FAM-OPH
in vitro Apoptosis Detection Reagents
Catalog #6354, 6355, 6356

FOR RESEARCH USE ONLY.
Not for use in diagnostic procedures.

INTRODUCTION

Apoptosis is an evolutionarily conserved process of programmed cell suicide that centers on a cascade of proteolytic enzymes called caspases that are triggered in response to pro-apoptotic signals. Once activated, caspases cleave protein substrates leading to the eventual disassembly of the cell[1].

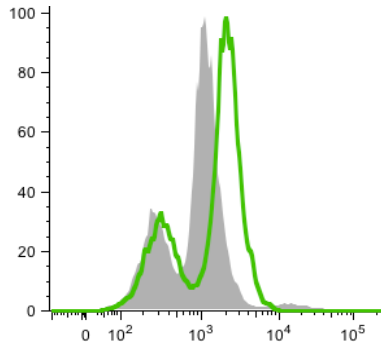
Early research studies using short peptide sequences linked to an aldehyde or fluoromethyl ketone (FMK) reactive group identified a number of peptide sequences preferentially targeted by different caspase enzymes. For example, the sequence Asp-Glu-Val-Asp (DEVD) is cleaved by caspase-3 and caspase-7, the sequence Val-Ala-Asp (VAD) demonstrates multiple caspase (caspase -1, -2, -3, -4, -5, -6, -7 -8, -9) recognition properties[2].

ImmunoChemistry Technologies utilized these findings to develop the often-cited FLICA® (Fluorescence Labeled Inhibitors of CAspases) caspase inhibitor reagents, which have been widely used for years as simple and reliable methods for screening apoptosis in live cells and tissues. These reagents were made by sandwiching target peptide sequences with varying caspase specificities between a green fluorescent label, carboxyfluorescein (FAM), and an FMK reactive moiety. The resulting green fluorescent, cell-permeant, non-cytotoxic inhibitor reagents can be added to cell culture media for *in vitro* use. They will cross the cell membrane and form irreversible covalent bonds with activated caspase enzymes present inside apoptotic cells, which can then be differentiated from non-apoptotic cells by their retained green fluorescence.

ImmunoChemistry Technologies has now released a novel set of inhibitor reagents that employ an O-phenoxy (OPH) reactive group instead of an FMK group. In a manner analogous to the FMK class of cysteine reactive compounds, the OPH inhibitors form a stable covalent thioether adduct with the reactive SH-site of caspase enzymes present in apoptotic cells[3]. OPH inhibitor compounds have the benefit of being extremely non-cytotoxic, display enhanced stability characteristics compared to the FMK analogs, and have been optimized to provide a high level of performance for *in vitro* applications[3-4].

FIGURE 1: CONSISTENTLY QUANTIFY APOPTOSIS

Jurkat cells (T lymphocytes) were treated with 1 µM staurosporine for 4 hours to induce apoptosis via caspase activity. Cells were then stained with one of ICT's green poly caspase inhibitor probes, FAM-VAD-OPH I (catalog #6354) or FAM-VAD-FMK FLICA® (catalog #92) at 10 µM for 1 hour. Cells were washed twice and read on a BD FACSCanto flow cytometer. Histograms were made of cell fluorescence intensity output. Staining results with the OPH inhibitor (grey solid) are consistent with those achieved using the FMK inhibitor (green outline). FAM-VAD-OPH inhibitor reagents mirror FAM-VAD-FMK analogs for *in vitro* apoptosis detection, and they may be less toxic and more stable. Data courtesy of Dr. Michael Olin, University of Minnesota (ICT-202).



REAGENTS

- FAM-VAD-OPH I (5-FAM-Val-Ala-Asp(OMe)-2,6-difluorophenoxymethyl ketone); poly caspase inhibitor
  #6354 (4/pack); MW = 829.8
- FAM-VAD-OPH II (5-FAM-Val-Ala-Asp(OMe)-Oph (2,6-diF)); poly caspase inhibitor
  #6355 (4/pack); MW = 771.7
- FAM-DEVD-OPH (5-FAM-Asp(OMe)-Glu(OMe)-Val-Asp(OMe)-Oph (2,6-diF)); caspase-3/7 inhibitor
  #6356 (4/pack); MW = 1001

STORAGE

Store unopened reagents at ≤ 2-8°C until the expiration date. Once reconstituted in DMSO, use immediately, or store at ≤-20°C for 6 months protected from light and thawed no more than twice during that time.

DETECTION EQUIPMENT

FAM-OPH reagents excite at 488-492 nm and emit at 515-535 nm. Use laser/filter pairings that best approximate these settings. The fluorescent signal may be analyzed by:

- Fluorescence microscope
- Fluorescence plate reader
- Flow cytometer

SAFETY

- See Safety Data Sheet (SDS) for any warnings.
- SDS are available at online at www.immunochemistry.com or by calling 1-800-829-3194 or 952-888-8788.
- For research use only.
- Not for use in diagnostic procedures.

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HOW TO USE

1. Prepare experimental samples 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. Just prior to staining, cells may need to be concentrated to 2-5 x 10^6 cells/mL as both microscopy and plate reader analysis methods require higher cell concentrations than flow cytometry. 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 290-300 µL per sample when ready to label.
2. Reconstitute FAM-OPH with 50 µL DMSO to form the 150X stock concentrate.
3. Dilute the 150X stock concentrate 1:5 by adding 200 µL PBS to create the 30X staining solution.
4. Add 10 µL of the 30X staining solution to each sample (~290 µL aliquot of cultured cells). For different volumes, add the reagent at 1:30. Some cells may require more or less reagent; perform an initial titration experiment to determine the ideal concentration.
5. Incubate approximately 1 hour.
6. Wash cells to remove any unbound reagent:
   a. Add 1-2 mL PBS or cell culture media and centrifuge at 200 x g for 5-10 minutes at room temperature (RT).
   b. Carefully remove and discard supernatants.
   c. Resuspend in 1-2 mL PBS or cell culture media and centrifuge at 200 x g for 5-10 minutes at RT.
   d. Carefully remove and discard supernatants.
   e. Gently vortex pellets to disrupt clumping.
   f. Resuspend cells in 300 µL PBS or cell culture media and gently mix.
7. If desired, label with additional stains, such as Hoechst, DAPI, PI, 7-AAD, or an antibody. Wash cells if necessary to remove excess stain from the media.
8. If desired, fix or mount cells.
9. Analyze with a fluorescence microscope, plate reader, or flow cytometer. FAM-OPH excites at 492 nm and emits at 520 nm.
10. For more information, contact ICT at 1-800-829-3194 or visit www.immunochemistry.com.

REFERENCES

1. Slee, E. A., Adrain, C. & Martin, S. J. Serial killers: ordering caspase activation events in apoptosis. Cell Death Differ 6, 1067-1074, doi:10.1038/sj.cdd.4400601 (1999).
2. Thornberry, N. A., et. al. A combinatorial approach defines specificities of members of the caspase family and granzyme B. Functional relationships established for key mediators of apoptosis. J Biol Chem 272, 17907-17911 (1997).
3. Chauvier, D., Ankri, S., Charriaut-Marlanque C., Casimir R. & Jacotot E. Broad spectrum caspase inhibitors: from myth to reality?. Cell Death Differ 14, 387-391, doi: 10.1038/sj.cdd.4402044 (2007).
4. Callus, B. A. & Vaux, D. L. Caspase inhibitors: viral, cellular, and chemical. Cell Death Differ 14:73-78 doi:10.1038/sj.cdd.4402034 (2007).

FIGURE 2: FLUORESCENCE MICROSCOPY IMAGING

Jurkat cells (T lymphocytes) were treated with 1 µM staurosporine for 3 hours at 37°C to induce apoptosis. Cells were then labeled with ICT's green fluorescent caspase-3/7 inhibitor FAM-DEVD-OPH (catalog #6356) at 5 µM for 60 minutes. Cells were washed twice and wet-mount slides were prepared. A grey differential interference contrast (DIC) image (left) was taken, and green fluorescence (right) was detected using a band pass filter (excitation at 488 nm, emission at 520 nm). FAM-DEVD-OPH reveals complete caspase-3/7 activation in the form of green fluorescence in all cells. Data courtesy of Ms. Tracy Hanson, ICT (ICT111709; FAM-DEVD-OPH_in_59_60).

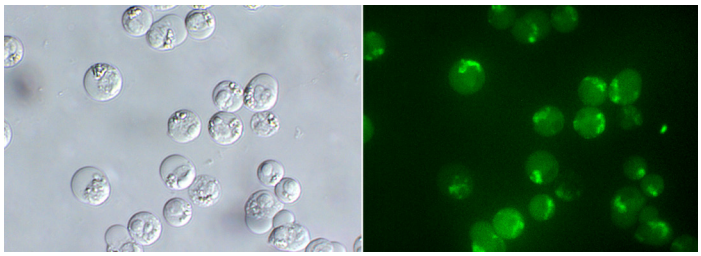
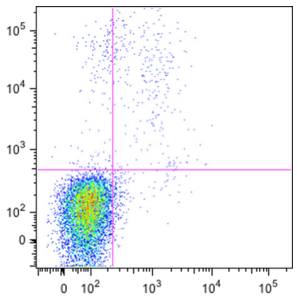


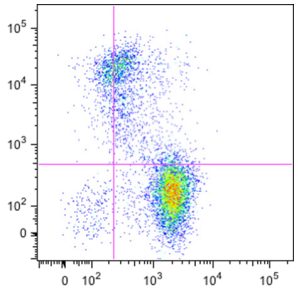
FIGURE 3: USE FAM-OPH WITH PI TO QUANTITATE 4 POPULATIONS OF APOPTOSIS AND NECROSIS WITHIN A SINGLE SAMPLE

ICT's green fluorescent poly caspase inhibitor reagent, FAM-VAD-OPH II (catalog #6355), was used with Propidium Iodide (PI, catalog #638), a red live/dead stain, to assess apoptosis and necrosis in Jurkat cells. Jurkat cells (T lymphocytes) were treated with DMSO as a control (left) or 1 µM staurosporine, an apoptosis inducer (right), for 3 hours at 37°C. Cells were then labeled with FAM-VAD-OPH II for 1 hour, washed, and dually stained with PI at 2.5 µg/mL. Cells were analyzed using two-color flow cytometry with a BD FACSCanto. Density plots were set up to detect caspase activity (green, FL-1) on the X-axis and necrosis (red, FL-2) on the Y-axis.

Four populations of cells were detected: unstained live cells do not fluoresce (lower left); cells in early apoptosis fluoresce green with FAM-VAD-OPH II (lower right); cells in late apoptosis (upper right) are dually stained with FAM-VAD-OPH II and PI: they fluoresce green (they have active caspases) and red (the cell membrane has permeabilized); and necrotic cells fluoresce red (upper left). Very few cells were killed when treated with the placebo (left plot), while many cells entered apoptosis and secondary necrosis when treated with staurosporine (right plot). Data courtesy of Dr. Michael Olin, University of Minnesota (ICT-202:49; 072110.pptx).



FL-1: Green FAM, Apoptosis Negative Control



FL-1: Green FAM, Apoptosis Induced

Thank you for using FAM-OPH! If you have any questions, or would like to share your data, please contact us at help@immunochemistry.com.

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ImmunoChemistry Technologies, LLC gratefully acknowledges the significant contributions made by one of its founders, Brian W. Lee, Ph.D in the development of this product, including the creation and illustration of its strategy and protocol.

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