# FAM Caspase Binding Assay

*For fluorescent detection of active caspases in apoptotic cells*

Catalog No. 30-1302 FAM Poly-Caspase Binding Kit, 100 tests
Catalog No. 30-1304 FAM Caspase 3 & 7 Binding Kit, 100 tests
Catalog No. 30-1306 FAM Caspase 8 Binding Kit, 100 tests
Catalog No. 30-1308 FAM Caspase 9 Binding Kit, 100 tests

## Instructions

Store at 2 to 8°C.

For research use only. Not for human, clinical, or diagnostic use.

**American Type Culture Collection**
P.O. Box 1549
Manassas, VA 20108 USA
www.atcc.org

800-638-6597 or 703-365-2700
Fax: 703-365-2750
E-mail: tech@atcc.org
Or contact your local distributor.

# FAM Caspase Binding Assay

FAM Poly-Caspase Binding Kit, Catalog No. 30-1302
FAM Caspase 3 & 7 Binding Kit, Catalog No. 30-1304
FAM Caspase 8 Binding Kit, Catalog No. 30-1306
FAM Caspase 9 Binding Kit, Catalog No. 30-1308

## Instruction Manual

## TABLE OF CONTENTS

## INTRODUCTION

### Background

Apoptosis is an evolutionarily conserved form of cell death 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 proapoptotic signals and result in the cleavage of protein substrates, causing the disassembly of the cell (1-6).

FAM Caspase Binding Kits use a novel approach to detect active caspases. The methodology is based on a fluorescently labeled inhibitor of caspases. These inhibitors are cell permeable and noncytotoxic. Once inside the cell, the labeled inhibitor binds covalently to the active caspase (7). For kits using green fluorescence, a carboxyfluorescein-labeled fluoromethyl ketone peptide inhibitor of caspases is used. (ATCC also offers a line of SR Caspase Binding Kits that use sulforhodamine-labeled inhibitors.)

For example, the FAM Poly-Caspase Binding Kit contains the green fluorescent-labeled inhibitor, FAM-VAD-FMK, which is a carboxyfluorescein (FAM) derivative of valylalanylaspartic acid (VAD) fluoromethyl ketone (FMK), a potent inhibitor of caspase activity. The FAM-VAD-FMK probe enters each cell and covalently binds to active caspase and inhibits further enzymatic activity, while unbound FAM-VAD-FMK reagent diffuses out of the cell and is washed away. The green fluorescent signal is a direct measure of the number of active caspase enzymes that are present in the cell. Cells that contain the bound probe can be analyzed by 96-well-plate fluorometry, fluorescence microscopy, or flow cytometry.

Because the FAM-VAD-FMK reagent irreversibly binds caspases 1, 3, 4, 5, 6, 7, 8, and 9, it can be used as a generic probe for caspase detection. In comparison, FAM-DEVD-FMK reagent binds primarily to caspase 3 and caspase 7 and is therefore used to measure the amount of active caspase 3 and 7 present in the cell. Other green fluorescence FAM Caspase Binding Kits are available to analyze specific caspases: FAM-LETD-FMK to primarily detect caspase 8 and FAM-LEHD-FMK to primarily detect caspase 9.

### Precautions

- Use gloves, protective clothing and eyewear when using the kit. The 10x Wash Buffer contains sodium azide, which is harmful if swallowed or absorbed through the skin. Propidium iodide and Hoechst stain are potential mutagens. Material safety data sheets are available upon request.
- FAM Reagent is reconstituted in DMSO, a powerful solvent that can rapidly penetrate intact skin. As a result there is potential hazard associated with using this compound. It is very important to avoid contact with DMSO and to safely dispose of any wastes containing DMSO.

## KIT COMPONENTS

Store all components at 2 to 8°C.

| Reagent | Quantity |
|---|---|
| FAM Reagent (lyophilized) | 4 vials |
| 10x Wash Buffer | 60 ml |
| Fixative | 6 ml |
| Propidium Iodide, 250 µg/ml | 1 ml |
| Hoechst Stain, 250 µg/ml | 1 ml |

## MATERIALS REQUIRED BUT NOT SUPPLIED

15-ml polystyrene centrifuge tubes (1 per sample)
Amber vials or polypropylene tubes (-20°C storage of 150x FAM Reagent aliquots)
600-ml graduated cylinder
Microscope slides
Hemocytometer
Centrifuge (400 x g)
$CO_2$ incubator at 37°C
Vortex mixer
Adjustable-volume pipettor with disposable tips
Reagents to induce apoptosis
Phosphate buffered saline (PBS) pH 7.4 (ATCC catalog no. 30-2200)
Dimethylsulfoxide (DMSO)
Deionized water

### Instrumentation Options

96-well fluorescence plate reader with **black** 96-well plates and appropriate filters
FAM: Excitation 488 nm, emission 520 nm

Fluorescence microscope with slides and appropriate filters
FAM: Excitation 490 nm, emission >520 nm
Propidium iodide: Excitation 490 nm, emission 635 nm
Hoechst stain: UV-filter with excitation 365 nm, emission at 480 nm

Flow cytometer equipped with a 15 mW, 488 nm argon excitation laser and appropriate filters
FAM: Excitation 490 nm, emission >520 nm
Propidium iodide: Excitation 490 nm, emission 635 nm

## PROTOCOLS

### Experimental Overview

The FAM Caspase Binding Assay can evaluate apoptotic events using three fluorescence detection methods: 96-well plate fluorometry for quantitation; fluorescence microscopy for qualitative analysis; and flow cytometry for quantitation. The FAM Reagent has an optimal excitation range from 488 to 492 nm and an emission range from 515 to 535 nm. Cells labeled with the FAM reagent may be read immediately or preserved for 24 hours using the fixative. Unfixed samples may be analyzed with propidium iodide or Hoechst stain.

### Use of Propidium Iodide and Hoechst Stain with Unfixed Samples

- Propidium iodide may be used to distinguish between live cells and dead cells. Propidium iodide stains necrotic, dead, and membrane-compromised cells. They may be viewed through a fluorescence microscope or analyzed on a flow cytometer.
- Hoechst stain can be used to label nuclei of dying cells after labeling with the FAM Reagent.

### Controls

Culture cells to a density optimal for apoptosis induction according to your specific induction protocol, but not to exceed $10^6$ cells/ml. (Cells grown to higher densities may begin to enter apoptosis naturally.) At the same time, culture a noninduced negative control cell population at the same density as the induced population for every labeling condition. All samples should contain a similar number of cells.

1. Unlabeled, induced and noninduced populations.
2. FAM labeled, induced and noninduced populations.
3. FAM and Hoechst (or propidium iodide) labeled, induced and noninduced populations.
4. Hoechst (or propidium iodide) labeled, induced and noninduced populations.

### Preparation of Reagents

#### 1x Wash Buffer

Reagent may be prepared ahead of time and stored at 2 to 8°C for up to 14 days.

| Step | Action |
|---|---|
| **1** | If necessary, gently warm the 10x concentrate to completely dissolve any salt crystals that may have come out of solution. |
| **2** | Dilute the 10x Wash Buffer 1:10 in deionized water. |
| **3** | Let the solution stir for 5 minutes or until all crystals have dissolved. |

**30x FAM Reagent**
The 30x Reagent must be prepared immediately before use. Protect from light during handling.

| Step | Action |
|---|---|
| **1** | Prepare 150x stock solution by reconstituting one vial of lyophilized reagent (25 reactions) with 50 µl of DMSO. Mix by swirling or tilting the vial at room temperature until completely dissolved.<br>**Note:** For later use, immediately aliquot 150x FAM stock solution into amber vials or polypropylene tubes and store at -20°C for 6 months. The 150x stock solutions may be thawed and used twice. |
| **2** | Prepare 30x FAM Reagent by diluting the stock 1:5 in PBS (pH 7.4). Mix by inverting or vortexing the vial at room temperature. |

**Fluorescence Plate Reader Protocol**

| Step | Action |
|---|---|
| **1** | Prepare populations of induced and noninduced cells, ensuring that both populations contain similar cell densities. If necessary, cells can be concentrated by centrifugation for 5 minutes at 400 x g at room temperature. You will need at least 5 x $10^5$ cells/100 ml aliquot per well before labeling. |
| **2** | Transfer 290 to 300 µl of each cell suspension to sterile tubes. Larger cell volumes can also be used; more of the FAM Reagent may be needed per sample. |
| **3** | Add 10 µl 30x FAM Reagent directly to the cell suspension. If a different cell volume was used, add the 30x FAM Reagent at a 1:30 ratio. |
| **4** | Mix the cells by lightly flicking the tubes. |
| **5** | Incubate cells for 1 hour at 37°C under 5% $CO_2$, protecting the tubes from light. Gently resuspend them by swirling cells once or twice during incubation. |
| **6** | Add 2 ml of 1x Wash Buffer to each tube and mix. |
| **7** | Centrifuge cells at <400 x g for 5 minutes at room temperature. |
| **8** | Carefully remove and discard supernatant. Gently vortex the cell pellet to disrupt any cell-to-cell clumping. |
| **9** | Resuspend the cell pellet in 1 ml of 1x Wash Buffer. |
| **10** | Centrifuge cells at <400 x g for 5 minutes at room temperature. |
| **11** | Carefully remove and discard supernatant. Gently vortex the cell pellet to disrupt any cell-to-cell clumping. |

| | |
|---|---|
| **12** | Resuspend the cell pellet in 1 ml of 1x Wash Buffer. |
| **13** | Determine the concentration of each cell population. |
| **14** | Centrifuge the remaining cells at <400 x g for 5 minutes at room temperature. Carefully remove and discard supernatant. |
| **15** | Resuspend cells in 400 µl PBS, or enough PBS to ensure the same cell density in each tube based on the counts in step 13. |
| **16** | Place 100 µl of the cell suspensions into each of 2 wells of a **black** microwell plate. Avoid bubbles. |
| **17** | Set the fluorescence plate reader to perform an endpoint read with excitation wavelength at 490 nm and the emission wavelength to 520 nm. Fluorescein has an optimal excitation range from 488 to 492 nm, and emission range from 515 to 535 nm. Select the filter pairing which most closely approximates this range and read the sample. |

**Fluorescence Microscopy Protocol for Adherent Cells**

| Step | Action |
|---|---|
| **1** | Seed about $10^4$ to $10^5$ cells onto a sterile glass coverslip in a 35-mm petri dish or onto chamber slides and grow to desired confluency. |
| **2** | Induce cells to undergo apoptosis. At the same time, culture an equal volume of noninduced cells for a negative control population. |
| **3** | Add the 30x FAM Reagent to the medium at a 1:30 ratio; mix well. |
| **4** | Incubate cells for 1 hour at 37° C under 5% $CO_2$ and remove the medium. |
| **5** | Optional: Add 1.5 µl Hoechst stain to 300 µl medium (0.5% v/v). Add this medium to the cells and incubate for 5 minutes at 37°C under 5% $CO_2$. |
| **6** | Wash cells twice with 2 ml of 1x Wash Buffer. |
| **7** | **To analyze immediately:** Mount a coverslip with cells facing down onto a microscope slide containing a drop of 1x Wash Buffer. Or, remove the plastic frame of the chamber slide, add a drop of 1x Wash Buffer onto the glass slide and cover with a coverslip.<br><br>**To fix the cells and analyze later**: Add fixative to wash buffer at a 1:10 ratio and place one drop onto a slide. Mount a coverslip with cells facing down onto the diluted fixative. Or, remove the plastic frame of the chamber slide, add a drop of drop of diluted fixative onto the glass slide and cover with a coverslip. Keep fixed cells at 2 to 8°C protected from light for up to 24 hours. |

| | |
|---|---|
| **8** | Observe cells under a fluorescence microscope using a bandpass filter (excitation 490 nm, emission >520 nm) to view green fluorescence. 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 Protocol for Suspension Cells**

| Step | Action |
|---|---|
| **1** | Prepare populations of induced and noninduced cells, ensuring that both populations contain similar cell densities. If necessary, cells can be concentrated by centrifugation for 5 minutes at 400 x g at room temperature. You will need at least 5 x $10^5$ cells/ml before labeling. |
| **2** | Transfer 290 to 300 µl of each induced and noninduced control cell population into fresh tubes. If desired, larger cell volumes can be used; more 30x FAM Reagent may be required. |
| **3** | Add 10 µl of the 30x working dilution FAM Reagent directly to each cell suspension. If a larger cell volume was used, add the 30x FAM Reagent at a 1:30 ratio. |
| **4** | Mix the cells by lightly flicking the tubes. |
| **5** | Incubate cells for 1 hour at 37°C under 5% $CO_2$, protecting the tubes from light. Gently resuspend by swirling cells once or twice during incubation time. |
| **6** | Optional: Add 1.5 µl Hoechst stain (0.5% v/v). Incubate for 5 minutes at 37°C under 5% $CO_2$. |
| **7** | Add 2 ml of 1x Wash Buffer to each tube and gently mix. |
| **8** | Centrifuge the cells at <400 x g for 5 minutes at room temperature. |
| **9** | Carefully remove and discard supernatant. Gently vortex the cell pellets to disrupt any cell-to-cell clumping. |
| **10** | Resuspend cells in 1 ml 1x Wash Buffer and gently mix. |
| **11** | Centrifuge the cells at <400 x g for 5 minutes at room temperature. |
| **12** | Carefully remove and discard supernatants. Gently vortex the cell pellets to disrupt any cell-to-cell clumping. |
| **13** | Resuspend cell pellets in 300 µl 1x Wash Buffer (more may be used with larger cell volumes). Place cells on ice.<br>• Proceed to Step 14 to stain with propidium iodide.<br>• Proceed to Step 15 to observe results immediately.<br>• Proceed to Step 16 to fix for future viewing. |

| | |
|---|---|
| **14** | To identify dead cells, 1.5 µl propidium iodide solution may be added at this point (0.5% v/v). Cells may then be viewed using a long pass filter with the excitation at 490 nm, emission >520 nm; propidium iodide has a maximum emission at 637 nm. |
| **15** | To view cells immediately, place one drop of the cell suspension onto a microscope slide and cover with a coverslip; go to Step 17. |
| **16** | If not viewing immediately, cells may be fixed for viewing up to 24 hours later. If cell pellets were resuspended in 300 µl 1x 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.<br>a. Incubate cells for 15 minutes at room temperature in the dark.<br>b. Dry cells onto a microscope slide.<br>c. Briefly wash the cells with PBS.<br>d. Cover cells with mounting medium and coverslip.<br>e. Store slides at 2 to 8°C up to 24 hours. Go on to Step 17. |
| **17** | Observe cells under a fluorescence microscope using a bandpass filter (excitation 490 nm, emission >520 nm) to view green fluorescence. 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. |

**Flow Cytometry Protocol (Single or Bicolor Staining)**

To set up electronic compensation and quadrant statistics for the flow cytometer, four types of samples are recommended for every labeling condition. For example, if labeling with FAM and propidium iodide, assemble eight populations:

1. Unlabeled, induced and noninduced populations.
2. FAM labeled, induced and noninduced populations.
3. FAM and propidium iodide labeled, induced and noninduced populations.
4. Propidium iodide labeled, induced and noninduced populations.

| Step | Action |
|---|---|
| **1** | Prepare populations of control and experimental cells, ensuring that all populations contain similar cell densities. If necessary, cells can be concentrated by centrifugation for 5 minutes at 400 x g at room temperature. Cells should be at approximately 1 x $10^6$ cells/ml before labeling. |
| **2** | Transfer 300 µl of each cell suspension to sterile tubes. Larger cell volumes can also be used; more of the FAM reagent may be needed per sample. |

| | |
|---|---|
| **3** | Add 10 µl 30x FAM Reagent directly to the 300 µl cell suspension. If a different cell volume was used, add the 30x FAM Reagent at a 1:30 ratio. |
| **4** | Mix the cells by lightly flicking the tubes. |
| **5** | Incubate cells for 1 hour at 37ºC under 5% $CO_2$, protecting the tubes from light. Gently resuspend by swirling cells once or twice during incubation time. |
| **6** | Add 2 ml of 1x Wash Buffer to each tube and mix the cells. |
| **7** | Centrifuge cells at <400 x g for 5 minutes at room temperature. |
| **8** | Carefully remove and discard supernatant. Gently vortex the cell pellet to disrupt any cell-to-cell clumping. |
| **9** | Resuspend the cell pellet in 1 ml 1x Wash Buffer. |
| **10** | Centrifuge cells at <400 x g for 5 minutes at room temperature. |
| **11** | Carefully remove and discard supernatant. Gently vortex the cell pellet to disrupt any cell-to-cell clumping. |
| **12** | Resuspend the cell pellet in 400 µl 1x Wash Buffer.<br>Go to Step 13 for single-color analysis.<br>Go to Step 14 for bicolor analysis. |
| **13** | Options for **single-color** analysis:<br>For immediate analysis, put samples on ice and measure fluorescein on the FL1 channel.<br>For analysis up to 24 hours later, add 40 µl fixative and mix. Or, if a different volume was used, add the fixative at a 1:10 ratio into the volume of cell suspension to be fixed. Keep fixed cells at 2 to 8ºC protected from light, then analyze as in Step 15. |
| **14** | If using **bicolor** analysis:<br>a. Add 2 µl of propidium iodide to a 400-µl aliquot of unlabeled and FAM–treated cells and mix well.<br>b. Set aside second aliquot of unlabeled and FAM-treated cells as non-PI controls.<br>c. Measure fluorescein on the FL1 channel and red fluorescence (propidium iodide) on the FL2 channel. |
| **15** | Analyze using a 15-mW argon ion laser at 488 nm. |

## INTERPRETATION OF RESULTS

### Fluorescence Plate Reader

Apoptosis can be quantified as the amount of green fluorescence emitted from FAM probes bound to caspases. Cell populations in more advanced stages of apoptosis will have a higher RFU intensity than cell populations in early stages.

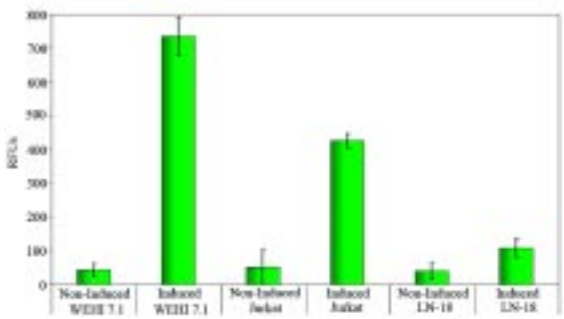


**Figure 1.** Detection of active caspases in WEHI 7.1, Jurkat and LN-18 cells (standard deviation of 3 wells). WEHI 7.1 (ATCC TIB-53) and Jurkat (ATCC TIB-152) cells were induced to undergo apoptosis by heat shock treatment, 2 minutes at 95°C. LN-18 (ATCC CRL-2610) cells were induced with 50 µM etoposide for 5 hours at 37°C. Cells were labeled with FAM-VAD-FMK solution for 1 hour (LN-18 cells) or 2 hours (WEHI 7.1 and Jurkat) at 37°C. The plate reader was set at 485 nm excitation and 530 nm emission. As the caspases became more active, indicating apoptosis, the amount of green fluorescence increased approximately 18, 7, and 3 fold in the induced WEHI 7.1, Jurkat, and LN-18 cells, respectively.

### Fluorescence Microscopy

Apoptotic cells appear green, while nonapoptotic cells are generally unstained. As apoptosis progresses, the amount of active caspase enzymes capable of binding the FAM probe increases and eventually reaches a maximum level. Therefore, cells in more advanced stages of apoptosis will appear brighter green than cells in earlier stages.

In Figure 2A, only one cell appears green; it is 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 and therefore is not apoptotic. The same cells, photographed at right under a different wave-length for Hoechst stain, appear blue. The lower cell in Figure 2B has a very brightly stained nucleus due to condensing DNA, a sign that the cell is dying. The upper cell does not have a brightly stained nucleus, therefore it is neither apoptotic nor necrotic.

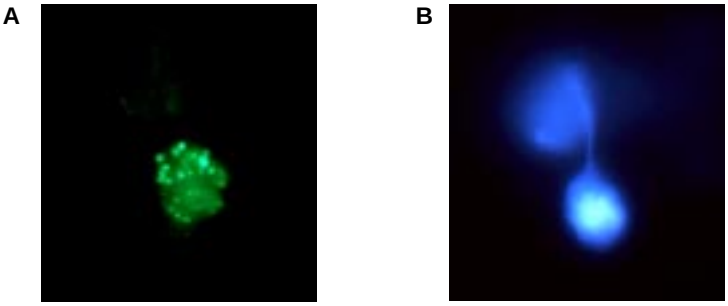


**Figure 2.** Apoptosis detection in adherent LN-18 cells (ATCC CRL-2610). Cells were incubated with 50 µM etoposide for 3 hours at 37°C to induce apoptosis, then labeled with FAM-VAD-FMK for 60 minutes at 37°C. Hoechst stain was added and incubated for 5 minutes, and the cells were then washed. **A.** Caspase activity was detected in one cell using a band pass filter (excitation at 480 nm, emission at 535 nm). **B.** Hoechst nuclear stain was analyzed using a UV-filter (excitation at 360 nm, emission at 460 nm).

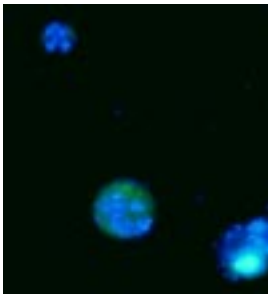

**Figure 3.** Apoptosis detection in suspension cells. Cells were incubated with 1 µM staurosporine for 3 hours at 37°C to induce apoptosis, then were labeled with FAM-VAD-FMK and Hoechst stain. Two photos were taken of the same cells and superimposed. Caspase activity (green) was detected using a band pass filter (excitation at 488 nm, emission at 520 nm). Hoechst nuclear stain (blue) was analyzed using a UV-filter (excitation at 365 nm, emission at 480 nm).

In Figure 3, only one cell of the three cells appears green; it 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 cells in Figure 3 stained with Hoechst stain. The top left cell is somewhat blue throughout the cell, indicating that the cell 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 very brightly stained blue nucleus indicating that it is dying. This cell did not stain green so 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).

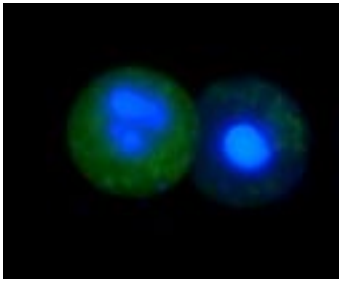

**Figure 4.** Apoptosis detection in suspension cells. Cells were prepared as in Figure 3 and photos superimposed. Both cells are apoptotic (green) and dying (blue nuclei). The cell at left is much brighter green than the right cell and thus has more active caspases.

**Flow Cytometry**

For single-color analysis, generate a log FL1 (X-axis) versus number of cells (Y-axis) histogram. On the histogram, two cell populations will be 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 (Figure 5). 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.

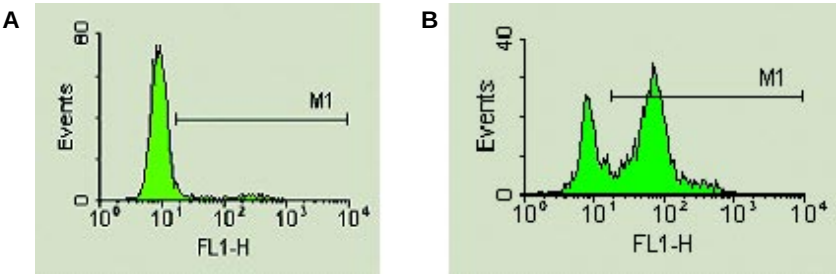


**Figure 5.** Caspase activity as detected by flow cytometry. Jurkat cells (ATCC TIB-152) were treated for 3 hours with (A) DMSO as a noninduced control or (B) camptothecin to induce apoptosis. Cells were labeled with FAM-VAD-FMK for 1 hour, washed, and analyzed.

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 (unlabeled cells), caspase-positive cells lay within the M1 region (FAM-labeled cells).

For bicolor analysis, generate a log FL1 (X-axis) versus log FL2 (Y-axis) dot plot. Add quadrant cursors. The four quadrant areas contain the following cell populations: (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 (living caspase positive) cells.

## APPENDIX

### Induction of Apoptosis

Here are several options for inducing apoptosis in cell cultures to serve as positive controls:

1. Jurkat cells (ATCC TIB-152) treated with 2 µg/ml camptothecin for 3 hours.
2. Jurkat cells treated with 1 µM staurosporine for 3 hours.
3. HL-60 cells (ATCC CCL-240) treated with 4 µg/ml camptothecin for 4 hours.
4. HL-60 cells treated with 1 µM staurosporine for 4 hours.

### Troubleshooting

**Problem:** Low intensity of fluorescence.

| Cause | Remedy |
|---|---|
| FAM reagent is light sensitive. | Protect FAM Reagent from light at all times. |
| 150x FAM stock was not stored properly. | Reconstitute new lyophilized stock, aliquot if necessary and promptly store at –20°C protected from light for up to 6 months with two freeze thaws during that time. |
| 30x FAM stock was not prepared immediately before use. | For best results, the 30x stock should be prepared immediately before use. |
| Cell density during 30x FAM labeling was low. | For fluorescence plate reader and fluorescence microscopy of suspension cells, cell density should be at least 5 x $10^5$ cells/100 µl.<br>For fluorescence microscopy of adherent cells,seed about $10^4$ to $10^5$ cells onto sterile coverslip or chamber slides prior to 24 hour growth.<br>For flow cytometry, cell density should be ~1 x $10^6$ cells/ml. |
| Cell volume other than 290 to 300 µl was used during 30x FAM labeling. | If a different cell volume was used, add 30x FAM Reagent at a 1:30 ratio.<br>Larger volume cell suspensions can be labeled using 25-$cm^2$ tissue culture flasks (laid flat) as incubator vessels. |
| Concentration of FAM Reagent not optimal. | Optimize FAM Reagent to accommodate cell line and research conditions. |

| | |
|---|---|
| Fixative inactivated FAM label. | Never add the formaldehyde fixative until the staining and final wash steps have been completed.<br>Do not use ethanol-based or methanol-based fixatives to preserve the cells. They will inactivate the FAM label. |
| Samples were not read immediately. | Cells labeled with FAM reagent must be read immediately using fluorescence plate reader.<br>Samples for fluorescence microscopy or flow cytometry may be preserved with fixative for analysis within 24 hours. |
| No apoptotic cells. | Make sure apoptosis induction protocol is optimal. Assess efficiency of apoptosis induction with either Hoechst stain or second assay (see Related Products). |

**Problem:** Fluorescence unreadable.

| Cause | Remedy |
|---|---|
| Incorrect excitation/emission pairs. | The FAM reagent has an optimal excitation range from 488 to 492 nm, and emission range from 515 to 535 nm. Use the excitation/emission pairs which best approximate this optimal range. |
| Clear plates used with fluorescence plate reader. | Use black microwell plates. |
| Flow cytometer: Electronic compensation and quadrant statistics was not performed for both induced and noninduced populations. | Apoptotic cells have varying light scattering properties. Calibrate flow cytometer to avoid spectral overlap using the recommended control samples. |
| Flow cytometer: Carryover of propidium iodide (PI) from sample injection port. | Rinse port with distilled water after each PI-containing sample. |
| Individual positive cells are seen with fluorescence microscopy while sensitivity with the fluorescence plate reader depends on the cell population. | For detection with fluorescence plate reader, increase the percentage of caspase-positive cells in the population by modifying the induction protocol.<br>Increase detection sensitivity on the fluorometer. |

**Problem:** Lack of distinct populations.

| Cause | Remedy |
|---|---|
| Cells were fixed, compromising membranes of all samples. | Do not fix cells if propidium iodide is to be used to distinguish between live cells and dead cells. PI stains necrotic, dead, and membrane-compromised cells. |
| No apoptotic cells. | Make sure apoptosis induction protocol is optimal. Assess efficiency of apoptosis induction with either Hoechst stain or second assay (see Related Products). |
| Cell density in the cell culture flasks exceeded optimal cell concentration and naturally entered apoptosis | Do not let density in the cell culture flasks exceed $10^6$ cells/ml. Optimal cell concentration will vary depending on the cell line. |

## REFERENCES


1. Slee EA et al. Serial killers: Ordering caspase activation events in apoptosis. Cell Death Differ. 6(11): 1067-1074, 1999.
2. Walker NP et al. Crystal structure of the cysteine protease interleukin-1β converting enzyme: A $(p20/p10)_2$ homodimer. Cell 78(6487): 343-352, 1994.
3. Wilson KP et al. Structure and mechanism of interleukin-1β converting enzyme. Nature 370(6487): 270-275, 1994.
4. Rotonda J et al. The three-dimensional structure of apopain/CPP32, a key mediator of apoptosis. Nat. Struct. Biol. 3(7): 619-625, 1996.
5. Kumar S. Mechanisms mediating caspase activation in cell death. Cell Death Differ. 6(11): 1060-1066, 1999.
6. Thornberry NA et al. A combinatorial approach defines specificities of members of the caspase family and granzyme B. Functional relationships established for key mediators of apoptosis. J. Biol. Chem. 272(29): 17907-17911, 1997.
7. Ekert PG et al. Caspase inhibitors. Cell Death Differ. 6(11): 1081-1086, 1999.

## RELATED PRODUCTS

| | | |
|---|---|---|
| **Apoptosis Detection Products** | | |
| Annexin V Fluorescent Detection Kit | 30-1236 | 100 tests |
| Anti-G3PDH Antibody | 30-1255 | 100 µl |
| Anti-PARP Monoclonal Antibody | 30-1253 | 50 µl |
| Anti-Phosphorylated Histone H2AX Polyclonal Antibody | 30-1251 | 20 µl |
| Anti-Phosphorylated Histone H2AX Polyclonal Antibody | 30-1252 | 100 µl |
| Apoptotic Cell DNA Laddering Kit | 30-1231 | 20 tests |
| CV Caspase 3 & 7 Substrate Kit | 30-1344 | 100 tests |
| FAM Caspase 3 & 7 Binding Kit | 30-1304 | 100 tests |
| FAM Caspase 8 Binding Kit | 30-1306 | 100 tests |
| FAM Caspase 9 Binding Kit | 30-1308 | 100 tests |
| FAM Poly-Caspase Binding Kit | 30-1302 | 100 tests |
| Mitochondrial Potential Assay Kit | 30-1233 | 100 tests |
| PARP Activity Assay Kit | 30-1235 | 50 tests |
| SR Caspase 3 & 7 Binding Kit | 30-1324 | 100 tests |
| SR Poly Caspase Binding Kit | 30-1322 | 100 tests |
| TdT Flow Cytometry Apoptosis Detection Kit | 30-1207 | 60 tests |
| TdT In Situ Apoptosis Detection Blue Kit | 30-1202 | 30 tests |
| TdT In Situ Apoptosis Detection DAB Kit | 30-1201 | 30 tests |
| TdT In Situ Replenisher Kit | 30-1205 | 30 tests |
| TdT Microplate Apoptosis Detection Kit | 30-1206 | 96 tests |
| **Stains and Reagents** | | |
| Erythrosin B Stain Solution | 30-2404 | 40 ml |
| Trypan Blue Stain Solution | 30-2402 | 40 ml |
| Dulbecco's Phosphate Buffered Saline (PBS) | 30-2200 | 500 ml |
| Molecular Grade Water | 60-2450 | 1 liter |
| **Clone Sets** | | |
| Human Apoptosis Clone Set | MBA-89 | |
| Mouse Apoptosis Clone Set | MBA-90 | |

ATCC also has apoptotic inducible cell lines, cell line model systems, and hybridomas, as well as a full line of products for cell culture. See our Web site at www.atcc.org for details.

Please refer to the Material Transfer Agreement and packing slip enclosed with the product for terms and conditions.

© 2004 ATCC. All rights reserved. ATCC is a registered trademark of the American Type Culture Collection.

30-FAM-M Revised March 2004

ATCC®

Manassas, VA USA

www.atcc.org