAD-FMK-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), it 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).

SECTION 13

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 pH 7.4. Using the suggested protocols here, each sample to be tested requires only 10 µl of 30X FAM-VAD-FMK solution (or 2µl 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). The FAM-VAD-FMK 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 FAM-VAD-FMK solution must be used the same day that it is prepared.

SECTION 14

STORAGE OF 150X FAM-VAD-FMK STOCK FOR FUTURE USE

If not all of the 150X FAM-VAD-FMK stock will be used the same time it is reconstituted, the unused portion may be stored at -20°C for 6 months. During that time, the 150X FAM-VAD-FMK stock may be thawed and used twice. 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 Section 15 below.

SECTION 15

PREPARATION OF 30X FAM-VAD-FMK SOLUTION FROM A FROZEN ALIQUOT

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

1. Thaw the 150X FAM-VAD-FMK 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 10µl of 150X FAM-VAD-FMK reagent with 40µl 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 it to the freezer. If the stock was thawed once before, discard it.
5. Go on to the labeling protocol (Section 14, 17, or 18).

SECTION 16

96-WELL FLUORESCENCE SPECTROSCOPY STAINING PROTOCOL

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

   When ready to label with the 30X FAM-VAD-FMK solution, cells should be at least 1 X 10^5 cells/100µl aliquot per microtiter plate well. Density can be determined by counting cell populations on a hemocytometer.
2. Induce apoptosis following your protocol.
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 ~ 1-2 X 10^7 cells/ml for FAM-VAD-FMK labeling. If necessary, cells can be concentrated by centrifugation for 5 minutes at 400 X g at RT.)

4. Once induction is completed, transfer 290-300µl of each cell suspension to sterile tubes. (Larger cell volumes can also be used as determined by each investigator, 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).
5. Add 10µl 30X FAM-VAD-FMK solution directly to the 290-300µl 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 2.9ml of cell suspension were used, add 100µl of the 30X FAM-VAD-FMK solution (forming a final volume of 3ml).

   Each investigator should titrate the FAM-VAD-FMK reagent to accommodate their particular cell line or research condition.
7. Mix the cells by slightly flicking the tubes.
8. Incubate cells for 1 hour at 37°C under 5% CO_2, protecting the tubes from light. As cells may settle on the bottom of the tubes, gently resuspend them by swirling cells once or twice during this incubation time. This will ensure an even distribution of the FAM-VAD-FMK reagent among all cells.
9. Add 2ml 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 1ml 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 1ml 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 50µl from each tube.
    b. Add to 450µl 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 400µl PBS
23. If it is not necessary to equilibrate the cell concentrations (Step 19d), resuspend the stimulated cells in 400µl PBS as well.
24. From Step 19d, if necessary, 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 100µl of the cell suspensions into each of 2 wells of a black microtiter plate. Avoid bubbles.
26. Measure the fluorescence intensity of fluorescein (excitation 490nm, emission 520nm) 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 16).

SECTION 17

96-WELL FLUORESCENCE PLATE READER SET UP

1. Set the plate reader to perform an endpoint read.
2. Set the excitation wavelength at 490nm and the emission wavelength to 520nm. Fluorescein has an optimal excitation range from 488-492nm and emission range from 515-535nm. Select the filter pairings which most closely approximate this range; the filter pairing used may differ slightly from these optimal settings.
3. Read the sample.

SECTION 18

96-WELL FLUORESCENCE SPECTROSCOPY SAMPLE DATA

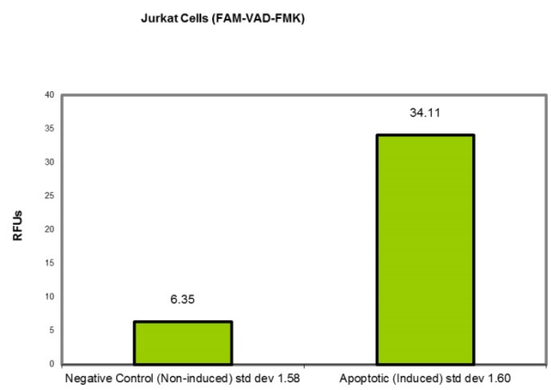

Figure 1. FAM-VAD-FMK fluorometric detection of active caspases in Jurkat cells (SD of 4 wells).

In **Figure 1**, 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 2 hours at 37°C. Cells were labeled with FAM-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 490nm excitation and 520nm emission using a 495nm cut-off filter. As the caspases became more active, indicating apoptosis, the amount of green fluorescence increased by over 500% in the Jurkat cells.

SECTION 19

FLUORESCENCE MICROSCOPY STAINING PROTOCOL FOR ADHERENT CELLS

1. Trypsinize cells.
2. Count cells.
3. Seed about 10^4-10^5 cells onto a sterile glass coverslip in a 35 mm petri dish or onto chamber slides.
4. Grow cells in their respective cell culture media formulation for 24 hours at 37°C.
5. Induce cells 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 titrate the FAM-VAD-FMK reagent to accommodate their particular cell line or research condition.
7. Mix well.
8. Incubate cells for 1 hour at 37° C under 5% CO2.
9. Remove the medium.
10. If cells are to be monitored using Hoechst stain, add 1.5 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 2ml 1X wash buffer.
12. At this point cells may be analyzed directly or fixed and analyzed later.
13. To analyze directly, mount the coverslip with cells facing down onto a microscope slide containing a drop of 1X wash buffer. Alternatively, 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 cells and analyze later, add fixative to wash buffer at a 1:10 ratio. For example, add 40 µl of fixative to 360 µl 1X wash buffer.

a. Mount a coverslip with cells facing down onto a microscope slide containing a drop of fixative plus wash buffer. Alternatively, remove the plastic frame of the chamber slide, add a drop of fixative plus wash buffer onto the glass slide and cover with a coverslip.

b. Keep fixed cells at 2°-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 490nm, emission >520nm) to view green fluorescence of caspase positive cells. If these filters are not available, select a filter combination that best approximates these settings.

SECTION 20

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 105 cells/ml.
3. Induce apoptosis following your protocol and sample at different time points following your specific protocol.
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.

   Cell density 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.

5. Transfer 290-300µl of each induced and negative control cell populations into fresh tubes. Or, if desired, larger cell volumes can be used, however more of the 30X FAM-VAD-FMK solution may be required. Larger volume cell suspensions label nicely using 25 cm2 tissue culture flasks (laid flat) as incubator vessels. (Cells should be at a density of at least 5 X 105 cells/ml.)

   When ready to label with the 30X FAM-VAD-FMK 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 10µl of the 30X working dilution FAM-VAD-FMK solution directly to each 290-300µl cell suspension.
7. Alternatively, if a larger cell volume was used, add the 30X FAM-VAD-FMK solution at a 1:30 ratio. For example, if 2.9ml of cell suspension were used, add 100µl of the 30X FAM-VAD-FMK solution (forming a final volume of 3ml).

   Each investigator should titrate the FAM-VAD-FMK reagent to accommodate their particular cell line or research condition.
8. Mix the cells by slightly flicking the tubes.
9. Incubate cells for 1 hour at 37°C under 5% CO2, protecting the tubes from light. As cells may settle on the bottom of the tubes, gently resuspend them by swirling cells once or twice during this incubation time. This will ensure an even distribution of the FAM-VAD-FMK 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% CO2
11. Add 2ml 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 1ml 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 300µl 1X wash buffer (higher volumes may be used if a larger staining cell volume was used).
22. Place cells on ice.
23. At this point, the cells may be stained with Propidium iodide (PI) for bicolor analysis (step 24), observed immediately (step 25), or fixed for future viewing (step 26).
24. To exclude dead cells from the analysis, 1.5 µl PI solution may be added at this point (0.05% v/v.) Cells may then be

viewed using a long pass filter with the excitation at 490nm, emission >520nm; PI has a maximum emission at 637nm.

25. To view cells immediately, place 1 drop of the cell suspension onto a microscope slide and cover with a coverslip; go to Step 27.
26. If not viewing immediately, cells may be fixed for viewing up to 24 hours later. If cell pellets were resuspended in 300µl wash buffer, add 30µl 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 3ml was used, add 300µl fixative.
    a. Incubate cells for 15 minutes at RT in the dark.
    b. Dry cells onto a microscope slide.
    c. Briefly wash the cells with PBS.
    d. Cover cells with mounting media and coverslip.
    e. Store slides at 2°-8°C up to 24 hours. Go on to Step 27.
27. Observe cells under a fluorescence microscope using a bandpass filter (excitation 490nm, emission >520nm) to view green fluorescence. Caspase positive cells bearing caspase enzyme covalently coupled to FAM-VAD appear green.

GLOBAL HEADQUARTERS
Enzo Life Sciences Inc.
10 Executive Boulevard
Farmingdale, NY 11735
Toll-Free:1.800.942.0430
Phone:631.694.7070
Fax: 631.694.7501
info-usa@enzolifesciences.com

EUROPE
Enzo Life Sciences (ELS) AG
Industriestrasse 17
CH-4415 Lausen
Switzerland
Phone:+41/0 61 926 89 89
Fax:+41/0 61 926 89 79
info-ch@enzolifesciences.com

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