APO LOGIX™

Carboxyfluorescein Caspase Detection Kits

Apoptosis Detection and in situ labeling of active caspase in live cells.

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I. Introduction

The **APO LOGIX™** Carboxyfluorescein (FAM) caspase detection kits (**APO LOGIX™ FAM**) detect active caspases in living cells through the use of a carboxyfluorescein (FAM) labeled peptide fluoromethyl ketone (FMK) caspase inhibitor (FAM-Peptide-FMK). The FAM-peptide inhibitor irreversibly binds to active caspases. Caspase positive cells are distinguished from caspase negative cells with the aid of flow cytometry or fluorescence microscopy.

Background

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 cleavage of protein substrates, causing the disassembly of the cell (1).
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 kD) and two small (~10 kD) subunits to form two heterodimers which associate in a tetramer (2-4). As is common with other proteases, caspases are synthesized as precursors that undergo proteolytic maturation, either autocatalytically or in a cascade by enzymes with similar specificity (5).
Caspase enzymes specifically recognize a 4 amino acid sequence (on their substrate) which necessarily includes an aspartic acid residue. This residue is the target for the cleavage reaction, which occurs at the carbonyl end of the aspartic acid residue(6). Caspases can be detected via immunoprecipitation, immuno-blotting techniques using caspase specific antibodies, or by employing fluorogenic substrates which become fluorescent upon cleavage by the caspase. **APO LOGIX™ FAM** uses a novel approach to detect active caspases (7-9). The methodology is based on carboxyfluorescein labeled fluoromethyl ketone (FMK)-peptide inhibitors of caspases. These inhibitors are cell permeable and non-cytotoxic. Once inside the cell, the inhibitor binds covalently to the active caspase (10). Cells that contain bound inhibitor can be analyzed by flow cytometry or fluorescence microscopy.

II. Warnings and Precautions

1. For Research Use Only. Not for use in diagnostic procedures.
2. Wash Buffer contains sodium azide; harmful if swallowed or absorbed through the skin. Sodium azide can react with lead and copper-containing sink drains forming explosive compounds. When disposing of excess Wash Buffer, flush sink drain with large amounts of water.
3. Propidium iodide is a potential mutagen. Use of gloves, protective clothing, and eyewear, as well as safe laboratory protocol is strongly recommended.

III. Storage and Shelf Life

1. Store the kit at 2ºC to 8ºC until first use.
2. Reconstituted FAM labeled inhibitors (FAM-100-1, FAM-100-2, FAM-200-1, FAM-200-2) (150X) may be aliquoted and stored at -20ºC, protected from light and moisture (preferably in a desiccator) for up to 6 months from date of reconstitution.
3. Avoid multiple freeze-thaw cycles.

4. 30X Working Dilutions of FAM labeled inhibitors should be made fresh prior to use. Diluted inhibitors should not be stored.
5. Keep FAM labeled inhibitors protected from light at all times.
6. 10X Wash Buffer may form a precipitate when stored at 2ºC to 8ºC. Incubate at 37ºC for 30 minutes prior to use or until precipitate is no longer visible.
7. The stability of this product is guaranteed for one year from the date of purchase if stored and handled properly.

IV. Principles of the Procedure and Kit Content

A. FAM-VAD-FMK

1.CAT#: FAM100-1= 25 Tests**.**
FAM-VAD-FMK 1 vial. Stroage –20C.
10X Wash Buffer 1 bottle, 15ml. Storage 4C. Store 1X at 4C.
Fixative Solution 1 bottle, 6ml. Storage Room Temp.
Propidium Iodide 1 vial, 0.550ml. Storage 4C.

2. **CAT#: FAM100-2=** 100 Tests**.**
FAM-VAD-FMK 4 vials. Stroage –20C.
10X Wash Buffer 1 bottle, 60ml. Storage 4C. Store 1X at 4C.
Fixative Solution 1 bottle, 6ml. Storage Room Temp.
Propidium Iodide 1 vial, 0.550ml. Storage 4C.

APO LOGIX™ Carboxyfluorescein caspase detection kits, FAM-VAD-FMK for general caspase detection. **FAM-VAD-FMK** is a carboxyfluorescein (FAM) analog of benzyloxycarbonylvalylalanylaspartic acid fluoromethyl ketone (zVAD-FMK) that is a potent inhibitor of caspase activity. The FAM-VAD-FMK reagent provided in the kit enters the cell and irreversibly binds to activated caspases (caspase-1, -2, -3, - 4, -5, -6, -7, -8 and -9).

B. FAM-DEVD-FMK

3. **CAT#: FAM200-1=** 25 Tests**.**
FAM-DEVD-FMK 1 vial. Stroage –20C.
10X Wash Buffer 1 bottle, 15ml. Storage 4C. Store 1X at 4C.
Fixative Solution 1 bottle, 6ml. Storage Room Temp.
Propidium Iodide 1 vial, 0.550ml. Storage 4C.

4. CAT#: FAM200-2= 100 Tests
FAM-DEVD-FMK 4 vial. Stroage –20C.
10X Wash Buffer 1 bottle, 60ml. Storage 4C. Store 1X at 4C.
Fixative Solution 1 bottle, 6ml. Storage Room Temp.
Propidium Iodide 1 vial, 0.55ml, Storage 4C

APO LOGIX™ Carboxyfluorescein caspase detection kits. FAM-DEVD-FMK for caspase 3 detection. FAM-DEVD-FMK is a carboxyfluorescein analog of benzyloxycarbonylaspartylglutamylvalylaspartic acid fluoromethyl ketone (zDEVD-FMK) that is a potent inhibitor of caspase-3 and caspase-3 like caspases. FAM-DEVD-FMK enters the cell and irreversibly binds to activated caspase-3 > caspase-8 > caspase-7 > caspase-10 > caspase-6 in the order of decreasing binding affinity (11).

C. FAM-VEID-FMK

5. **CAT#: FAM500-1=** 25 Tests**.**
FAM-VEID-FMK 1 vial. Stroage –20C.
10X Wash Buffer 1 bottle, 15ml. Storage 4C. Store 1X at 4C.
Fixative Solution 1 bottle, 6ml. Storage Room Temp.
Propidium Iodide 1 vial, 0.550ml. Storage 4C.

6. **CAT#: FAM500-2=** 100 Tests**.**
FAM-VEID-FMK 4 vials. Stroage –20C.
10X Wash Buffer 1 bottle, 60ml. Storage 4C. Store 1X at 4C.
Fixative Solution 1 bottle, 6ml. Storage Room Temp.
Propidium Iodide 1 vial, 0.55ml, Storage 4C

APO LOGIX™ Carboxyfluorescein caspase detection kits. FAM-VEID-FMK for caspase 6 detection. FAM-VEID-FMK is a carboxyfluorescein analog of benzyloxycarbonylvalylglutamylisoleucylaspartic acid fluoromethyl ketone (zVEID-FMK) that is apotent inhibitor of caspase-6. FAM-VEID-FMK enters the cell and irreversibly binds to activated caspase-6 and with lower efficiency to caspase-3, -7, and -8 (12).

D. FAM-YVAD-FMK

7. **CAT#: FAM600-1=** 25 Tests**.**
FAM-YVAD-FMK 1 vial. Stroage –20C.
10X Wash Buffer 1 bottle, 15ml. Storage 4C. Store 1X at 4C.
Fixative Solution 1 bottle, 6ml. Storage Room Temp.
Propidium Iodide 1 vial, 0.550ml. Storage 4C.

8. **CAT#: FAM600-2=** 100 Tests**.**
FAM-YVAD-FMK 4 vial. Stroage –20C.
10X Wash Buffer 1 bottle, 60ml. Storage 4C. Store 1X at 4C.
Fixative Solution 1 bottle, 6ml. Storage Room Temp.
Propidium Iodide 1 vial, 0.55ml, Storage 4C

APO LOGIX™ Carboxyfluorescein caspase detection kits. FAM-YVAD-FMK for caspase 1 detection
FAM-YVAD-FMK is a carboxyfluorescein analog of benzyloxycarbonyltyrosylvalylalanylaspartic acid fluoromethyl ketone (zYVAD-FMK) that is a potent inhibitor of caspase-1. FAM-YVAD-FMK enters the cell and irreversibly binds to activated caspase-1 and with lower affinity to caspase-3, -4 and-6.

E. FAM-LEHD-FMK

9. **CAT#: FAM400-1=** 25 Tests**.**
FAM-LEHD-FMK 1 vial. Stroage –20C.
10X Wash Buffer 1 bottle, 15ml. Storage 4C. Store 1X at 4C.
Fixative Solution 1 bottle, 6ml. Storage Room Temp.
Propidium Iodide 1 vial, 0.550ml. Storage 4C.

10. **CAT#: FAM400-2=** 100 Tests**.**
FAM-LEDH-FMK 4 vial. Stroage –20C.
10X Wash Buffer 1 bottle, 60ml. Storage 4C. Store 1X at 4C.
Fixative Solution 1 bottle, 6ml. Storage Room Temp.
Propidium Iodide 1 vial, 0.550ml. Storage 4C.

APO LOGIX™ Carboxyfluorescein caspase detection kits. FAM-LEHD-FMK for caspase 9 detection FAM-LEHD-FMK is a carboxyfluorescein analog of benzyloxycarbonylleucylglutamylhistidylaspartic acid fluoromethyl ketone (zLEHD-FMK) that is a potent inhibitor of caspase-9. FAM-LEHD-FMK enters the cell and irreversiblybinds to activated caspase-9 as well as to caspase-4, and -5.

F. FAM-VDVAD-FMK

11. **CAT#: FAM700-1=** 25 Tests**.**
FAM-VDVAD-FMK 1 vial. Stroage –20C.
10X Wash Buffer 1 bottle, 15ml. Storage 4C. Store 1X at 4C.
Fixative Solution 1 bottle, 6ml. Storage Room Temp.
Propidium Iodide 1 vial, 0.550ml. Storage 4C.

12. **CAT#: FAM700-2=** 100 Tests**.**
FAM-VDVAD-FMK 4 vials. Stroage –20C.
10X Wash Buffer 1 bottle, 60ml. Storage 4C. Store 1X at 4C.
Fixative Solution 1 bottle, 6ml. Storage Room Temp.
Propidium Iodide 1 vial, 0.550ml. Storage 4C.

APO LOGIX™ Carboxyfluorescein caspase detection kits. FAM-VDVAD-FMK for caspase 2 detection FAM-VDVAD-FMK is a carboxyfluorescein analog of benzyloxycarbonylvalylaspartylvalylalanylaspartic acid fluoromethyl ketone (zVDVAD-FMK) that is a potent inhibitor of caspase-2. FAM-VDVAD-FMK enters the cell and irreversibly binds to activated caspase-2, -3 and -7.

G. FAM-AEVD-FMK

13. **CAT#: FAM800-1=** 25 Tests**.**
FAM-AEVD-FMK 1 vial. Stroage –20C.
10X Wash Buffer 1 bottle, 15ml. Storage 4C. Store 1X at 4C.
Fixative Solution 1 bottle, 6ml. Storage Room Temp.
Propidium Iodide 1 vial, 0.550ml. Storage 4C.

14. **CAT#: FAM800-2=** 100 Tests**.**
FAM-AEVD-FMK 4 vial. Stroage –20C.
10X Wash Buffer 1 bottle, 60ml. Storage 4C. Store 1X at 4C.
Fixative Solution 1 bottle, 6ml. Storage Room Temp.
Propidium Iodide 1 vial, 0.550ml. Storage 4C.

APO LOGIX™ Carboxyfluorescein caspase detection kits. FAM-AEVD-FMK for caspase 10 detection FAM-AEVD-FMK is a carboxyfluorescein analog of benzyloxycarbonylalanylglutamylvalylaspartic acid fluoromethyl ketone (zAEVD-FMK) that is a potent inhibitor of caspase-10. FAM-AEVD-FMK enters the cell and irreversibly binds to activated caspase-10 and caspase-8.

H. FAM-LETD-FMK

15. **CAT#: FAM300-1=** 25 Tests**.**
FAM-LETD-FMK 1 vial. Stroage –20C.
10X Wash Buffer 1 bottle, 15ml. Storage 4C. Store 1X at 4C.
Fixative Solution 1 bottle, 6ml. Storage Room Temp.
Propidium Iodide 1 vial, 0.550ml. Storage 4C.

16. **CAT#: FAM300-2=** 100 Tests**.**
FAM-LETD-FMK 4 vial. Stroage –20C.
10X Wash Buffer 1 bottle, 60ml. Storage 4C. Store 1X at 4C.
Fixative Solution 1 bottle, 6ml. Storage Room Temp.
Propidium Iodide 1 vial, 0.550ml. Storage 4C.

APO LOGIX™ Carboxyfluorescein caspase detection kits. FAM-LETD-FMK for caspase 8 detection FAM-LETD-FMK is a carboxyfluorescein analog of benzyloxycarbonylleucylglutamylthreonylaspartic acid fluoromethyl ketone (zLETD FMK) that is a potent inhibitor of caspase-8. FAM-LETD-FMK enters the cell and irreversibly binds to activated caspase-8 and with greatly reduced efficiency to caspase-1, - 6, and -10 (11).

V. Materials Required But Not Supplied

1. Solutions
a. Phosphate-Buffered Saline (PBS)
b. Dimethyl Sulfoxide (DMSO)
2. Equipment
a. Flow cytometer, equipped with a 15 mW, 488 nm argon excitation laser, with appropriate filters.
b. Fluorescence microscope with appropriate filters.

VI. Experimental Preparation and Setup

Working Dilution of FAM-Peptide-FMK
1. Reconstitute each lyophilized vial of FAM-Peptide-FMK (FAM-100-1, FAM-100-2, FAM-200-1, FAM-200-2, FAM-300-1, FAM300-2, FAM400-1, FAM400-2, FAM500-1, FAM500-2, FAM600-1, FAM600-2, FAM7001-, FAM700-2, FAM800-1, FAM800-2) with 50 µL DMSO resulting in a 150X concentration.
2. Mix contents at room temperature until completely dissolved. Aliquots may be made and stored frozen at -20ºC until ready to use.
3. Prior to use, make 30X Working Dilution. Dilute the 150X solution 1:5 in PBS, pH 7.4 (1 part 150X FAM-Peptide-FMK and 4 parts PBS). Mix the vial contents thoroughly to assure that a homogeneous solution is obtained.
4. Use diluted FAM-Peptide-FMK solution immediately for best results.
5. Protect from light at all times.

1X Working Dilution Wash Buffer from 10X Concentrate
1. Place 10X Wash Buffer in a 37ºC water bath for 30 minutes to redissolve precipitated protein and buffer salts.
2. Mix thoroughly and visually verify that the 10X Wash Buffer contents are completely in solution.
3. Dilute 10 mL 10X Wash Buffer in 90 mL deionized H_2O and mix thoroughly.

VII. Staining of Activated Cells With FAM-Peptide-FMK.

1. Induce cells and negative control samples at time points according to your specific protocol. Cells maybe cultured in a tissue culture plate or culture tube. Cells should be cultured in a volume of 300µL and at a maximum density of 10^6 cells/mL.

2. Cell Labeling

a. Add 10 µL 30X Working Dilution FAM-Peptide-FMK directly to the cell suspension. Slightly flicking the tissue culture plates or culture tubes will sufficiently mix the cells. Cells are then incubated for 1 hour at 37ºC under 5% CO2 (or for 1 hour according to your experimental conditions) protecting the tubes from light.

Each investigator should titrate the FAM-Peptide-FMK to accommodate their particular cell line or research conditions.

3. Washings

a. Add 2 mL of 1X Working Dilution Wash Buffer to the labeling mix. Gently mix. Spin down the cells at 400 x g for 5 minutes at room temperature.

b. Decant supernatant.

c. Gently vortex the cell pellet to disrupt cell to cell clumping.

d. Resuspend the cell pellet in 2 mL 1X Working Dilution Wash Buffer followed by a second centrifugation step.

e. Repeat steps b & c.

f. Resuspend the cell pellet in 400 µL of 1X Working Dilution Wash Buffer. Put samples on ice.

g. Analyze samples directly via flow cytometry, or fix and analyze cells within 24 hours. Note: *Do not fix cells that are to be analyzed by PI screening procedure (see Bicolor Fluorescent Staining protocol below).*

4. Cell Fixation

Add 40 µL 10X Fixative Solution to the 400 µL cell suspension (step f of previous paragraph), mix well, and keep at 2ºC to 8ºC in the dark for up to 24 hours until flow cytometry analysis can be completed.

Note: Do not fix cells that are to be labeled with PI.

VIII. Cell Analysis

1. Single color Flow cytometry

Flow cytometry analysis is done using 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. Eventsfalling to the right of the vertical cursor should be counted as caspase positive (+).

2. Bicolor Flow cytometry

Controls For Flow CytometryThree types of control samples are recommended for flow cytometry to aid insetting up electronic compensation and quadrant statistics. For bicolor experiments, these are: 1) cells stained with *FAM-Peptide-FMK* only; 2) cells stained with *PI* only; and 3) unstained cells. To distinguish between live cells and dead cells, either caspase negative (-) or caspase positive (+), a propidium iodide (PI) / fluorescein bicolor analysis is performed. PI stains necrotic, dead, and membrane compromised cells red. For bicolor analysis, 5 µL PI solution is added to the 400µL cell suspension of non-fixed cells prior to flow cytometry analysis. Measure fluorescein on the FL1 channel and red fluorescence (PI) on the FL3 channel. Generate a log FL1 (X-axis) versus log FL3 (Y-axis) dot plot. Put in the quadrant cursors. The 4 quadrant areas contain the following cell populations: (i) upper left quadrant, fluorescein negative PI positive cells; (ii) upper right quadrant, fluorescein positive PI positive cells; (iii) lower left quadrant,

fluorescein negative PI negative cells; (iv) lower right quadrant, fluorescein positive PI negative cells. The cell population in the lower right quadrant consists of living caspase positive (+) cells.

3. **Fluorescence microscope**

Place one drop of the cell suspension onto a microscope slide and cover with a cover slip. Observe cells under a fluorescence microscope using a bandpass filter (excitation 490 nm, emission 520 nm) to view green fluorescence. To view green fluorescence and PI simultaneously a long pass filter (excitation 490 nm, emission >520 nm) can be used. Caspase positive (+) cells appear green and PI positive cells stain red.

IX. Adherent Cells

a. Trypsinize cells, count, and seed cells onto a sterile glass cover slip in a 35 mm petri dish or onto chamber slides. Grow cells for 24 hours.
b. Induce cells at time points according to your specific protocol.
c. Add 10 µL 30X Working Dilution FAM-Peptide-FMK per 300 µL medium. Mix well.
Each investigator should titrate the FAM-Peptide-FMK to accommodate their particular cell line or research conditions.
d. Incubate cells for 1 hour at 37ºC under 5% CO_2. Protect from light.
e. Remove the medium and wash cells twice with 2 mL 1X Working Dilution Wash Buffer. For flow cytometry analysis proceed to step f. For fluorescence microscope proceed to step g.
f. If cell are going to be analyzed by flow cytometry, they should be trypsinized and washed once with 2mL of 1X Wash Buffer prior to flow analysis (above).
g. Observe cells under a fluorescence microscope using a bandpass filter (excitation 490 nm, emission 520 nm) to view green fluorescence. To view green fluorescence and PI simultaneously, a long pass filter (excitation 490 nm, emission >520 nm) can be used. Caspase positive (+) cells appear green and PI positive cells stain red.

X. APO LOGIX Fluorescence Plate Reader High Throughput Screening Protocol.

A. Adherent Cells

1. Seed cells in 96 well plates (black plates clear flat bottom or any other suitable black plate, tissue culture grade). Culture cells until confluent or until the desired cell density is reached. The total volume of media should be 300uL or less.

2. Induce your experimental protocol and incubate for the desired time point.

3. At the desired time point add 10uL of the 30X caspase detection reagent (FAM-Peptide-FMK, from: **VI Experimental Preparation and Setup step 3**) to your sample.

Note: 10uL of 30X caspase detection reagent is added per 300uL of sample. Sample volume maybe reduced e.g. 150uL of sample + 5uL of 30X caspase detection reagent, or any ratio of 3.33 uL 30X caspase detection reagent per 100uL of sample volume. Each investigator should titrate out the caspase detection reagent to optimize their results.

4.Incubate the samples for 1 to 2 hours according to your experimental conditions (i.e. CO2 incubator at 37C).

5.After the desired incubation time decant the media by gently inverting and flicking the plate or alternatively gently aspirating out the media from each well.
Note: If your experimental protocol results in a large number of cells detaching form the surface of the 96 well plate, we recommend spinning the plates in a centrifuge before flicking or aspirating the media.

6. Gently add 300 uL of the 1X Wash Buffer and repeat step 5. You may need to repeat step 5 twice. This will depend on the cell lines used and each investigator should determine this empirically.

7. After the final wash add 100 uL of Wash Buffer. Plates are ready for analysis. We recommend using a fluorescence plate reader that is set up for cell cultures i.e. bottom reading. Consult your plate reader manufacture regarding instrument set up. Read FAM with excitation at 488 nm and measure emission at 515 - 530 nm. Read Sulforhodamine with Excitation at 535 - 560 nm and measure emission at 590 - 600 nm.

8. Cells maybe fixed at this time point prior to analysis. Add 10uL of our Fixative solution to each sample. After fixation samples should be analyzed within 24 hours.
Note: Fixation may result in a higher background signal. Store samples after fixation in the 96 well plate (with lid) in a plastic bag (with a wet paper towel) at 4C. This prevents evaporation of samples.

B. Suspension Cells

1.Proceed as above to step 5.

2. Before removing the media, gently spin the 96 well plates in a centrifuge to pellet the cells.

3.After centrifugation gently aspirate each well to remove the media.

4. Vortex the plate to remove cell-to-cell clumping. Add 300 uL of the Wash Buffer and centrifuge the 96 well plates.

5. Repeat step 3.

6. Add 100 uL of Wash Buffer. Cells are ready for analysis. Proceed to step 7 above (adherent cells).

XI. Frozen Section Protocol

1. Do not use fixatives on tissue samples as they will inactivate the caspase enzyme. Sections can be snap frozen (avoid any organic solvents).
2. Make frozen sections of tissue. Cut sections between 5 to 20 cell layers thick.
3. Mount sections on slides.
4. Flood sections with PBS+ FBS (2-5%).
5. Dilute the caspase reagent to 1X in media (use media that you would typically use in the culture conditions of your cells or sections e.g. RPMI + 10% FCS). This 1X dilution can be accomplished by the following: reconstitute the caspase detection reagent, in DMSO, according to the protocol (Section VI). Then dilute this reconstituted reagent 1:30 in media (as stated above). This is your 1X working solution. Remove the PBS + FBS from the sections and flood the tissue sections with this 1X solution.
6. Incubate the sections, for 1-2 hours in a humidified chamber at 37C + 10%CO2, or any other suitable condition for your cell/ tissue section culture conditions.
7. After the 1-2 hour incubation period, wash the sections in PBS. This wash step can be accomplished by: flooding the slides with PBS and incubating for 10-15 minutes. This incubation step will allow the excess caspase detection reagent to be washed out of the sections. Repeat this step 2 times.
8. The sections can be mounted and/or fixed in a suitable mounting medium and viewed under a fluorescent microscope. Avoid alcohol fixatives like methanol. Instead use formalin based fixatives.

9. Caspase positive cells can be visualized by excitation at 488nm and measuring fluorescence at 515-530nm (FITC filter).

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