Version 1 Last updated 24 January 2020

ab270770 Caspase Assay Kit (orange, VAD-FMK)

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For the detection of apoptosis in cultured cells.

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Table of Contents

1. Overview 1
2. Materials Supplied and Storage 2
3. Materials Required, Not Supplied 3
4. General guidelines, precautions, and troubleshooting 4
5. Experimental Preparation 4
6. Controls 5
7. Apoptosis Induction 6
8. Preparation of SR-VAD-FMK 6
9. Preparation of 1X Apoptosis Buffer 7
10. Fixative 7
11. Hoechst 33342 8
12. Staining Protocol for Suspension Cells 9
13. Staining Protocol for Adherent Cells 10
14. Microscopy Analysis 13
15. Fluorescent Plate Reader Analysis 14
16. Flow Cytometry Analysis 14
17. Notes 17

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1. Overview

Caspases play important roles in apoptosis and inflammation. Caspase Assay Kit (orange, VAD-FMK) (ab270770) is used by researchers seeking to quantitate apoptosis via caspase activity in cultured cells and tissues.

The SR-VAD-FMK reagent enters each cell and irreversibly binds to activated caspases. Because the SR-VAD-FMK reagent becomes covalently coupled to the active enzymes, it is retained within the cell, while any unbound SR-VAD-FMK reagent diffuses out of the cell and is washed away. The remaining red fluorescent signal is a direct measure of the active caspase enzyme activity present in the cell at the time the reagent was added. Cells that contain the bound can be analyzed by a fluorescence plate reader, fluorescence microscopy or flow cytometry. Cells labeled with the reagent may be read immediately or preserved for 16 hours using the fixative included in the kit. Unfixed samples may also be analyzed with Hoechst 33342 to detect changes in nuclear morphology.

2. Materials Supplied and Storage

Store kit at +4°C immediately on receipt.

Δ Note: Once reconstituted with DMSO, use SR-VAD-FMK immediately, or store at ≤-20°C for 6 months protected from light and thawed no more than twice during that time.

Δ Note: Do not freeze Acridine Orange or Hoechst 33342.

Item	Quantity		Storage temperature
	25 tests	100 tests	
Fixative	6 mL	6 mL	+4°C
Hoechst 33342, 200 µg/mL	1 mL	1 mL	+4°C
10X Apoptosis Wash Buffer	15 mL	60 mL	+4°C
Caspase inhibitor reagent SR-VAD-FMK	25 tests	100 tests	+4°C

3. Materials Required, Not Supplied

These materials are not included in the kit, but will be required to successfully perform this assay:

- DMSO, 50 µL per vial to reconstitute SR-VAD-FMK
- DiH_2O, 135 -540 mL to dilute 10X Apoptosis wash buffer
- Phosphate buffered saline (PBS) pH 7.4, 100 mL
- FBS and/or BSA to add to buffer when handling cells
- Cultured cells treated with the experimental conditions ready for staining
- Reagents to induce apoptosis and create controls, such as staurosporine or camptothecin
- Centrifuge at 200 *x g*
- Hemocytometer
- 15 mL polypropylene centrifuge tubes (1 per sample)
- Black 96-well microtiter plate, flat bottom, non-treated, non-sterile. If using a bottom reading instrument, use a plate with black walls and a clear bottom. If culturing cells in the plate, use a sterile black tissue culture plate.
- Fluorescence microscope
- Fluorescence plate reader
- Flow cytometer

4. General guidelines, precautions, and troubleshooting

Please observe safe laboratory practice and consult the safety datasheet.

For general guidelines, precautions, limitations on the use of our assay kits and general assay troubleshooting tips, particularly for first time users, please consult our guide:
www.abcam.com/assaykitguidelines

For typical data produced using the assay, please see the assay kit datasheet on our website.

5. Experimental Preparation

5.1 Staining apoptotic cells with SR-VAD-FMK can be completed within a few hours. However, SR-VAD-FMK 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 be allotted for the experimental treatment or apoptosis induction process which typically requires a 2-6 hr incubation at 37°C based on the cell line and concentration.

5.2 Create cell populations, such as:
a. Cells that were exposed to the experimental condition or treatment
b. A placebo population of cells that received a blank treatment instead of the experimental treatment

5.3 As SR-VAD-FMK detects the presence of catalytically active forms of caspase enzymes, plan the experiment so that SR-VAD-FMK will be diluted and administered at the time when caspases are expected to be activated in the cells

5.4 The recommended volume of 30-60X SR-VAD-FMK is 5-10 µL per 300 µL of cells at 3-5 x 10^5 cells/mL, but the amount may vary based on the experimental conditions and the instrument used for analysis. Each investigator should adjust the amount of SR-VAD-FMK to accommodate the particular cell line and research conditions.

5.5 Culture cells to a density optimal for the specific experiment or apoptosis induction protocol. Cell density should not exceed

10^6 cells/mL. Cells cultivated in excess of this concentration may begin to naturally enter apoptosis.

5.6 An initial experiment may be necessary to determine when and how much SR-VAD-FMK to use as the resulting positive signal is a direct measurement of caspase activity occurring during the incubation period.

6. Controls

Create experimental samples and control cell populations:

- Treated experimental population(s): cells exposed to the experimental condition(s).
- Negative control: non-treated cells grown in a normal culture environment.
- Positive control: cells induced to undergo apoptosis using a known caspase induction protocol.

Δ Note: The induced positive cell population and negative control cell population tubes should come from a common pool of cells and contain similar quantities of cells.

Create negative controls by culturing an equal volume of noninduced cells for every labeling condition. For example, if labeling with SR-VAD-FMK and Hoechst 33342 (which is optional), make eight populations:

Control #	
1 and 2	Unlabeled: induced and non-induced
3 and 4	SR-VAD-FMK-labeled: induced and non-induced
5 and 6	SR-VAD-FMK-labeled and Hoechst-labeled: induced and non-induced
7 and 8	Hoechst-labeled: induced and non-induced

7. Apoptosis Induction

Prior to commencing the experiment, determine a reproducible method for obtaining a positive control by triggering caspase activity. This process varies significantly with each cell line.

For example, apoptosis may be induced with 2-4 µg/mL camptothecin or 1-2 µM staurosporine for >4 hours.

8. Preparation of SR-VAD-FMK

- SR-VAD-FMK is supplied as a lyophilized powder that may be slightly visible as an iridescent sheen inside the vial.
- Protect from light and use gloves when handling. Because the 30-60X SR-VAD-FMK solution must be used immediately, prepare it just before staining.

8.1 Reconstitute each vial of FLICA with 50 µL DMSO to form the 150-300X stock concentrate. The stock solution should be pink to red in color. Once reconstituted, it may be stored at ≤-20°C for 6 months protected from light and thawed no more than twice during that time.

8.2 Immediately prior to addition to the samples and controls, dilute SR-VAD-FMK 1:5 by adding 200 µL PBS to each vial to form the 30-60X SR-VAD-FMK solution. Use 30-60X SR-VAD-FMK within 30 mins of dilution into aqueous buffers.

9. Preparation of 1X Apoptosis Buffer

10X Apoptosis Wash Buffer is an isotonic solution used to wash cells following exposure to SR-VAD-FMK. It contains mammalian proteins to stabilize cells stained with SR-VAD-FMK and sodium azide to retard bacterial growth (1X Apoptosis Wash Buffer contains 0.01% w/v sodium azide). Cell culture media containing FBS and other additives may be used instead of Apoptosis Wash Buffer.

- 10X Apoptosis Wash Buffer may form precipitates during cold storage. If this happens, gently warm it until all crystals have dissolved. Do not boil.
- Dilute 10X Apoptosis Wash Buffer 1/10 in diH_2O. For example, add 15 mL 10X Apoptosis Wash Buffer to 135 mL diH_2O for a total of 150 mL.

Δ Note: 1X Apoptosis Wash Buffer may be stored at 4°C and used within 1 week or frozen and used within 6 months.

10. Fixative

The Fixative is a formaldehyde solution designed to crosslink and aggregate intracellular components. If the stained cell populations cannot be evaluated immediately after labeling with SR-VAD-FMK, add Fixative at a ratio of 1/5-1/10. For example, to use Fixative at 1/10, add 100 µL Fixative to 900 µL cells.

Never add Fixative until all the staining and final wash steps have been completed. Fixed cells may be stored on ice or at 4°C for up to 16 hours, protected from light.

Fixative will not interfere with the sulforhodamine (SR) label.

Δ Note: Do not use absolute ethanol- or methanol-based fixatives, as they may inactivate the SR-VAD-FMK label.

11. Hoechst 33342

Hoechst 33342 is a cell-permeant nuclear stain that emits blue fluorescence when bound to double stranded DNA. It is used to stain the nuclei of living or fixed cells, to distinguish condensed pyknotic nuclei in apoptotic cells, and for cell cycle studies.

Hoechst 33342 is provided ready-to-use at 200 µg/mL. It can be used with Magic Red to label nuclei.

When bound to nucleic acids, it has a maximum absorbance at 350 nm and a maximum emission at 480 nm. It is revealed under a microscope using a UV-filter with excitation at 365 nm and emission at 480 nm.

- Hoechst 33342 contains a low concentration of Bis benzimide H 33342 trihydrochloride (CAS 23491-52-3) which is below the threshold for reporting. Hoechst is a suspected mutagen at high concentrations. Prolonged skin contact may cause redness and irritation. Because of the small quantity of product, the health hazard is small. See SDS for further information.

12. Staining Protocol for Suspension Cells

12.1 Prepare experimental and control cell populations. Ideally, cell concentration should be 3-5 x 10^5 cells/mL. The concentration should not exceed 10^6 cells/mL, as cells cultivated in excess of this concentration may begin to naturally enter apoptosis.

12.2 Just prior to staining with SR-VAD-FMK, cells may need to be concentrated to 2-5 x 10^6 cells/mL as both microscopy and plate reader analysis methods (Sections 14 and 15) require high cell concentrations. Start with a larger volume of cells at 3-5 x 10^5 cells/mL (which is a typical density for cell culture) and then concentrate cells and resuspend to 300 µL per sample when ready for SR-VAD-FMK staining.

12.3 Expose cells to the experimental and control conditions. Include the following controls:

a. Positive control cells induced to undergo apoptosis.

b. Negative control cells not induced to undergo apoptosis.

12.4 If analyzing with a fluorescence microscope or plate reader, concentrate cells to 2-5 x 10^6 cells/mL just prior to SR-VAD-FMK staining. Fluorescence microscopy requires an excess of 2 x 10^6 cells/mL to obtain 5-20 cells per image field. Flow cytometry can efficiently analyze samples at 3-5 x 10^5 cells/mL.

12.5 Transfer 290-295 µL cells into fresh tubes.

12.6 Add 5-10 µL 30-60X SR-VAD-FMK solution, forming a final volume of 300 µL. If different cell volumes were used, add SR-VAD-FMK at a ratio of 1/30-1/60. Mix by gently flicking the tubes. The amount of SR-VAD-FMK should be optimized for each cell line and experimental condition. However, 1/60 is generally sufficient for flow cytometry applications, and 1/30 is recommended for fluorescence microscopy or plate reader applications.

12.7 Incubate cells at 37°C protected from light. The incubation period may range from 30 mins to several hours and should be optimized for each cell line and experimental condition. As cells may settle on the bottom of the tubes, gently resuspend by swirling cells every 10-20 mins to ensure an even distribution of SR-VAD-FMK.

12.8 If cells are to be analyzed with a microscope, cells may be dually stained with Hoechst. Do not stain with Hoechst if using a plate reader (Section 15) or flow cytometer (Section 16).

Add Hoechst at 0.5% v/v and incubate 5 mins at 37°C. For example, if the cell suspension is at 300 µL, add 1.5 µL Hoechst.

12.9 Add 2 mL 1X Apoptosis Wash Buffer and gently mix.

12.10 Centrifuge at 200 *x g* for 5-10 mins at room temperature (RT).

12.11 Carefully remove and discard supernatants. Gently vortex pellets to disrupt clumping. Resuspend in 1 mL 1X Apoptosis Wash Buffer and gently mix.

12.12 Centrifuge cells at 200 *x g* for 5-10 mins at RT.

12.13 Carefully remove and discard supernatants. If analyzing by fluorescence microscopy or fluorescence plate reader, repeat wash process a third time. If using a flow cytometer, two wash steps are generally sufficient.

12.14 Gently vortex pellets to disrupt clumping.

- If analyzing with a fluorescence microscope, go to Section 14.
- If using a fluorescence plate reader, go to Section 15.
- If using a flow cytometer, go to Section 16.

13. Staining Protocol for Adherent Cells

Adherent cells need to be handled carefully to avoid the loss of any cells that round up and come off the culture surface. In microscopy or plate reader applications where trypsinization is not required, adherent cells can be stained and washed directly on the chamber slide, well, or culture surface. To avoid losing cells that are no longer adherent during washing, spin down all overlay media and wash buffer and recombine washed cell pellets with the adherent samples prior to analysis.

Cells may be trypsinized to create suspensions, which may be labeled with SD-VAD-FMK before or after trypsinization. Avoid trypsinizing cells prior to labeling with a live/dead DNA dye, like 7-AAD. Cell membranes exposed to trypsin could be transiently permeable to live/dead dyes for a variable time depending upon the cell line.

13.1 Culture cells in T25 flasks, culture dishes, or chamber slides and expose to the experimental or control conditions.

13.2 If staining cells while adherent, go to Step 13.4. If suspension cells are required for the final analysis:

a. Remove overlay media. Spin to pellet any loose cells.

b. Trypsinize adherent cells. Alternatively, SR-VAD-FMK labeling can be performed first, followed by washing and trypsinization steps.

c. Neutralize with trypsin inhibitor, as found in cell culture media with 20% FBS.

d. Add 2-5 mL media. Expose cells to the experimental conditions and create positive and negative controls (Sections 6 and 7).

13.3 Prepare trypsinized cells for staining:

a. Centrifuge at 200 x *g* for 5-10 mins at RT.

b. Remove all but ~100 µL supernatant.

c. Resuspend cells in 300-500 µL cell culture media containing 10-20% FBS.

d. If necessary, count cells and adjust the concentration and volume of cell suspension to fit the experiment.

13.4 Add the SR-VAD-FMK working solution to samples at a v/v ratio of 1/30-1/60 and mix the cell suspension to disperse the SR-VAD-FMK reagent. If staining adherent cells, add SR-VAD-FMK directly to the overlay media. The concentration of SR-VAD-FMK should be optimized for each cell line, experimental condition, and method of analysis:

- Flow cytometry analysis may provide the sensitivity to detect SR-FLICA when used at 1/60.
- For analysis by fluorescence microscopy, fluorescence plate reader, or applications where a higher staining concentration is needed, it is recommended that the SR-VAD-FMK working solution be used at 1/30. For example, if staining cells at 1/30, add 10 µL SR-VAD-FMK working solution to 290 µL cells, forming a final volume of 300 µL.

13.5 Incubate 30-60 mins at 37°C, mixing gently every 10-20 mins to disperse the reagent.

13.6 Wash cells. For trypsinized adherent (suspension) cells, go to Step 13.7. For adherent cells, go to Step 13.8.

13.7 Wash trypsinized adherent (suspension) cells:
a Add 2 mL 1X Apoptosis Wash Buffer and gently mix.
b. Centrifuge at *200 x g* for 5-10 mins at RT.
c. Carefully aspirate supernatant.
d. Resuspend samples in 1-2 mL wash buffer and gently mix.
e. Centrifuge a second time at 200 x *g* for 5-10 mins at RT.
f. Carefully aspirate supernatant.
g. For flow cytometry analysis, two wash steps are generally sufficient. For microscopy or fluorescence plate reader analysis, repeat wash procedure a third time (resuspend samples, gently pellet by centrifugation, and carefully remove supernatants). Go to Step 13.9.

13.8 Wash adherent cells. To avoid losing cells that are no longer adherent during washing, spin down all overlay media and wash buffer and recombine washed cell pellets with the adherent samples prior to analysis.
a. Carefully remove overlay media containing SR-FLICA and replace with 1X Apoptosis Wash Buffer.
b. Incubate 10 mins at 37°C to allow any unbound SR-FLICA to diffuse out of cells.
c. Carefully remove and replace wash buffer with fresh wash buffer and incubate another 10 mins at 37°C.
d. Gently remove overlay buffer and replace for a third wash step. Incubate 10 mins at 37°C.
e. Gently remove overlay buffer. Go to Step 13.9

13.9 If desired and using a microscope, cells may be counter-stained with ancillary dyes like the nuclear stain Hoechst 33342 or other compatible fluorescent dye.

- Resuspend cells or replace overlay buffer, add Hoechst 33342 at 0.5% v/v, and incubate 5 mins at 37°C. For example, if the cell suspension or overlay volume is at 1 mL, add 5 µL Hoechst 33342.
- Live/dead cell stains should not be used after trypsinization. Cell membranes exposed to trypsin could be transiently permeable to live/dead dyes for a variable time depending upon the cell line. To identify dead cells with a live/dead stain, perform the SR-VAF-FMK and ancillary dye staining and wash steps prior to using trypsin. To avoid false positives, include another wash step to remove excess live/dead dye prior to trypsinization.

- If using any compatible ancillary dyes, follow the manufacturer's specific instructions for staining samples.

13.10 Read cells within 4 hours or fix.

- If analyzing with a fluorescence microscope, go to Section 14.
- If using a fluorescence plate reader, go to Section 15
- If using a flow cytometer, go to Section 16.

14. Microscopy Analysis

Follow Section 12 or Section 13.

14.1 Resuspend cells or replace overlay media in 300-500 µL 1X Apoptosis Wash Buffer and place on ice. At this point, the cells may be stained with other dyes, fixed for future viewing (Step 2), or observed immediately (Step 14.3). Expose cells to the experimental conditions and create positive and negative controls (Section 6).

14.2 If not viewing immediately, cells may be fixed for viewing up to 16 hours later.

a. Add Fixative at a v/v ratio of 1/5-1/10.
b. Incubate 15 mins at RT in the dark.
c. Place cells on a microscope slide and allow to dry.
d. Briefly rinse cells with PBS.
e. Cover with mounting media and coverslip.
f. Store slides at 4°C for up to 16 hrs.

14.3 To view cells immediately, place 1 drop of cell suspension onto a microscope slide and cover with a coverslip.

14.4 Observe cells with a fluorescence microscope using a bandpass filter (excitation 550 nm, emission >580 nm) to view red fluorescence. Cells bearing active caspase enzymes covalently coupled to SR-VAD-FMK appear red. Hoechst 33342 can be seen using a UV-filter with excitation at 365 nm and emission at 480 nm.

15. Fluorescent Plate Reader Analysis

Follow Section 12 or Section 13, but omit optional nuclear staining with Hoechst 33342.

15.1 Determine the concentration and compare the cell density of each sample. The non-induced population may have more cells than the induced population, as some apoptotic cells in the induced samples may be lost during the wash steps. Adjust the volume of the cell suspensions to equalize the cell density. When ready to read, cells should be >3 x 10^6 cells/mL. Adherent cells should be cultured to 80-90% confluency. Δ No Δ Note: Some cell lines will not tolerate confluency levels >60%; adjust as necessary for the particular cells being used.

15.2 If using suspension cells, pipette 100 µL stained and washed cells per well into a black microtiter plate. Do not use clear plates. If using a bottom-reading instrument, use a plate with black walls and a clear bottom. Analyze at least 2 aliquots per sample. Avoid bubbles.

15.3 Perform an endpoint read. Set the excitation wavelength at 550 nm and the emission wavelength at 595 nm; if possible, use a 570 nm cut-off filter. SR-VAD-FMK is excited at 550-580 nm (excitation peak is 570 nm) and the emission optima is at 590-600 nm (emission peak is 590 nm).

16. Flow Cytometry Analysis

Flow cytometry with SR-VAD-FMK is best performed using a laser that can efficiently excite the fluorophore, such as a yellow-green 561 nm laser. A green 532 nm laser with a 610/20 filter pairing has also been shown to be effective. If yellow-green or green laser options are unavailable, a standard 488 nm blue excitation laser paired with an appropriate emission filter set, such as 585/40 (often FL-2/PE channel), can be used.

When using SR-FLICA in combination with other fluorochromes for bi-color analyses, compensation may be necessary. Correcting for fluorescence spill over is especially important when using a sub-optimal excitation laser to excite SR-VAD-FMK in combination with a fluorochrome with significant spectral overlap with the SR-VAD-FMK detector. For instance, some green dyes, like FITC or Green Live/Dead Stain, can spill

over from FL-1 into FL-2. If using such a dye to co-label SR-FLICA stained cells, consider reading the SR-VAD-FMK reagent in an alternate channel with reduced spectral overlap, such as 670LP (often FL-3/PerCP), or similar.

Because of its orangy-red profile, SR-VAD-FMK can be measured in flow cytometry with the red live/dead stain, 7-AAD, to detect necrosis concurrently (Figure 7). Careful compensation and gating of SR-VAD-FMK and 7-AAD fluorophores (using the FL-2 and FL-3 channels, respectively) will distinguish the red 7-AAD live/dead fluorescence signal from the orangy-red SR-VAD-FMK caspase positive signal within each sample. This will reveal four populations of cells:

- Live unstained cells display only normal background or autofluorescence (revealed in lower left quadrant).
- Early apoptotic cells fluoresce orangy-red with SR-VAD-FMK (revealed in lower right quadrant).
- Late apoptotic cells are dually stained SR-VAD-FMK and 7-AAD: they fluoresce orangy-red (they have active caspases) and red (the cell membrane has permeabilized). This population will be revealed in the upper right quadrant.
- Necrotic cells fluoresce red with 7-AAD (revealed in the upper left quadrant).

Follow Section 12 or Section 13, but omit optional nuclear staining with Hoechst 33342.

16.1 Resuspend cells in 300 µL 1X Apoptosis Wash Buffer and place on ice.

16.2 Cells may be fixed for analysis up to 16 hrs later. Add Fixative at a v/v ratio of 1/5-1/10. Store samples at 4°C and protected from light.

16.3 Run the unstained control. If possible, adjust voltages to place the unstained sample in the first decade of the FL dot plots.

16.4 For single-color analysis, a 488 nm blue argon laser or comparable can be used with the emission filter pairing that best approximates 585/40 (often FL-2/PE channel). If other options are available, use the laser/ filter pairing that most closely resembles the excitation and emission optima of sulforhodamine B, i.e. 570 nm and 590-600 nm, respectively.

16.5 Generate a histogram with the log FL-2 on the X-axis versus the number of cells on the Y-axis. Casapse negative (SR-VAD-FMK -) cells will fall within the lower log fluorescence output decades of the FL-2 X-axis, whereas caspase-positive (SR-VAD-FMK +) cells will appear as a shoulder or as a separate peak on the right side of the negative peak histogram.

16.6 For bi-color analyses, run each single color control. Adjust compensation to remove spectral overlap from interfering FL channels. When the data have been correctly compensated, the median fluorescence intensity (MFI) values in non-primary detectors of any given single-stained control sample should be the same as an unstained control sample (e.g. an SR-VAD-FMK stained sample being read in FL-2 should have the same MFI in FL-3 as an unstained sample). For example, if reading SR-VAD-FMK in FL-2 and 7-AAD in FL-3:

a. Subtract a percentage of the fluorescence in the SR-VAD-FMK channel from the fluorescent channel used for 7-AAD (e.g. FL-3 - %FL-2).

b. Subtract a percentage of the fluorescence in the channel used for 7-AAD from the fluorescence in the SR-VAD-FMK channel (e.g. FL-2 - %FL-3).

16.7 Run the bi-color experimental samples and analyze.

17. Notes

Technical Support

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