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ab270783
Poly Caspase Staining Kit (Far Red)

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Poly Caspase Staining Kit (Far Red) datasheet:

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For the detection of caspase activity in cultured cells.

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1. Overview

Caspases play important roles in apoptosis and inflammation. Poly Caspase Staining Kit (Far Red) is used by researchers seeking to quantitate apoptosis via caspase activity in cultured cells. The fluorescent 660-VAD-FMK poly caspase inhibitor reagent allows researchers to assess caspase activation.

The fluorescent reagent 660-VAD-FMK enters each cell and irreversibly binds to activated Caspases. Because the 660-VAD-FMK reagent becomes covalently coupled to the active enzymes, it is retained within the cell, while any unbound 660-VAD-FMK reagent diffuses out of the cell and is washed away. The remaining far 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 660-VAD-FMK can be analyzed by fluorescence microscopy or flow cytometry. Cells labeled with the 660-VAD-FMK reagent may be read immediately or preserved for 16 hours using the fixative. Unfixed samples may also be analyzed with Hoechst stain to detect changes in nuclear morphology.

The target for this kit is poly Caspases.

NOTE: The number of tests per kit depends on the application being tested. This kit provides 50 tests for flow and 25 for imaging.

The 660-VAD-FMK poly caspase inhibitor reagent contains the preferred binding sequence for all caspases, Val-Ala-Asp (VAD). This preferred poly caspase tri-peptide binding sequence is labeled at the amino terminus end with a far-red fluorescent 660 dye and linked at the carboxyl end to a fluoromethyl ketone (FMK) reactive entity. The resulting cell permeant, fluorescent molecule, 660-VAD-FMK, optimally excites at 660 nm and emits between 685-690 nm. A conventional red HeNe laser with a 633 nm excitation provides excellent excitation efficiency, enabling cells labeled with 660-VAD-FMK to be analyzed with most flow cytometers and fluorescence microscopes equipped with electronic grey scale image capabilities.

2. Materials Supplied and Storage

Store the kit at +4°C upon receipt.

Δ Note: Once reconstituted with DMSO, use FLICA 660 immediately, or store at ≤-20°C for up to 6 months, protected from light. Avoid repeated freeze/thaw cycles.

Item	Quantity	Storage Temperature
10X Apoptosis Wash Buffer	1 x 15 mL	+4°C
Fixative	1 x 6 mL	+4°C
660-VAD-FMK poly caspase inhibitor reagent	1 x 1 vial	+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 660-VAD-FMK poly caspase inhibitor reagent
- DiH_2O, 135 mL to dilute 10X Apoptosis Wash Buffer
- Phosphate buffered saline (PBS) pH 7.4, up to 100 mL, to dilute FLICA and handle cells
- FBS and/or BSA to add to the buffer when handling cells
- Cultured cells treated with the experimental conditions ready to be labeled
- Reagents to induce apoptosis or trigger caspase activity, such as staurosporine or camptothecin
- Hemocytometer
- Centrifuge at 200 *x g*
- 15 mL polystyrene centrifuge tubes (1 per sample)
- Hoechst 33342 or DAPI for optional nuclear staining
- Fluorescence microscope (Use band or long pass filter set pairings that best approximate excitation at 660 nm and emission at 685-690 nm. Due to the long wavelength emission properties of 660-VAD-FMK (>650 nm), use a fluorescence microscope with electronic grey scale image capture capabilities)
- Flow Cytometer (Use a standard 633 nm excitation laser and 675/25 emission filter set, or similar (often FL-4)

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 Apoptosis assessment with 660-VAD-FMK can be completed within a few hours. However, since 660-VAD-FMK is used to label living cells, adequate time must be allotted for the cultivation of cell samples and the experimental treatment or apoptosis induction process. The optimal cell concentrations and sample volumes will vary based on the experimental conditions and method of analysis. As 660-VAD-FMK detects the presence of the catalytically active forms of caspase enzymes, plan the experiment so that 660-VAD-FMK will be diluted and added at the time when caspases are expected to be activated in the cells.

5.2 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. Carefully monitor the density of adherent cell monolayers to avoid excessive levels of confluency.

5.3 Calculate how much 660-VAD-FMK is needed. Initial experiments may be necessary to assess the optimal concentration of 660-VAD-FMK and incubation period to adequately label the samples. **660-VAD-FMK should not be reconstituted and diluted until the cells are ready to be labeled.**

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 cell culture environment.

- Positive control: cells induced to undergo apoptosis using a known apoptosis induction protocol.

Flow cytometry controls: additional controls should be established for instrument compensation and gating:

- Unlabeled cells induced to activate caspases

- Unlabeled cells not induced to activate caspases
- Cells labeled with 660-VAD-FMK and induced to activate caspases
- Cells labeled with 660-VAD-FMK not induced to activate caspases
- Cells stained only with the secondary dye (if applicable) and induced to activate caspases
- Cells stained only with the secondary dye (if applicable) not induced to activate caspases
- Cells stained with both 660-VAD-FMK and the secondary dye (if applicable) and induced to activate caspases
- Cells stained with both 660-VAD-FMK and the secondary dye (if applicable) not induced to activate caspases

7. Apoptosis Induction

Determine a reproducible method for stimulating serine protease activity to obtain a positive control prior to commencing the experiment. For example, apoptosis may be induced with:

- 2-4 µg/mL camptothecin for >4 hours.
- 1-2 µM staurosporine for >4 hours.

8. Preparation of FLICA 660

– 660-VAD-FMK is supplied as a lyophilized powder that is dried onto the base of the amber glass vial. To minimize hydrolysis of the reactive FMK group, 660-VAD-FMK should not be prepared until the samples are ready to be stained. Add it to the samples immediately after diluting it with the aqueous PBS solution. Protect from light and use gloves when handling.

8.1 Reconstitute each vial of 660-VAD-FMK with 50 µL DMSO to form the stock concentrate. The stock concentrate should appear as a clear, blue-green solution. Once reconstituted in DMSO, the stock concentrate 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 660-VAD-FMK 1/5 by adding 200 µL PBS to form the 30-60X FLICA working solution. Add the working solution to the samples and controls within 15 mins of preparation to minimize hydrolysis of the FMK reactive group. The working solution is used at approximately 1/30–1/60 in suspension cell samples at 2-5 x 10^5 cells/mL. This calculates to 5 to 10 µL of FLICA 660 working solution per 300 µL cell sample.

8.3 The optimal cell concentrations and volumes will vary based on the experimental conditions and method of analysis. Flow cytometry typically requires a lower cell concentration and less 660-VAD-FMK reagent than fluorescence microscopy. For analysis by flow cytometry or applications where a lower staining concentration is needed, use the 660-VAD-FMK working solution at 1/60. For analysis by fluorescence microscopy or applications where a higher staining

concentration is needed, use the 660-VAD-FMK working solution at 1/30.

9. Preparation of 1X Apoptosis Wash Buffer

10X Apoptosis Wash Buffer is an isotonic solution used to wash cells following exposure to FLICA. It contains mammalian proteins to stabilize cells stained with FLICA and sodium azide to retard contamination (1X Apoptosis Wash Buffer contains 0.01% w/v sodium azide). Cell media containing FBS and other additives may be used instead of 1X 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 2-8°C and used within 1 week or frozen and used within 6 months.

10. Fixative

- The Fixative is a formaldehyde solution designed to cross-link and aggregate intracellular components. If the stained cell populations cannot be evaluated immediately after labeling with 660-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 protected from light on ice or at 4°C for up to 16 hours.
- The Fixative will not interfere with the 660-VAD-FMK label. If using absolute ethanol or methanol-based fixatives, caution is recommended as they have been shown to inhibit the fluorescence output of other fluorescent labels, like carboxyfluorescein, and may affect the fluorescence potential of the 660-VAD-FMK label.

11. Staining Protocol

11.1 Expose cells to the experimental condition and prepare control cell populations. If analyzing with a flow cytometer, be sure to include all gating and compensation controls.

11.2 Initial cell concentration should be between 2-5 x 10^5 cells/mL but should not exceed 10^6 cells/mL; cells cultivated in excess of this concentration may begin to naturally enter apoptosis and trigger pan-caspase activity. For analysis by fluorescence microscopy, concentrate cells by centrifugation to 2-5 x 10^6 cells/mL just prior to staining with 660-VAD-FMK. Fluorescence microscopy requires a higher concentration of cells to provide an adequate cell density within the field of vision at higher magnifications. For example, an excess of 2 x 10^6 cells/mL is required to obtain 5-20 cells per image field. Flow cytometry has lower cell density requirements, and thus, a concentration as low as 1 x 10^5 cells/mL is sufficient for flow analysis.

11.3 Transfer 290-295 µL cells into fresh tubes. Different sample volumes may be used; however, this changes the amount of 660-VAD-FMK needed for optimal staining and alters the number of tests per vial.

11.4 Add 5-10 µL of the 30-60X 660-VAD-FMK working solution. The concentration of 660-VAD-FMK should be optimized for each cell line, experimental condition, and method of analysis. Microscopy analysis may require more reagent than flow cytometry. Flow cytometry analysis may provide the sensitivity to detect 660-VAD-FMK when used at 1/60. For example, to stain cells at 1/30, add 10 µL 660-VAD-FMK working solution to 290 µL cells, forming a final volume of 300 µL. To stain cells at 1/60, add 5 µL 660-VAD-FMK working solution to 295 µL cells, forming a final volume of 300 µL. Mix the cell suspension to disperse the 660-VAD-FMK reagent.

11.5 Incubate cells at 37°C protected from light. The incubation period may range from 15 mins to several hours, depending upon the cell line and experimental conditions. For best results, resuspend the cells every 20 mins to ensure an even distribution of 660-VAD-FMK.

11.6 If cells are to be analyzed with a microscope, cells may be counterstained with a nuclear stain such as Hoechst 33342 or DAPI.

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

11.8 Centrifuge at 200 *x g* for 5-10 mins at room temperature.

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

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

11.11 Carefully remove and discard supernatants. Gently vortex pellets to disrupt clumping. If analyzing by fluorescence microscopy, repeat wash process a third time. If using a flow cytometer, two wash steps are generally sufficient.

12. Microscopy analysis

Follow section 11.

12.1 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 12.2), or observed immediately (Step 12.3).

12.2 If not viewing immediately, cells may be fixed and viewed later. Fixed cells may be stored protected from light on ice or at 2-8°C for up to 16 hrs.

12.3.1 Add Fixative at a v/v ratio of 1:5-1:10.

12.3.2 Incubate 15 mins at RT in the dark.

12.3.3 Place cells onto a microscope slide and allow to dry.

12.3.4 Briefly wash cells with PBS.

12.3.5 Cover cells with mounting media and coverslip.

12.3.6 Store slides at 2-8°C for up to 16 hrs.

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

12.4 Observe cells under a fluorescence microscope equipped with excitation band pass filter optics capable of efficiently transmitting 660 nm excitation light and a long pass emission filter >680 nm to view far-red fluorescence. Cells bearing active caspase enzymes that are covalently bound to 660-VAD-FMK will show elevated levels of fluorescence >680 nm. Because the human eye is not adept at seeing emission wavelength light greater than 650 nm, the use of electronic gray scale imaging equipment is strongly recommended.

12.5.3 DAPI nuclear stain exhibits an optimal dsDNA-bound excitation of 358 nm and an emission maximum of 461 nm.

12.5.4 Hoechst 33342 (blue) can be seen using a UV-filter with excitation at 365 nm and emission at 480 nm.

13. Flow Cytometry analysis

Follow Section 11, but omit the optional nuclear staining steps.

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

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

13.3 For single-color analyses, use a 633 nm (peak emission) 15 mW helium-neon ion laser or comparable >640 nm laser illumination source. Measure 660-VAD-FMK emission on the FL4 channel or with emission filters compatible with light emission between 680-690 nm.

13.4 Generate a histogram with the log FL4 on the X-axis versus the number of cells on the Y-axis. Caspase-negative (660-VAD-FMK -) cells will fall within the lower log fluorescence output decades of the FL4 X-axis, whereas caspase-positive (660-VAD-FMK +) cells will appear as a shoulder on the right side or as a separate peak on the right side of the negative peak histogram.

13.5 For dual-color analyses, run each single-color control. Adjust compensation to remove spectral overlap from interfering FL channels. Depending on the instrument and the software used, compensation might be set within the instrument hardware before samples are run or within the software after data collection. 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. a 660-VAD-FMK stained sample being read in FL-4 should have the same MFI in FL-3 as an unstained sample).

13.6 Run the dual-color experimental samples and analyze.

14. Notes

Technical Support

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