Red FLICA™ Poly Caspases Assay Kit

Detect active caspases with the red SR FLICA™ Poly-Caspases Assay Kit. This in vitro assay employs the fluorescent inhibitor probe SR-VAD-FMK to label active caspase enzymes in living cells or tissue samples. Analyze the fluorescent signal using fluorescence microscopy, a fluorescent plate reader, or by flow cytometry (green or yellow laser required).
FLICA (Fluorescent Labeled Inhibitor of Caspases) probes are comprised of an inhibitor peptide sequence that binds to active caspase enzymes, a fluoromethyl ketone (FMK) moiety that results in an irreversible binding event with the enzyme, and a fluorescent tag (either carboxyfluorescein or sulforhodamine B) reporter. For a poly-caspase inhibitor, the multi-enzyme recognition sequence is valine-alanine-aspartic acid (VAD). The FLICA probe interacts with the enzymatic reactive center of an activated caspase via the recognition sequence, forming a covalent thioether adduct with the enzyme through the FMK moiety.
FLICA probes are cell permeant and non-cytotoxic. Unbound SR- FLICA reagent is washed away; the remaining red fluorescent signal is a direct measure of caspase activity at the time the probe was added. Detection of nuclear morphology is also possible with the additional kit component, Hoechst 33342.

Background

Apoptosis is an evolutionarily conserved form of cell suicide mediated by a cascade of proteolytic enzymes called caspases. Pro-apoptotic signals activate the enzymatic cascade resulting in the cleavage of protein substrates, leading to the disassembly of the cell (1-4). Caspases have been identified in organisms ranging from C. elegans to humans. Members of the mammalian caspase family of cysteinyl aspartate-specific proteases play distinct roles in apoptosis and inflammation.

There are two types of caspases; the initiators (caspases 8, 9, and 10) and the effector caspases (caspases 1, 2, 3, 4, 6, 7, 12, and 13). The initiator caspases 8 and 10 are also referred to as the extrinsic apoptosis pathway that originates upon activation of cell surface death receptors. Caspases 8 and 10 are monomers that bind to death receptor proteins through their death effector domain (DED) structure. Caspase 9 is also called the intrinsic pathway that results from the mitochondrial release of cytochrome c. The initiator caspase 9 monomer binds other proteins through their caspase activation and recruitment domain (CARD). The initiator caspase -protein interaction results in dimerization of the initiator caspases that leads to their activation. These activated initiator caspases then cleave the effector pro-caspases at specific aspartic acid residues to yield large (20 kDa) and small (10 kDa) subunits that then assemble into the heterotetrameric, catalytically active form of the caspase effector enzymes (5, 6). Active caspase enzymes exhibit catalytic and substrate specificities comprised of short tetra-peptide amino acid sequences that must contain an aspartate in the P1 position (7 - 9). These preferred tetra-peptide sequences have been used to derive peptides that specifically compete for caspase binding (4 - 6). In addition to the distinctive aspartate cleavage site at P1, the catalytic domains of the caspases require typically four amino acids to the left of the cleavage site with P4 as the prominent specificity-determining residue (9). In contrast to this tetrapeptide specificity, the tri-peptide VAD is able to bind to the active site of every caspase family member studied. Furthermore, addition of a fluoromethyl ketone (FMK) to the tri-peptide results in an irreversible linkage and permanent inactivation of the cysteine protease enzyme (10). Accordingly, the Z-VAD-FMK inhibitor has been shown in numerous studies to effectively inhibit the induction of apoptosis by blocking caspase activation (9, 11). Furthermore, substitution of the amino terminal benzyloxycarbonyl blocking group (Z-) with a detection moiety, such as a fluorescent dye, yields a probe that allows for the detection of caspase activity (12 - 14).

FLICA™: Fluorescent-Labeled Inhibitors of Caspases

The FLICA methodology of caspase detection is available in kit form for assessing individual or poly-caspase activity in cultured cells and tissues. Upon addition to a cultured cell sample, the cell-permeant poly-caspases FLICA™ reagent SR-VAD-FMK will enter each cell and form irreversible

bonds with activated intracellular caspases. Because FLICA reagent becomes covalently coupled to the active enzyme, it is retained within the cell during wash steps, while any unbound FLICA reagent diffuses out of the cell and is washed away. The remaining red fluorescent signal is a direct measure of the amount of caspase activity present in the cell at the time the reagent was added.

Cells that contain the bound FLICA SR-VAD-FMK reagent can be analyzed by 96-well-plate based fluorometry, fluorescence microscopy, or flow cytometry (when a green laser is available). The sulforhofamine (SR) FLICA reagents have an optimal excitation range of 560 - 570 nm, and emission range from 590 - 600 nm. Cells labeled with FLICA may be read immediately or preserved for 24 hours using the fixative. Unfixed samples may be subsequently analyzed with Hoechst stain or 7-AAD to detect changes in nuclear morphology or necrosis respectively.

Other FLICA™ Caspase Detection Kits, containing the preferred caspase recognition amino acid sequences for caspases 1, 2, 3, 6, 8, 9, 10, and 13, are also available with green or red fluorescence.

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Protocols

Sample Protocol:

FLICA™, Fluorescent-Labeled Inhibitor of Caspases, is a simple yet accurate method to measure apoptosis via caspase activity in whole cells. Four sample protocols are outlined below.

Suspension Cells

Culture your cells up to 1 x 106 cells/mL.
Follow experimental protocol where caspase activity will be investigated; create positive and negative controls for caspase activity.
Reconstitute the reagent with 50µL DMSO to form the stock concentrate (can be frozen for future use).
Dilute the stock concentrate with 200µL 1X PBS to form the working solution.
Add ~10µL of the working solution directly to a 300-500µL aliquot of your cell culture for labeling.
Incubate 30 minutes -1 hour.

Wash and spin cells two or three times, or let incubate for 1 hour with fresh media or 1x apoptosis wash buffer.
If desired, label cells with Hoechst stain.
If desired, fix cells.
Analyze data using a fluorescence microscope, plate reader, or flow cytometer.
Frozen Tissues

Prepare frozen tissues according to the experiment.
Allow slides to air-dry.
Fix slides with acetone for 1 minute.
Rehydrate slides by washing (twice for 5 min) in TBS-tween (TBSt) or PBS-tween (PBSt).
Block slides for 20 minutes (such as 20% Aquablock in media with 0.2% tween).
Dilute 150X FLICA stock 1:50 in PBS to form a 3X working solution. For example, add 50 µL 150X stock to 2450 µL PBS (2.5 mL total).
Add 50 µL of 3X FLICA™ and incubate >1hr protected from light.
Wash with TBSt or PBSt (twice for 5 min) by setting slides in slide incubation dish containing 1X wash buffer.
Develop with DAPI and coverslip.
Store samples at 2-8°C for short term storage, staining will last at -20° C for long periods.
Adherent Cells

Adherent cells need to be carefully washed to avoid the loss of any cells which round up and come off the plate surface. Loose cells may be harvested from the plate or slide surface and treated as suspension cells, while those remaining adherent to the surface should be washed as adherent cells. If the adherent cells are trypsinized, the loose cells can be recombined with the trypsinzed pool, or the washed loose cells can then be recombined with the adherent portion when the analysis is performed. If growing adherent cells on a tissue culture plate, the entire plate may be gently spun as part of the wash process to sediment any loose floating cells. Avoid any attempts to trypsinize cells prior to labeling with a vital dye such as PI. Trypsin exposed cell membranes could become transiently permeant to vital dyes for a variable time period, depending upon the cell line. Cells may be labeled with FLICA™ before or after trypsinization.

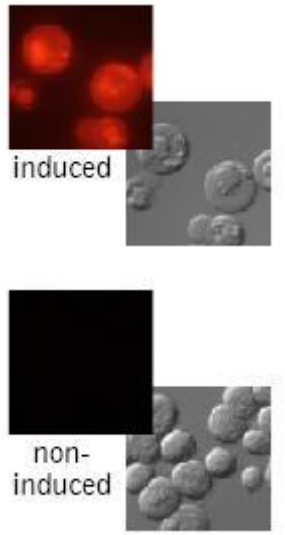
Adherent Cells: Trypsinization prior to FLICA™ labeling and FACS analysis:

Culture cells in T25 flasks and expose to the experimental conditions.
Apoptotic cells may detach and begin to float into the media. Save and spin to pellet and include these cells in your analysis.
Trypsinize adherent cells; neutralize with trypsin inhibitor present in 20% FBS-cell culture media; pool cells with any pellets created in #2; add a few mL media.
Spin ~5 minutes at 220 x g and remove all but ~100 µL supernatant.
Count cells and adjust volume of cell suspension to fit the experiment (typically 300-500 µL). Transfer cells into a 15 mL tube.
Add 10 – 17 µL of 30X FLICA.
Incubate at 37°C, 30-60 minutes, mixing gently every 10 minutes.
Wash by adding ~10mL media and incubate at 37°C for 60 minutes to allow any unbound FLICA™ to diffuse out of the cells.
Spin at 220 x g for 5 minutes; aspirate supernatant.
Add ~300µL 1X apoptosis wash buffer. Put cells on ice, and protect from light.
If desired, add 30 µL fixative.
Analyze cells with a flow cytometer.
Adherent Cells: FLICA™ label prior to trypsinizing, and FACS analysis:

Seed 5-8 x 104 cells in a 24-well plate in a final volume of 600 µL and let attach for 24 hours.
Expose cells to the experimental conditions.
Add 1-4 µL of FLICA™ 150X stock concentrate and incubate 1-3 hours at 37°C.
Remove supernatant containing any rounded up cells and set aside in labeled tube.
Wash adherent cell monolayer by gently adding PBS to cover the adherent cell monolayer.
Remove PBS and combine with cells previously set aside in step 4.
Add trypsin – versene to barely cover the attached cell monolayer.

Allow cells to detach and remove detached cells by adding 1 mL of cell culture media + 20% FBS to the trypsinized cells in the wells.
Add detached cells from the trypsinization step to supernatant from step 4.
Add 2 mL of cell culture media + 20% FBS to each tube containing trypsinized cells.
Spin cells at 220 x g for 5 min. Remove supernatant and discard. Add 1mL 1x apoptosis wash buffer.
Spin cells at 220 x g for 5 min. Remove supernatant. Add 1mL 1x apoptosis wash buffer.
Spin cells at 220 x g for 5 min. Remove supernatant and resuspend in 300 µL 1X apoptosis wash buffer.
If desired, add 30µL fixative.
Analyze on FACS immediately.

Data



Jurkat cells were treated with 1µM staurosporine to induce caspase activity (top), or a negative control (bottom) for 3 hours, incubated with ICT's red poly caspases inhibitor probe, SR-VAD-FMK, for 1 hour, washed twice, and examined under a fluorescence microscope (DIC images were also taken). The color image of induced cells (upper left) reveals experimental cells which fluoresce red, therefore they all have some degree of caspase activity. The non-induced DIC image (lower right) reveals many control cells, however the corresponding fluorescence image (lower left) is dark; none of these cells have active caspases (Dr. Brian W. Lee, ICT).

Specifications

Target: poly caspases
Excitation / Emission: 565 nm / 586 nm
Method of Analysis: Flow Cytometer, Fluorescence Microscope, Fluorescence Plate Reader
Types of Samples: cell culture, tissue
Kit Contents:
Kit #916 (trial size, 25 tests):

FLICA Poly Caspases Reagent (SR-VAD-FMK), 1 vial
10x Apoptosis Wash Buffer, 15mL
Fixative, 6mL
Hoechst 33342 Stain, 1mL
Kit #917 (regular size, 100 tests):

FLICA Poly Caspases Reagent (SR-VAD-FMK), 4 vials
10x Apoptosis Wash Buffer, 60mL
Fixative, 6mL
Hoechst 33342 Stain, 1mL

Citations

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FAQ

How many tests can be run with the trial size and regular size kits?
The trial size FLICA kit provides enough reagent to test 7.5mL of cell culture samples - approximately 25 tests. The regular size FLICA kit provides reagent for testing 30mL of cell culture samples - approximately 100 tests.

What is one "test"?
One "test" is a 300uL aliquot of cells grown at 1X10^6 cells/mL and analyzed on a fluorescence plate reader or microscope. Plate readers tend to require the most reagent, flow cytometers the least.

How is FLICA™ different from other caspase detection kits?
The FLICA assay kits are used with whole, living cells; no lysis or permeabilization is necessary. FLICA is not an ELISA and does not involve the use of any antibodies. Because active caspase enzymes bind to FLICA, there is no interference from pro-caspases nor inactive forms of the enzyme. The fluorescent signal can be analyzed by fluorescence microscopy, plate reader, or flow cytometer.

Can I read SR-FLICA™ labeled cells using a flow cytometer?
We recommend that the investigator use a flow cytometer that includes a green or yellow laser excitation option for flow cytometry analysis of SR-FLICA stained cells. SR-FLICA labeled cells may be detected using the conventional blue laser (488 nm) excitation setting common to most flow cytometers, however, the efficiency of sulforhodamine B excitation at this wavelength is very low, leading to a dramatic reduction in assay