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APO LOGIX™

Sulforhodamine Caspase Detection Kits

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

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Introduction

The **APO LOGIX™** Sulforhodamine (SR) caspase labeling kits (**APO LOGIX™ SR**) detects active caspases in living cells through the use of a sulforhodamine (SR) labeled peptide fluoromethyl ketone (FMK) caspase inhibitor (SR-Peptide-FMK). The SR-peptide inhibitor irreversibly binds to active caspases. Caspase positive cells are distinguished from caspase negative cells with the aid of fluorescence microscopy or fluorescence plate reader.

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™ SR** uses a novel approach to detect active caspases (7-9). The methodology is based on sulforhodamine 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 fluorescence microscopy or fluorescence plate reader.

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.

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III. Storage and Shelf Life

1. Store the kit at 2ºC to 8ºC until first use.
2. Reconstituted SR labeled inhibitors (SR-100-1, SR-100-2, SR-200-1, SR-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 SR labeled inhibitors should be made fresh prior to use. Diluted inhibitors should not be stored.
5. Keep SR 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. SR-VAD-FMK

1.CAT#: SR100-1= 25 Tests**.**
SR-VAD-FMK 1 vial. Storage –20C.
10X Wash Buffer 1 bottle, 15ml. Storage 4C. Store 1X at 4C.
Fixative Solution 1 bottle, 1ml. Storage Room Temp.

2. **CAT#: SR100-2=** 100 Tests**.**
SR-VAD-FMK 4 vial. Storage –20C.
10X Wash Buffer 1 bottle, 60ml. Storage 4C. Store 1X at 4C.
Fixative Solution 1 bottle, 6ml. Storage Room Temp.

APO LOGIX™ Sulforhodamine caspase detection kits, SR-VAD-FMK for general caspase detection. **SR-VAD-FMK** is a sulforhodamine (SR) analog of benzyloxycarbonylvalylalanylaspartic acid fluoromethyl ketone (zVAD-FMK) that is a potent inhibitor of caspase activity. The SR-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. SR-DEVD-FMK

3. **CAT#: SR200-1=** 25 Tests**.**
SR-DEVD-FMK 1 vial. Storage –20C.
10X Wash Buffer 1 bottle, 15ml. Storage 4C. Store 1X at 4C.
Fixative Solution 1 bottle, 1ml. Storage Room Temp.

4. CAT#: SR200-2= 100 Tests
SR-DEVD-FMK 4 vial. Storage –20C.
10X Wash Buffer 1 bottle, 60ml. Storage 4C. Store 1X at 4C.
Fixative Solution 1 bottle, 6ml. Storage Room Temp.

APO LOGIX™ Sulforhodamine caspase detection kits. SR-DEVD-FMK for caspase 3 detection. SR-DEVD-FMK is a sulforhodamine analog of benzyloxycarbonylaspartylglutamylvalylaspartic acid fluoromethyl ketone (zDEVD-FMK) that is a potent inhibitor of caspase-3 and caspase-3 like caspases. SR-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).

V. Materials Required But Not Supplied

1. Solutions

a. Phosphate-Buffered Saline (PBS)

b. Dimethyl Sulfoxide (DMSO)

2. Equipment

a. Fluorescence microscope with appropriate filters. Excitation filter 550nm and emission filter range 590 – 600 nm.

b. Fluorescence plate reader. Excitation filter 550nm and emission filter range 590 – 600 nm.

c. Black flat bottom 96 well ELISA plates.

d. Slides and cover slipes.

VI. Experimental Preparation and Setup

Working Dilution of SR-Peptide-FMK

1. Reconstitute lyophilized SR-Peptide-FMK (SR-100-1, SR-100-2, SR-200-1, SR-200-2) 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 SR-Peptide-FMK and 4 parts PBS). Mix the vial contents thoroughly to assure that a homogeneous solution is obtained.
4. **Use diluted SR-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 SR-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 SR-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% CO_2 (or according to your experimental conditions) protecting the tubes from light.

Each investigator should titrate the SR-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 100-150 µL of 1X Working Dilution Wash Buffer. Put samples on ice.
g. Analyze samples directly via fluorescence microscope or fluorescence plate reader, or fix and analyze cells within 24 hours.

4. Cell Fixation

Add 10-15 µL 10X Fixative Solution to the 100-150 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 analysis can be completed.

VIII. Cell Analysis

1. Fluorescence Plate Reader

a. Determine the concentration of cells from step f above. This can be accomplished by counting cells using a hemocytometer or any other cell counting device.
b. After counting, compare the density of cells in each sample. Some samples may have a lower cell density than other samples. This cell loss is expected in samples that have undergone apoptosis. Adjust the cell concentration in all samples so that they match the non-induced (control) sample. This adjustment step may not be need if your cell treatment does not result is a significant cell loss.
Note: Each investigator should determine the optimal cell concentration so that the difference between the induced and non-induced signal is maximum.
d. Place 100µL of the cell suspension in black 96 well ELISA plates
e. Measure fluorescence: excitation: 550nm emission: 590-600nm. Filter pairs can be selected that closely matches these settings.

2. Fluorescence microscope

Place one drop of the cell suspension (from step VII 3f.) onto a microscope slide and cover with a coverslip. Observe cells under a fluorescence microscope using a bandpass filter (excitation 550nm nm, emission 590-600 nm) to view red fluorescence.

IX. Adherent Cells

Note; Some adherent cell lines maybe incompatible with the SR-Peptide-FMK active caspase detection reagents. This incompatibility is due to the Sulforhodamine binding non-specifically to the extra cellular matrix secrete by some of these adherent cell lines.
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 SR-Peptide-FMK per 300 µL medium. Mix well.
Each investigator should titrate the SR-Peptide-FMK to accommodate their particular cell line or research conditions. Some adherent cell lines will produce a high background signal.
d. Incubate cells for 1 hour at 37ºC under 5% CO_2 (or according to your experimental conditions). Protect from light.
e. Remove the medium and wash cells twice with 2 mL 1X Working Dilution Wash Buffer. Cell are ready for analysis (Part V111 above).

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

Note; Some adherent cell lines maybe incompatible with the SR-Peptide-FMK active caspase detection reagents. This incompatibility is due to the Sulforhodamine binding non-specifically to the extra cellular matrix secrete by some of these adherent cell lines.

A. Adherent Cells

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

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 (SR-Peptide-FMK) 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 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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