Product Manual

CV-Caspase 3&7 detection kit

Catalog # BML-AK118

96 Well Plate Kit

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Enzo

Product Manual

Please read entire booklet before proceeding with the assay.

Carefully note the handling and storage conditions of each kit component.

Please contact Enzo Life Sciences Technical Support if necessary.

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INTRODUCTION

Enzo Life Sciences' CV-Caspase 3&7 assay kit enables researchers to detect and monitor apoptosis over time via intracellular caspase activity *in vitro*. The $CR(DEVD)_2$ reagent is a non-cytotoxic substrate that fluoresces upon cleavage by active caspase 3&7 enzymes. It measures the intracellular process of apoptosis instead of a side-effect, such as the turn-over of phosphatidyl serine.

To use $CR(DEVD)_2$, add the reagent directly to the cell culture media, incubate, and analyze. Because $CR(DEVD)_2$ is cell-permeant, it easily penetrates the cell membrane and the membranes of the internal cellular organelles - no lysis or permeabilization steps are required. $CR(DEVD)_2$ will enter the cell in a non-fluorescent state. If caspase 3&7 enzymes (DEVDases) are active, they will cleave $CR(DEVD)_2$ and release the cresyl violet fluorophore, which will begin to fluoresce red. The red fluorescent product will stay inside the cell and will often aggregate inside lysosomes (Figures 1 and 3); caspases are not lysosomal. As protease activity progresses and more $CR(DEVD)_2$ substrate is cleaved, the red fluorescent signal will intensify, enabling researchers to watch the color develop over time (Figure 2) and quantify apoptosis (Figures 6 and 9).

There is no interference from pro-caspases nor inactive forms of the enzymes. If the treatment is causing cell death via apoptosis, apoptotic cells will have elevated levels of caspase 3&7 activity relative to non-apoptotic or negative control cells.

Up-regulation and initiation of the caspase enzyme cascade is the central driving force behind apoptosis[3]. Although a number of other intracellular enzyme families, including the cathepsins, calpains, and granzymes, participate in the cell break-down mechanism, the caspase cascade occupies the central effector role in the cell suicide process[21,25,8,12]. Like other intracellular proteases, caspases are initially synthesized as inactive zymogen precursors that can be rapidly activated upon auto and heterologous enzymatic processing at specific sites containing an aspartic acid[17]. Caspase 3 is the predominant effector caspase in apoptosis (with few exceptions, such as MCF-7 cells, which are deficient in caspase 3; Figures 1, 3, and 4).

Caspase enzymes cleave proteins and are classified as cysteine proteases based on the mechanism of substrate hydrolysis at their active site. Caspases specifically recognize a 3 or 4 amino acid sequence which must include an aspartic acid residue (D)

in the P1 position. This C–terminal residue is the target for the cleavage reaction at the carbonyl end[15]. Enzo Life Sciences' CV-caspase 3&7 probe contains a 4 amino acid sequence, aspartylglutamylvalanyl aspartic acid (DEVD), which is the optimal target sequence for caspase 3 as well as caspase 7[22]. Two copies of this sequence are coupled to a photostable red fluorophore, cresyl violet, to create the CV-caspase 3&7 substrate $CR(DEVD)_2$. The intact $CR(DEVD)_2$ substrate reagent is not fluorescent; the fluorescent signal is generated by the cresyl violet fluorophore once $CR(DEVD)_2$ is cleaved by caspase 3 or caspase 7. $CR(DEVD)_2$ has been shown to work in human, rat, and mouse cells, among other species. A baseline level of DEVDase activity is present in all cell lines. Apoptotic cells will fluoresce red and have pronounced red lysosomes and mitochondria. Healthy and nonapoptotic cells will exhibit very low levels of background red fluorescence evenly distributed throughout the cell (Figures 1-3, 6-8). This background level of substrate activity could be the result of constitutively synthesized serine proteases that target analogous aspartic acid sequences for hydrolysis. Cells in more advanced stages of apoptosis, containing peak levels of DEVDase activity, will appear brighter red than cells in earlier stages of apoptosis.

The fluorophore, cresyl violet, fluoresces red when excited at 550-590 nm[23]. The red fluorescent signal can be monitored with a fluorescence microscope (Figures 1-3, 6-8) or plate reader (Figure 9). It has an optimal excitation of 592nm and emission of 628 nm[2]. At these higher excitation wavelengths, the amount of cell- mediated auto-fluorescence is minimal[22]. The excitation peak is rather broad allowing good excitation efficiency at 540-560 nm. Hoechst stain is included with the kit to concurrently label nuclei after labeling with $CR(DEVD)_2$ (Figures 1 and 6). Acridine orange (AO) is also included in the kit to identify lysosomes and other intracellular organelles (Figures 4 and 5).

MATERIALS PROVIDED

1. 1 vial of CV-Caspase 3&7 Substrate Reagent ($CR(DEVD)_2$), approximately 25 tests, BML-KI224
2. 1 vial of Hoechst 33342, 200 µg/mL (1 mL), BML-KI189
3. 1 vial of Acridine Orange, 1 mM (0.5 mL), BML-KI223

STORAGE

Store the unopened kit and each unopened component at 2-8°C until the expiration date. Once reconstituted with DMSO, use $CR(DEVD)_2$ immediately, or store at ≤-20°C up to 6 months, protected from light and thawed no more than twice during that time.

OTHER MATERIALS NEEDED

- DMSO, 100-400 µL to reconstitute $CR(DEVD)_2$
- diH_2O, 400-1600 µL to dilute $CR(DEVD)_2$
- Phosphate buffered saline (PBS) pH 7.4, 100 mL
- Cultured cells treated with the experimental conditions ready for staining
- Reagents to induce apoptosis and create controls, such as staurosporine (BML-EI156) or camptothecin (ALX-350-015)
- Hemocytometer
- Centrifuge at <200g
- 15 mL polystyrene centrifuge tubes
- Sterile black 96-well microtiter tissue culture plates, round or flat bottom.
- Slides and coverslips
- Ice or refrigerator

DETECTION EQUIPMENT

$CR(DEVD)_2$ excites at 550 nm and emits >610 nm. It has an optimal excitation and emission wavelength tandem of 592 nm and 628 nm, respectively. Hoechst stain can be visualized using a UV-filter with excitation at 365 nm and emission at 480 nm (Section 5). AO excites at 480 nm and emits >540 nm (Section 6). Select a filter combination that best approximates these settings.

Fluorescence microscope: Use an excitation filter of 550 nm (540-560 nm) and a long pass >610 nm emission/barrier filter pairing. Using this excitation/emission filter pairing, cells should stain red with brightly stained vacuoles and lysosomes (Figures 2 and 3). If the samples were stained with both $CR(DEVD)_2$ and Hoechst, the dual staining properties can be examined using a multi- wavelength filter (Figures 1 and 6). Hoechst stain can be seen using a UV-filter with excitation at 365 nm and emission at 480 nm. As AO exhibits a very broad emission range, one of several filter pairings may be used. The same excitation/emission filter pairings used to view $CR(DEVD)_2$ may be used for AO: a 550 nm (540 – 560 nm) excitation filter with a long pass >610 nm emission/barrier filter. With this pairing, lysosomes appear red. When illuminating AO with a blue light (480 nm) excitation filter, a green light (540-550 nm) emission/barrier filter combination works well. Lysosomes will appear yellowish green instead of red (Figures 4 and 5).

Fluorescence plate reader: $CR(DEVD)_2$ has an optimal excitation and emission wavelength tandem of 592 nm and 628 nm, respectively. Use a fluorescence plate reader with excitation at 590 nm and emission at 630-640 nm. If available, use a cut-off filter at 630 nm to filter out shorter wavelength excitation interference (Figure 9).

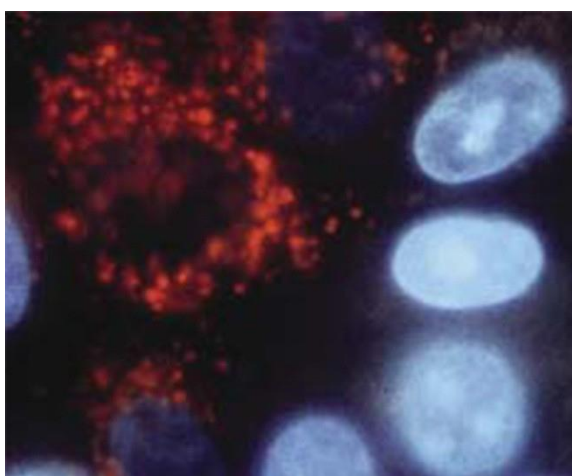
PROTOCOL

1. Experimental Preparation

Staining apoptotic cells with $CR(DEVD)_2$ can be completed within a few hours. However, $CR(DEVD)_2$ 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 procedure or apoptosis induction (which typically requires a 3-6 hour incubation at 37°C).

As $CR(DEVD)_2$ detects caspase-mediated apoptosis, plan the experiment so that it will be diluted and administered at the time when caspases are expected to be activated in the cells. The recommended volume of the $CR(DEVD)_2$ staining solution is 0-20 µL per 300 µL of cells at 10^6 cells/mL, but the ideal amount may vary based on the experimental conditions and method of analysis. Culture cells to a density optimal for the specific experiment or apoptosis induction protocol. Cell density should not exceed 10^6 cells/mL as cells cultivated in excess of this concentration may begin to naturally enter apoptosis due to nutrient deprivation or the accumulation of cell degradation products in the media.

FIGURE 1: DUAL STAINING OF MCF-7 CELLS



Apoptotic MCF-7 cells were dually stained using Enzo Life Sciences' $CR(DEVD)_2$ fluorogenic caspase 3&7 substrate and Hoechst 33342 nuclear stain (BML-AK118-0001). MCF-7 cells were exposed to 0.15 µM camptothecin (ALX-350-015) for 24 hours at 37°C, then stained with $CR(DEVD)_2$ for 30 minutes at 37°C, washed twice in PBS, and supravitally stained with 1 µg/mL Hoechst stain for about 10 minutes. Using the Nikon Microphot FXA system with multi-wavelength filter pairs (UV for Hoechst stain and green light for $CR(DEVD)_2$), apoptotic cells bearing orange-red lysosomal bodies with less intense blue nuclei are intermixed with non-apoptotic cells bearing bright blue nuclei and absent or reduced orange- red lysosomal staining. Photo provided by Dr. Zbigniew Darzynkiewicz at Brander Cancer Research Center Institute, New York City, NY.

Cells undergoing apoptosis will generate a stronger red fluorescence with $CR(DEVD)_2$ than non-apoptotic cells of the same lineage. To optimize this assay, determine the greatest difference in the fluorescent signal between induced and non-induced cell populations. Adjust the amount of $CR(DEVD)_2$ substrate used to stain cells and the incubation time. Hoechst stain can be used with $CR(DEVD)_2$ to label nuclei (Figures 1 and 6). Because of the overlap in emissions, dual staining of cells with both $CR(DEVD)_2$ and AO will yield confusing results and is not recommended; these dyes should be used separately. Do not use $CR(DEVD)_2$ with paraffin-embedded tissues as the chemicals used for paraffin-embedding may denature and inactivate the substrate.

2. Controls

It is highly recommended that two sets of controls be run: one positive population of cells that was induced to undergo apoptosis or trigger caspase 3/7 activity; and a placebo population of cells that received just the vehicle used to deliver the apoptosis- inducing agent (Figure 6). Create positive controls by inducing apoptosis (Section 3). Create negative controls by culturing an equal volume of non-induced cells for every labeling condition. The negative control and induced positive control populations should contain similar quantities of cells. For example, if labeling with $CR(DEVD)_2$, Hoechst stain, and Acridine Orange, make 10 control populations:

a. Unlabeled, induced and non-induced populations.

b. $CR(DEVD)_2$-labeled, induced and non-induced populations.

c. $CR(DEVD)_2$- and Hoechst-labeled, induced and non-induced populations.

d. Hoechst-labeled, induced and non-induced populations.

e. AO-labeled, induced and non-induced populations.

FIGURE 2: WATCH CASPASE ACTIVITY IN REAL TIME

REC:MYC immortalized rat fibroblasts were seeded in a 12-well plate at $1x10^4$ cells/mL and irradiated (4 Gy, X-ray) the following day. Enzo Life Sciences' Caspase 3&7 CR(DEVD)$_2$ Substrate Reagent (BML-KI224) was added, and cells were photographed for 16 hours using an inverted Nikon TE2000 microscope with a CCD camera from Hamamatsu and PCI software from Compix. The red fluorescence became brighter as caspase activity and apoptosis progressed. Data courtesy of Dr. Martin Purschke, Massachusetts General Hospital.

3. Apoptosis Induction

CR(DEVD)$_2$ works with your current apoptosis protocols - induce apoptosis or trigger DEVDase activity as you normally would, then label cells with CR(DEVD)$_2$. For example, apoptosis may be induced with:

a. 2-4 µg/mL camptothecin for >4 hours

b. 1-2 µM staurosporine for >4 hours

4. CR(DEVD)2 Substrate Reagent

CR(DEVD)$_2$ is supplied as a lyophilized powder that may be slightly visible as an iridescent sheen inside the vial. It must first be reconstituted in DMSO, forming the stock concentrate, and then diluted 1:5 in diH_2O to form the final staining solution. For best results, the staining solution should be prepared immediately prior to use. However, the stock concentrate may be stored at ≤20°C for future use. Protect from light and use gloves when handling.

1. Create the stock solution by reconstituting CR(DEVD)$_2$.

a) Use 100 µL DMSO to reconstitute the vial BML-KI224.

2. Gently vortex or swirl the vial, allowing the DMSO to travel around the base of the vial until completely dissolved. At room temperature (RT), this should take just a few minutes. The stock solution should appear red. Once reconstituted, it may be stored at ≤-20°C up to 6 months protected from light and thawed no more than twice during that time. If using immediately, dilute

in diH_2O to form the staining solution. If not diluting within 1 hour, aliquot and freeze.

3. Immediately prior to staining the samples, dilute the stock solution 1:5 in diH_2O to form the staining solution. Use the staining solution within 15 minutes of dilution to prevent substrate hydrolysis.

b) Add 400 µL diH_2O to dilute the vial BML-KI224. The vial contains 100 µL of the stock (1a); this yields 500 µL of the staining solution. Use immediately.

c) For other amounts, dilute the stock 1:5 in diH_2O. For example, add 10 µL stock to 40 µL diH_2O; this yields 50 µL of the staining solution. Use immediately.

d) Mix by inverting or vortexing the vial at RT.

5. Hoechst Stain

Hoechst 33342 is a cell-permeant nuclear stain that emits blue fluorescence when bound to double stranded DNA. It is used: a) to stain the nuclei of living or fixed cells; b) to distinguish condensed pyknotic nuclei in apoptotic cells; and c) for cell cycle studies. When bound to nucleic acids, the maximum absorption is 350 nm and the maximum emission is 480 nm. It is revealed under a microscope using a UV-filter with excitation at 365 nm and emission at 480 nm. Hoechst Stain (BML-KI189) is provided ready- to-use at 200 µg/mL. Hoechst Stain can be used concurrently with the $CR(DEVD)_2$ to label nuclei (Figures 1 and 6).

Warning: Hoechst Stain is a potential mutagen. Gloves, protective clothing, and eye wear are strongly recommended. When disposing, flush sink with copious amounts of water. See MSDS for further information.

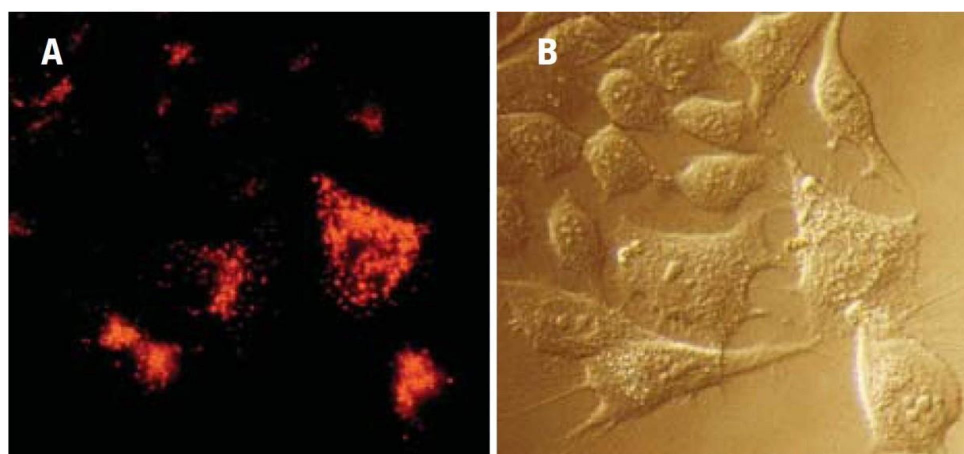
6. Acridine Orange

Acridine Orange (AO) is a chelating dye and can be used to reveal lysosomes, nuclei, and nucleoli (Figures 4 and 5). 0.5 mL of AO is provided at 1 mM (ML-KI223). AO may be used neat or diluted in diH2O or media prior to pipetting into the cell suspension. Always protect AO from bright light.

Lysosomal structures can be visualized by staining with AO at 0.5- 5.0 µM. This concentration range can be obtained by diluting the AO reagent stock 1:2,000-1:200 (0.05-0.5% v/v)

into the final cell suspension. For example, if using AO at 1.0 µM in the final cell suspension, it must be diluted 1:1,000. First dilute it 1:100 in PBS; e.g., put 10 µL AO into 990 µL PBS. Pipette the diluted AO into the cell suspension at approximately 1:10; e.g., put 50 µL diluted AO into 450 µL cell suspension.

FIGURE 3: CASPASE ACTIVITY IN MCF-7 CELLS



DEVDase activity was detected in apoptotic MCF-7 cells using Enzo Life Sciences' CR(DEVD)$_2$ caspase 3&7 fluorogenic substrate. Apoptosis was induced in MCF-7 cells by treating them with 0.15 µM camptothecin (ALX-350-015) for 24 hours at 37°C. Cells were then exposed to CR(DEVD)$_2$ for 60 minutes at 37°C. DEVDase activity is demonstrated by the appearance of orange-red lysosomal bodies within the cytoplasm of the cell (A). The photograph was taken on a Nikon Microphot FXA system at 541-551 nm excitation with a long pass >640 nm barrier filter (compare with AO staining in Figure 4). Photo B shows the corresponding interference contrast image of the cells. Data courtesy of Dr. Zbigniew Darzynkiewicz (Brander Cancer Research Center Institute, New York City, NY).

As AO exhibits a very broad emission range, one of several filter pairings on the fluorescence microscope can be used to view this stain. The same excitation/emission filter pairings used to view CR(DEVD)$_2$ may be used: an excitation filter of 550 nm (540-560 nm) and a long pass >610 nm emission/barrier filter pair. With this pairing, the lysosomes appear red instead of yellowish green.

When illuminating with a blue light (480 nm) excitation filter, a green light (540-550 nm) emission/barrier filter combination works well. Lysosomes will appear yellowish green. As this filter combination is very close to the maximum emission of AO, the slide may appear too bright. Excess AO may be removed by washing cells prior to viewing.

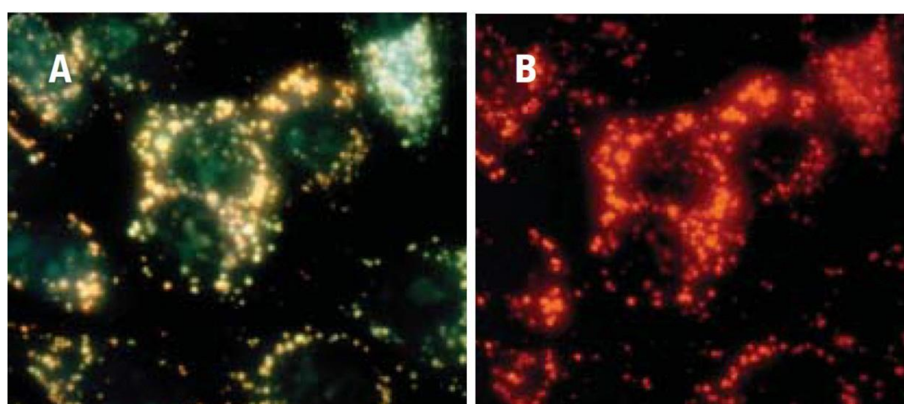
Because of the overlap in emissions, dual staining of cells with both CR(DEVD)$_2$ and AO will yield confusing results. Therefore, these dyes should be used separately. Warning:

AO is a potent mutagen and probable carcinogen. Use gloves, protective clothing, and eye wear. When disposing, flush sink with copious amounts of water. See MSDS for further information.

7. Microscopy Analysis of Suspension Cells

1. Prepare cell populations. Initial cell concentrations should be 3-5 x 10^5 cells/mL and should not exceed 7 x 10^5 cells/ mL, as cells cultivated in excess of this concentration may begin to naturally enter apoptosis.
2. Expose cells to the experimental conditions and create positive and negative controls (Section 2) or induce cells to undergo apoptosis (Section 3).
3. When ready to label with the staining solution, cell concentrations should be 1-2 x 10^6 cells/mL for best viewing. Density can be determined by counting cell populations on a hemocytometer. If necessary, concentrate cells by gentle centrifugation at 200 x g for 3-8 minutes. Remove the supernatant and resuspend with cell culture media or PBS.
4. Transfer 300 µL into 12 x 75 mm glass or polypropylene tubes. If desired, larger cell volumes can be used, but additional CR(DEVD)$_2$ staining solution may be required.

FIGURE 4: ACRIDINE ORANGE STAINING OF MCF-7 CELLS



Apoptosis was induced in MCF-7 cells by treating them with 0.15 µM camptothecin (ALX-350-015) for 24 hours at 37°C. Cells were stained with AO in PBS for 30 minutes, then washed twice in PBS. Cells were photographed with a Nikon Microphot-FXA epifluorescence microscope at 40X using either a blue light excitation (492 nm) with a 540-550 nm emission filter (A, lysosomes appear yellowish green), or green light excitation (540 nm) with a long pass >640 nm barrier filter (B, lysosomes appear red; compare with CR(DEVD)$_2$ staining of MCF cells in Figure 3, and

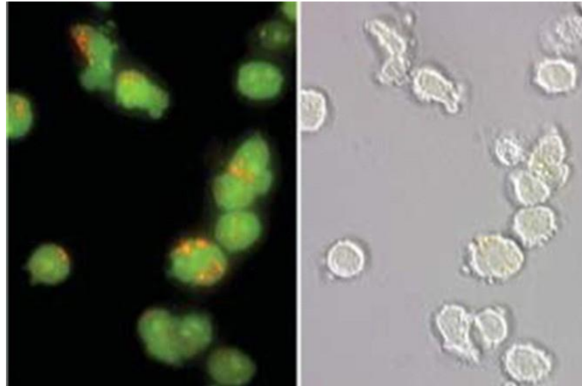
with Jurkat cells in Figure 5). Experiment performed in the laboratory of Dr. Zbigniew Darzynkiewicz (Brander Cancer Research Center Institute, New York City, NY).

5. Reconstitute $CR(DEVD)_2$ to form the stock solution (Section 4):
   a. Use 100 µL DMSO to reconstitute the vial (BML-KI224).
6. When ready to stain cells, dilute the stock 1:5 in diH_2O to form the $CR(DEVD)_2$ staining solution (Section 4):
   a. Add 400 µL diH_2O to dilute the vial (BML-KI224).
7. Add 10 µL of the staining solution to each 300 µL cell suspension and mix thoroughly. If different cell volumes are used, add the $CR(DEVD)_2$ staining solution at a ratio of approximately 1:30. For example, add 35 µL $CR(DEVD)_2$ staining solution to 1,000 µL of cell suspension forming a final volume of 1,035 µL. Do not add $CR(DEVD)_2$ to cells that are to be labeled with AO; add a placebo instead, such as diH_2O (Step 10).
8. Incubate cells for 1 hour at 37°C and protect from light. Cells may settle on the bottom of the tubes; gently resuspend them by swirling cells every 20 minutes during the incubation to ensure even distribution of $CR(DEVD)_2$ substrate. After the incubation, cells can be stained with Hoechst Stain (Section 9), or unstained cells may be labeled with AO (Section 10).
9. If cells are to be labeled with Hoechst Stain, add it at approximately 0.5% v/v. Add 1.55 µL Hoechst to 310 µL cell suspension. Incubate 5 minutes at 37°C. Go to Step 11.
10. Because of the overlap in emissions, dual staining of cells with both $CR(DEVD)_2$ and AO is not recommended; the dyes should be used separately. To stain cells with AO:

    a. Dilute AO to 1:2,000-1:200 (which is 0.05-0.5% v/v) into the final cell suspension. For example, if using AO at 1.0 µM in the final cell suspension, first dilute it 1:100 in PBS; e.g., put 10 µL AO into 990 µL PBS. Pipette the diluted AO into the cell suspension at 1:10; e.g., add 35 µL to 315 µL cell suspension.

    b. Incubate 30 minutes at 37°C.

FIGURE 5: ACRIDINE ORANGE STAINING OF JURKAT CELLS



Jurkat cells were stained with Acridine Orange (AO) in PBS for 60 minutes at 37°C. Jurkat cells stained with AO show orange lysosomal staining (photo A). Photomicrographs were taken using a Nikon Eclipse E800 photomicroscope using a 460- 500 nm excitation filter and a 505- 560 nm emission / barrier filter set at 300X. AO-stained lysosomes appear in photo A; photo B shows the corresponding DIC image of the cells (compare with Figure 4).

c. If viewing under the same filters used for CR(DEVD)$_2$ (excitation at 550 nm; emission >610 nm), cells may be viewed immediately after staining without a wash step - go to Step 11.

d. If viewing under blue (480 nm) excitation and green (540-550 nm) emission wavelengths, any excess AO may have to be washed away as the cells may appear too bright at this range. Brightness will depend on the type of microscope used and the cell line. To wash cells:

i) Gently pellet cells at 200 x g for 3-8 minutes at RT.

ii) Remove and discard supernatant.

iii) Resuspend cells in 300 µL or a similar volume of PBS in which the cells were originally suspended.

11. Place 15-20 µL of cell suspension onto a microscope slide and cover with a coverslip.

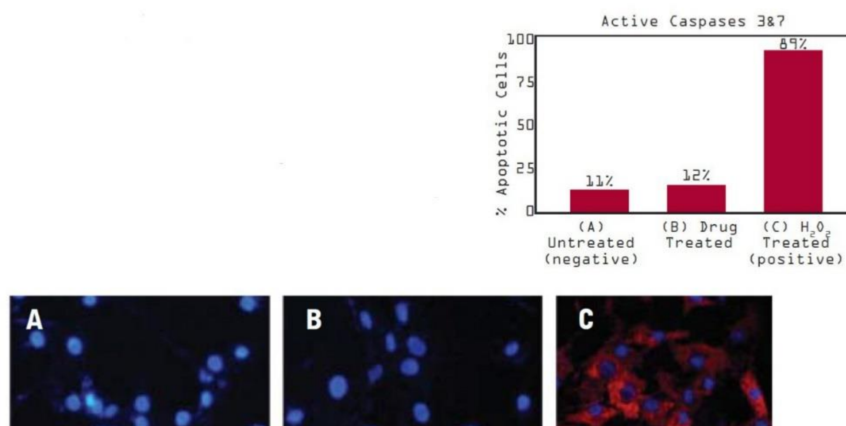
12. Observe cells using a fluorescence microscope equipped with an excitation filter of 550 nm (540- 560 nm) and a long pass >610 nm emission/barrier filter pairing. Select a filter combination that best approximates these settings. Using these filters, positive cells will appear red with brightly stained vacuoles and lysosomes.

    If the samples were stained with both CR(DEVD)$_2$ and Hoechst, and if a multi-wavelength filter option is

available on the fluorescence microscope, the dual staining properties can be examined. Hoechst Stain can be seen using a UV-filter with excitation at 365 nm and emission at 480 nm.

As AO exhibits a very broad emission range, one of several filter pairings may be used. The same excitation/emission pairing filters used to view $CR(DEVD)_2$ may be used: a 550 nm (540-560 nm) excitation and long pass >610 nm emission/barrier filter pairing. With this pairing, the lysosomes appear red. When illuminating with a blue light (480 nm) excitation filter, a green light (540-550 nm) emission/barrier filter combination works well. Lysosomes will appear yellowish green instead of red.

FIGURE 6: QUANTIFYING THE APOPTOTIC EFFECTS OF A DRUG AND CONTROLS ON PULMONARY ARTERY SMOOTH MUSCLE CELLS



Enzo Life Sciences' $CR(DEVD)_2$ (BML-KI224), was used to quantify apoptosis via caspase 3&7 activity in human pulmonary artery smooth muscle cells (PASMC). Cells were treated with: a negative control condition (A); a drug that inhibits proliferation of PASMCs (B); or 1 mM H_2O_2 as a positive control to induce apoptosis (C). Cells were then labeled with $CR(DEVD)_2$ to detect caspases 3&7 and with Hoechst to stain nuclei blue. Cells in red ($CR(DEVD)_2$) contain active caspases 3&7 and have less intense blue nuclei (Hoechst) than healthy cells, which have bright blue nuclei. Only 11% of untreated cells (A) and 12% of drug-treated cells (B) were apoptotic (red) compared with 89% of cells treated with H_2O_2 (C). Although this drug inhibits proliferation of PASMCs, it does not induce apoptosis. Research done by Dr. Frederic Perros, et al., Universite Paris-Sud 11; INSERM U764, Clamart; INSERM U841, Hopital Henri-Mondor, Creteil, France[16]

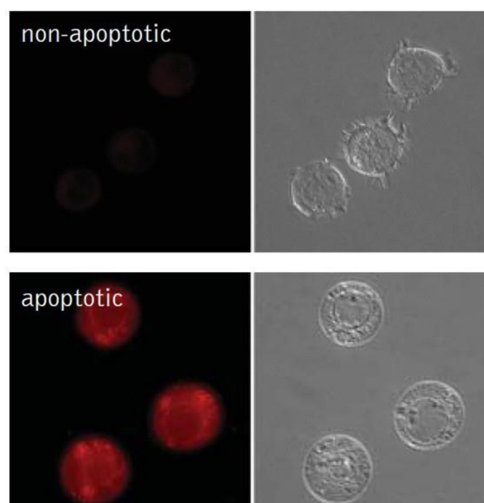
8. Microscopy Analysis of Adherent Cells

1. Seed 104-105 cells onto a sterile coverslip in a 35 mm petri dish or onto chamber slides, or grow in a plate (Figure 2).
2. Grow cells until 80% confluent. This usually takes about 24 hours but will vary with each cell line.
3. Expose cells to the experimental conditions and create positive and negative controls (Section 2) or induce cells to undergo apoptosis (Section 3).
4. Reconstitute $CR(DEVD)_2$ to form the stock solution (Section 4):
   a. Use 100 µL DMSO to reconstitute the vial BML-KI224.
5. When ready to stain cells, dilute the stock 1:5 in diH2O to form the $CR(DEVD)_2$ staining solution (Section 4):
   a. Add 400 µL diH_2O to dilute the vial BML-KI224.
   b. Add $CR(DEVD)_2$ staining solution at approximately 1:30 and gently mix to ensure an even distribution of $CR(DEVD)_2$. For example, add 10 µL staining solution to 300 µL cells forming a final volume of 310 µL. Do not add $CR(DEVD)_2$ to cells that will be stained with AO: add a placebo instead, such as diH2O (Step 10).

7. Incubate 30-60 minutes at 37°C.

8. Remove the media from the cell monolayer surface and rinse twice with PBS, 1 minute per rinse. At this point, cells can be analyzed (Step 12) or stained with Hoechst (Step 9) or unstained cells can be labeled with AO (Step 10).

FIGURE 7: NEGATIVE VS. APOPTOTIC THP-1 CELLS



Using Enzo Life Sciences' CV-Caspase 3&7 Substrate Kit (BML-AK118-0001) to detect DEVDase activity in THP-1 cells, there is a clear differential between nonapoptotic (negative, top) and apoptotic (positive, bottom) cells. THP-1 cells were incubated with DMSO (top) or 1 µM staurosporine (BML-EI156; bottom) for 3 hours at 37°C to induce DEVDase activity. Cell cultures were subsequently stained with CR(DEVD)$_2$ for 1 hour at 37°C. Left panels contain fluorescence images obtained using a Nikon Eclipse E800 photomicroscope equipped with a 100 W mercury lamp and excitation (510-560 nm) and emission (570-620 nm) filter pairings. Right panels contain the corresponding differential-interference- contrast (DIC) image (compare with Jurkat cells in Figure 8).

9. If cells are to be labeled with Hoechst Stain, add it at approximately 0.5% v/v. Add 1.55 µL Hoechst to 310 µL cells labeled with CR(DEVD)$_2$ and control samples. Incubate 5-10 minutes at 37°C. Go to Step 11.

10. Because of the overlap in emissions, dual staining of cells with both CR(DEVD)$_2$ and AO is not recommended; the dyes should be used separately. To stain cells that have not been exposed to CR(DEVD)$_2$:

    a. Dilute AO at 1:2,000-1:200 (which is 0.05-0.5% v/v) into the final cell volume. For example, if using AO at 1.0 µM in the final cell volume, it must be diluted 1:1,000. First dilute it 1:100 in PBS; e.g., add 10 µL AO to 1,000 µL PBS. Pipette the diluted AO to the cells at 1:10; e.g., add 35 µL diluted AO to 315 µL cell media.

    b. Incubate 30 minutes at 37°C.

    c. Remove the media from the cell monolayer surface. Rinse twice with PBS, 1 minute per rinse.

11. Mount the coverslip with cells facing down onto a drop of PBS. If a chamber-slide was used, pull off the plastic frame and add a drop of PBS to the cell surface and cover with a coverslip.

12. Observe cells using a fluorescence microscope equipped with an excitation filter of 550 nm (540- 560 nm) and a long pass >610 nm emission/barrier filter pair. Select a filter combination that best approximates these settings. Using these filters, positive cells stained with CR(DEVD)$_2$ will appear red with more brightly stained vacuoles and lysosomes.

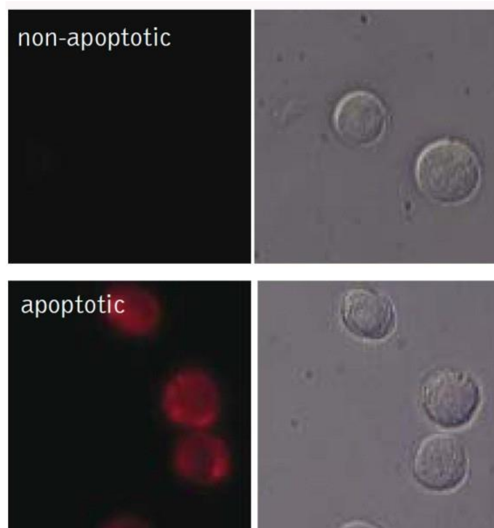
If samples were stained with both CR(DEVD)$_2$ and Hoechst, and if a multi-wavelength filter option is available on the fluorescence microscope, the dual

staining properties of the sample can be examined. Hoechst Stain can be seen using a UV-filter with excitation at 365 nm and emission at 480 nm.

As AO exhibits a very broad emission range, one of several filter pairs may be used. The same excitation/emission pairing filters used to view $CR(DEVD)_2$ may be used: an excitation filter of 550 nm (540-560 nm) and a long pass >610 nm emission/barrier filter. With this pairing, the lysosomes appear red.

When illuminating with a blue light (480 nm) excitation filter, a green light (540-550 nm) emission/barrier filter combination works well. Lysosomes will appear yellowish green instead of red.

FIGURE 8: DEVDASE ACTIVITY IN JURKAT CELLS



Following the same protocol outlined in Figure 7, Jurkat cells were analyzed to detect DEVDase activity. Negative cells do not fluoresce (top), while apoptotic cells fluoresce red using $CR(DEVD)_2$ (bottom). Left panels contain the fluorescence image; right panels contain the DIC image (compare with THP-1 cells in Figure 7).

9. Fluorescence Plate Reader Staining Protocol

1. Prepare cell populations. Cell concentrations should be 2-8 x 10^6 cells/mL. If this is too dense for the cell line, induce apoptosis first, then concentrate the cells and stain with $CR(DEVD)_2$. Cell concentration can be achieved by low speed centrifugation (<400 x g at RT) for 5 minutes.

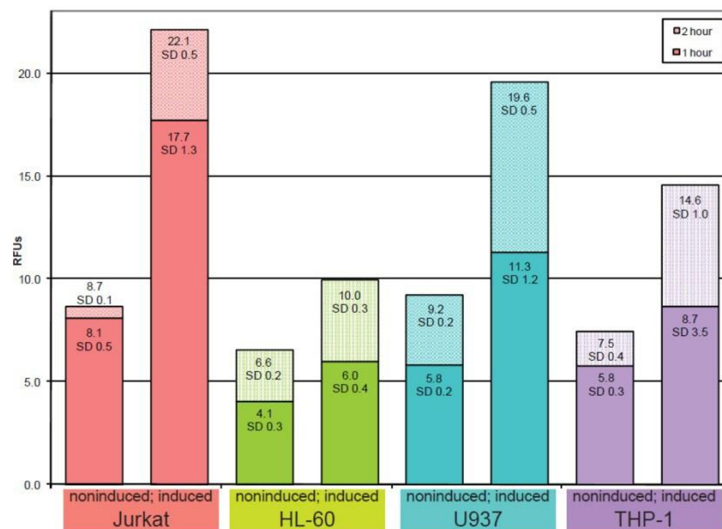
2. Expose cells to the experimental conditions and create

positive and negative controls (Section 2) or induce cells to undergo apoptosis (Section 3).

3. Transfer 300 µL cell suspension into sterile tubes or a black microtiter plate. Do not use clear plates. Avoid bubbles. Larger cell volumes may also be used, but additional $CR(DEVD)_2$ substrate will be required per sample.

4. When ready to label with the $CR(DEVD)_2$ staining solution, cells should be at least 2×10^5 cells/100 µL aliquot (equal to 2×10^6 cells/mL) for each microtiter plate well.

FIGURE 9: QUANTIFICATION OF CASPASE ACTIVITY IN 4 CELL LINES USING A FLUORESCENCE PLATE READER



DEVDase activity in Jurkat, HL-60, U937, and THP-1 cells was quantified using Enzo Life Sciences' CV- Caspase 3&7 fluorogenic substrate, $CR(DEVD)_2$ (BML- KI224), and analyzed with a fluorescence plate reader. Cells were incubated with 1 µM staurosporine (BML-EI156) or DMSO control for 3 hours (Jurkat cells) or 4 hours (HL-60, U937, THP-1 cells) at 37º C to induce caspase activity. Cells were incubated with $CR(DEVD)_2$ for 1-2 hours to reveal the increase in intracellular DEVDase activity associated with apoptosis induction. DEVDase activity in each cell line is shown after 1 hour (solid bars) and 2 hour (spotted bars) exposures to the $CR(DEVD)_2$. Cells were analyzed using a Molecular Devices Gemini XS fluorometric plate reader set at 590 nm excitation, 640 nm emission, with a 630 nm cut-off filter.

5. Reconstitute $CR(DEVD)_2$ to form the stock solution (Section 4):
   a. Use 100 µL DMSO to reconstitute the vial BML-KI224.
6. When ready to stain cells, dilute the stock 1:5 in diH_2O to form the $CR(DEVD)_2$ staining solution (Section 4):

a. Add 400 µL diH_2O to dilute the vial BML-KI224.

7. Add 20 µL $CR(DEVD)_2$ staining solution directly to 300 µL cell sample. If different cell volumes are used, add $CR(DEVD)_2$ staining solution at approximately 1:15. Due to sensitivity limitations, plate readers require a higher concentration of $CR(DEVD)_2$ for detection compared with microscopes.
8. Gently mix the cells. This can be done by gently aspirating and expelling the cells with a pipette. To minimize cell shearing, cut the tip of the pipette to enlarge the hole.
9. Incubate cells for at least 60 minutes at 37°C protected from light. As cells settle to the bottom, gently resuspend them approximately every 20 minutes to ensure $CR(DEVD)_2$ is evenly dispersed among all cells.
10. Read the 300 µL sample as one sample or split it into 3 wells of 100 µL each. If cells were stained in a tube, transfer 100-300 µL into a well of a black microtiter plate.
11. Measure the fluorescence intensity of the red fluorescent cresyl violet fluorophore. Set the plate reader to perform an endpoint read. $CR(DEVD)_2$ has an optimal excitation and emission wavelength tandem of 592 nm and 628 nm, respectively. Select the filter pairings that best approximate these settings. If available, use a cut-off filter at 630 nm to filter out shorter wavelength excitation interference (Figure 9).

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Product Manual

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