Magic Red®Caspase-3/7 Assay Kit

Catalog #935 & #936

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Not for use in diagnostic procedures.

1. INTRODUCTION

ICT's Magic Red® Caspase-3/7 Assay Kit enables researchers to detect and monitor in vitro apoptosis over time via intracellular caspase activity. The Magic Red (MR) 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 Magic Red, add the substrate directly to the cell culture media, incubate, and analyze. Because MR is cell-permeant, it easily penetrates the cell membrane and the membranes of the internal cellular organelles - no lysis nor permeabilization steps are required. If caspase-3/7 enzymes (DEVDases) are active, they will cleave the intact (quenched) substrate and release the cresyl violet fluorophore, which will greatly enhance the cresyl violet fluorescence potential[1]. The red fluorescent product will often aggregate inside lysosomes[2] (Figures 2 and 4); caspases are not lysosomal enzymes. As protease activity progresses and more MR substrate is cleaved, the red fluorescent signal potential will intensify, enabling researchers to watch it increase over time (Figure 3) and quantify apoptosis (Figures 7 and 10-12).

There is no interference from pro-caspases or inactive forms of the enzymes. If the treatment or experimental condition 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[4-7]. 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[8]. Caspase-3 is the predominant effector caspase in apoptosis with few exceptions, such as MCF-7 cells, which are deficient in caspase-3 (Figures 2, 4, and 5).

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[9]. ICT's MR caspase-3/7 substrate contains a 4 amino acid sequence, aspartylglutamylvalanylaspartic acid (DEVD), which is the optimal target sequence for caspases 3 and 7[10].

Two copies of this sequence are coupled to a photostable red fluorophore, cresyl violet, to create the Magic Red caspase-3/7 substrate

Assess caspase-3/7 activity in whole cells over time using MR-$(DEVD)_2$.

MR-$(DEVD)_2$. In the intact MR-(DEVD)2 substrate, the fluorescence has been quenched. Maximum fluorescence potential is achieved upon cleavage of both DEVD side chains by activated caspases 3 and 7.

MR 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 non-apoptotic cells will exhibit very low levels of background red fluorescence evenly distributed throughout the cell (Figures 2-4, 7-9). 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 display brighter red fluorescence than cells in earlier stages of apoptosis.

The MR fluorophore, cresyl violet, fluoresces red when excited at 550-590 nm[11]. The red fluorescent signal can be monitored with a fluorescence microscope (Figures 2-4, 7-9), plate reader (Figure 10), or flow cytometer (Figures 11 and 12). It has an optimal excitation of 592 nm and emission of 628 nm in aqueous solutions[12]. At these higher excitation wavelengths, the amount of cell-mediated auto-fluorescence is minimal[10]. The excitation peak is rather broad, allowing good excitation efficiency at 540-560 nm. In flow cytometry applications, optimal results can be achieved using an orange 594 nm laser and a 685/35 filter pairing, or similar. However, good results have also been generated using a more common 640 nm red laser excitation with a 675/25 filter pairing.

To detect caspase-3/7 activity using an inhibitor, use ICT's FLICA® kits. To learn more about all of ICT's products, please visit www.immunochemistry.com or call 1-800-829-3194.

For microscopy usage, Hoechst 33342 is included with the kit to concurrently label nuclei after labeling with MR (Figures 2 and 7). Acridine orange (AO) is also included in the kit to identify lysosomes and other intracellular organelles (Figures 5 and 6).

In addition to the Magic Red Caspase-3/7 Assay Kit, ICT offers 3 other Magic Red assay kits to detect cathepsins B, K, or L. ICT also offers FLICA® caspase assays to detect caspase-3/7 using a green, red, or far red inhibitor. Magic Red is for research use only. Not for use in diagnostic procedures.

2. KIT CONTENTS

Kit #935, Trial Size (25-50 tests), contains:				
Quantity	**Reagent**	**Part#**	**Size**	**Storage**
1 vial	Magic Red® Caspase-3/7 Substrate, MR-$(DEVD)_2$	6131	25-50 tests / vial	Refrigerate or freeze
1 vial	Hoechst 33342	639	1 mL at 200 µg/mL	Refrigerate
1 vial	Acridine Orange	6130	0.5 mL at 266 µg/mL (1 mM)	Refrigerate
Kit #936, Standard Size (100-200 tests), contains:				
Quantity	**Reagent**	**Part#**	**Size**	**Storage**
1 vial	Magic Red® Caspase-3/7 Substrate, MR-$(DEVD)_2$	6132	100-200 tests / vial	Refrigerate or freeze
1 vial	Hoechst 33342	639	1 mL at 200 µg/mL	Refrigerate
1 vial	Acridine Orange	6130	0.5 mL at 266 µg/mL (1 mM)	Refrigerate

3. STORAGE

- Store the unopened kit and each unopened component at 2-8°C until the expiration date.
- Store the unopened Magic Red reagent refrigerated or frozen.
- Once reconstituted with DMSO, use Magic Red reagent immediately, or store ≤-20°C up to 6 months, protected from light and thawed no more than twice during that time.
- Store Hoechst refrigerated; do not freeze.
- Store Acridine Orange (AO) refrigerated; do not freeze.

4. SAFETY DATA SHEETS (SDS)

Safety data sheets are available online at www.immunochemistry.com or by calling 1-800-829-3194 or 952-888-8788.

5. RECOMMENDED MATERIALS

- DMSO, 100-400 µL to reconstitute Magic Red reagent
- diH_2O, 400-1600 µL to dilute Magic Red reagent
- Phosphate buffered saline (PBS) pH 7.4, 100 mL
- Cultured cells treated with the experimental conditions ready for staining; do not use paraffin-embedded tissues
- Reagents to induce apoptosis and create controls, such as staurosporine (catalog #6212) or camptothecin (catalog #6210)
- Green Live/Dead Stain (catalog #6342)
- Hemocytometer
- Centrifuge at 200 x g
- 15 mL polypropylene centrifuge tubes (1/sample)
- Black 96-well microtiter plate, flat bottom, non-treated, non-sterile (ICT catalog #266). 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.
- Slides and coverslips
- FACS tubes
- Ice or refrigerator
- 12 x 75 mm glass or polypropylene tubes

6. DETECTION EQUIPMENT

- Magic Red excites at 550-590 nm and emits >610 nm. It has an optimal excitation and emission wavelength tandem in aqueous solutions of 592 nm and 628 nm, respectively (Figure 1).
- Hoechst 33342 can be visualized using a UV-filter with excitation at 365 nm and emission at 480 nm (Section 11).
- AO excites at 480 nm and emits >540 nm (Section 12). 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 3 and 4).

  If the samples were stained with both MR-$(DEVD)_2$ and Hoechst, the dual staining properties can be examined using a multi-wavelength filter (Figures 2 and 7). Hoechst 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 pairing used to view MR-$(DEVD)_2$ 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 5 and 6).

- **Fluorescence Plate Reader:**

  Magic Red 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 interference (Figure 10).

  Cells are typically analyzed in a black 96-well microtiter plate. If transferring cells, use a flat-bottom, non-treated, non-sterile plate, such as Costar #3915 (ICT catalog #266). 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.

- **Flow Cytometer:**

  Use laser/filter pairings that best approximate excitation and emission optima.

  A yellow-green laser with 561 nm excitation or an orange laser with 594 nm excitation and emission filter pairings >600 nm are preferred.

  A common 640 nm excitation laser paired with a 675/25 emission filter set can also produce good results.

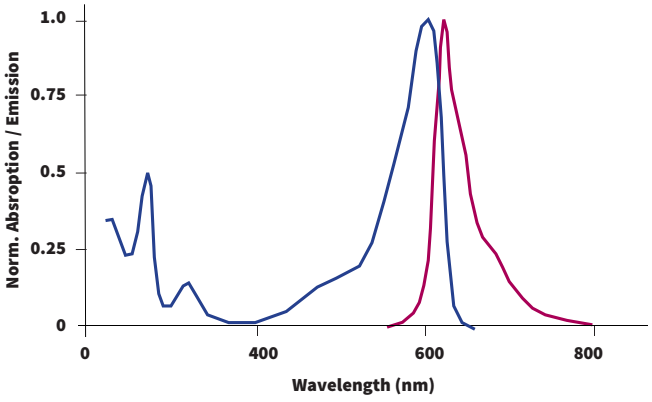
Figure 1: Cresyl Violet Perchlorate Excitation and Emission Spectra in ETOH

Absorption λ_{max}: 603 nm, 320 nm

Emission λ_{max}: 622 nm

Solvent: EtOH

Molar Abs. Coefficient: 83,000 $M^{-1}cm^{-1}$.



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7. EXPERIMENTAL PREPARATION

Staining apoptotic cells with Magic Red can be completed within a few hours. However, Magic Red 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 MR-$(DEVD)_2$ 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 Magic Red staining solution is 10-20 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.

Cells undergoing apoptosis will generate a stronger red fluorescence with MR-$(DEVD)_2$ than non-apoptotic cells of the same lineage. To optimize this assay, adjust the amount of Magic Red substrate used to stain cells and the incubation time. Determine which parameters produce the greatest difference in the fluorescent signal between induced and non-induced cell populations.

- Do not use Magic Red with paraffin-embedded tissues as the chemicals used for paraffin-embedding may denature and inactivate the substrate.
- Because of the overlap in emissions, dual staining of cells with both Magic Red and Acridine Orange (AO) will yield confusing results and is not recommended; these dyes should be used separately.
- Hoechst 33342 can be used with Magic Red to label nuclei (Figures 2 and 7).

8. CONTROLS

It is highly recommended that two sets of controls be run:

1: One positive population of cells that was induced to undergo apoptosis or trigger caspase-3/7 activity.

2: A placebo population of cells that received just the vehicle used to deliver the apoptosis-inducing agent.

Create positive controls by inducing apoptosis (Section 9). 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 MR-$(DEVD)_2$, Hoechst 33342, and Acridine Orange, make 10 control populations:

1&2: Unlabeled, induced and non-induced populations.

3&4: MR-$(DEVD)_2$-labeled, induced and non-induced populations.

5&6: MR-$(DEVD)_2$-and Hoechst-labeled, induced and non-induced populations.

7&8: Hoechst-labeled, induced and non-induced populations.

9&10: AO-labeled, induced and non-induced populations.

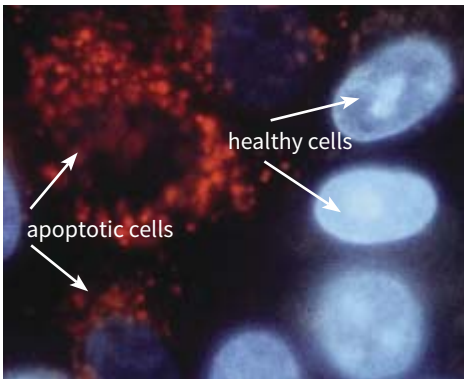
9. APOPTOSIS INDUCTION

Prior to commencing the experiment, determine a reproducible method for obtaining a positive control by triggering caspase -3 and -7 activity. This process varies significantly with each cell type.

For example, apoptosis via caspases -3 and -7 may be induced in Jurkat cells with 2-4 µg/mL camptothecin or 1-2 µM staurosporine for >4 hours.

Figure 2: Dual Staining of MCF-7 Cells

Apoptotic MCF-7 cells were dually stained using ICT's Magic Red® MR-$(DEVD)_2$ fluorogenic caspase-3/7 substrate and Hoechst 33342 nuclear stain (catalog #936). MCF-7 cells were exposed to 0.15 µM camptothecin (catalog #6210) for 24 hours at 37°C, then stained with MR-$(DEVD)_2$ 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 MR-$(DEVD)_2$), 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, NY.



10. PREPARATION OF MAGIC RED® SUBSTRATE

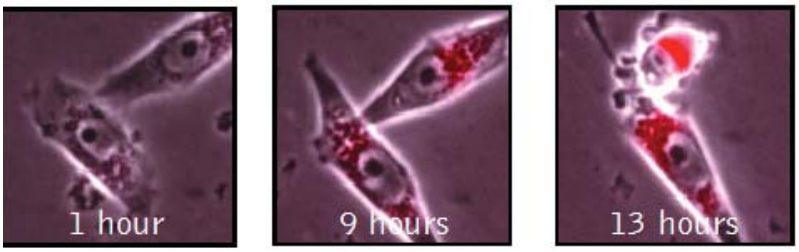
Magic Red Caspase-3/7 Substrate is supplied as a lyophilized powder that is dried onto the base of the amber glass vial. It must first be reconstituted in DMSO, forming the stock concentrate, and then diluted 1:5 in diH_2O to form the final staining solution. For best results, the staining solution should 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 Magic Red in DMSO. It is vialed in 2 sizes: trial and standard. Trial kits contain a 25-50 test size vial; standard kits contain a 100-200 test size vial. The reconstitution volume varies based on the vial size:
   - Reconstitute the trial size vial (#6131) with 100 µL DMSO.
   - Reconstitute the standard size vial (#6132) with 400 µL DMSO.
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. Mix by inverting or vortexing the vial at RT. Use the staining solution within 15 minutes of dilution to prevent non-specific substrate hydrolysis.
   - The reconstituted trial size vial (#6131) contains 100 µL of the stock. Dilute it 1:5 by adding 400 µL diH_2O; this yields 500 µL of the staining solution.
   - The reconstituted standard size vial (#6132) contains 400 µL of the stock. Dilute it 1:5 by adding 1,600 µL diH_2O; this yields 2 mL of the staining solution.
   - For other volumes, 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.

Figure 3: 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. ICT's Magic Red® Caspase-3/7 Substrate, MR-$(DEVD)_2$ (catalog #936), 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.



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 (#639) is provided ready-to-use at 200 µg/mL. Hoechst can be used concurrently with the Magic Red substrate to label nuclei (Figures 2 and 7).

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.

- **Warning:** Hoechst 33342 is a potential mutagen. Gloves, protective clothing, and eye wear are strongly recommended. When disposing, flush sink with copious amounts of water. See SDS for further information.

12. ACRIDINE ORANGE

Acridine Orange (AO) is a chelating dye that can be used to reveal lysosomes, nuclei, and nucleoli (Figures 5 and 6). AO is provided at 1 mM (#6130). It may be used neat or diluted in diH_2O 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.

As AO exhibits a very broad emission range, several filter pairings can be used to view this stain. The same excitation/emission filter pairing used to view MR-$(DEVD)_2$ 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. 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 MR-$(DEVD)_2$ 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 SDS for further information.

13. 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 (Sections 8 and 9).
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 5 - 10 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 MR-$(DEVD)_2$ staining solution may be required.
5. Reconstitute MR-$(DEVD)_2$ with DMSO to form the concentrated stock solution (Section 10):
   - Reconstitute the trial size vial (#6131) with 100 µL DMSO.
   - Reconstitute the standard size vial (#6132) with 400 µL DMSO.
6. When ready to stain cells, dilute the stock 1:5 in diH_2O to form the MR-$(DEVD)_2$ staining solution (Section 10):
   - Dilute the trial size vial (#6131) by adding 400 µL diH_2O.
   - Dilute the standard size vial (#6132) by adding 1,600 µL diH_2O.
7. Add 10 µL of the MR-$(DEVD)_2$ staining solution to each 300 µL cell suspension and mix thoroughly. If different cell volumes are used, add the MR-$(DEVD)_2$ staining solution at a ratio of approximately 1:30. For example, add 35 µL MR-$(DEVD)_2$ staining solution to 1,000 µL of cell suspension forming a final volume of 1,035 µL. Do not add MR-$(DEVD)_2$ 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 to ensure even distribution of the Magic Red substrate. After the incubation, cells can be stained with Hoechst (Section 11), or unstained cells may be labeled with AO (Section 12).
9. If cells are to be labeled with Hoechst, add it at approximately 0.5% v/v. For example, if the cell suspension is 310 µL, add 1.55 µL Hoechst. Incubate 5 minutes at 37°C. Go to Step 11.

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Figure 4: Caspase Activity in MCF-7 Cells

DEVDase activity was detected in apoptotic MCF-7 cells using ICT's Magic Red® Caspase-3/7 fluorogenic substrate, MR-$(DEVD)_2$. Apoptosis was induced in MCF-7 cells by treating them with 0.15 µM camptothecin (catalog #6210) for 24 hours at 37°C. Cells were then exposed to MR-$(DEVD)_2$ 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 5). Photo B shows the corresponding interference contrast image of the cells. Data courtesy of Dr. Zbigniew Darzynkiewicz (Brander Cancer Research Center Institute, New York, NY).

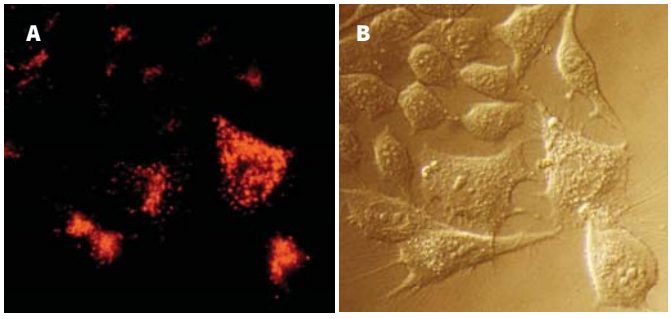
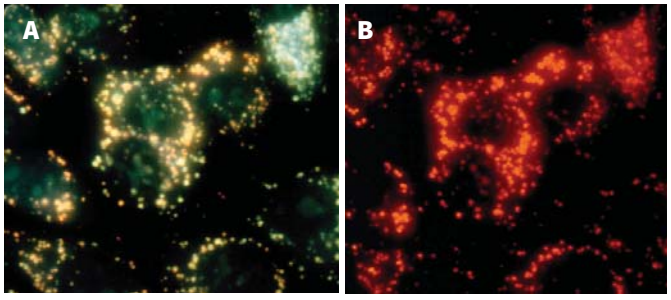


Figure 5: Acridine Orange Staining of MCF-7 Cells

Apoptosis was induced in MCF-7 cells by treating them with 0.15 µM camptothecin (catalog #6210) 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 a green light excitation (540 nm) with a long pass >640 nm barrier filter (B, lysosomes appear red; compare with MR staining of MCF-7 cells in Figure 4 and with Jurkat cells in Figure 6). Experiment performed in the laboratory of Dr. Zbigniew Darzynkiewicz (Brander Cancer Research Center Institute, New York, NY).



10. Because of the overlap in emissions, dual staining of cells with both MR-$(DEVD)_2$ and AO is not recommended; the dyes should be used separately. To stain cells with AO:
    a. Dilute the 1 mM AO reagent 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, 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.
    c. If viewing under the same filters used for MR-$(DEVD)_2$ (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 5 - 10 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 MR-$(DEVD)_2$-stained 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 MR-$(DEVD)_2$ 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 filters used to view MR-$(DEVD)_2$ 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: 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 (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 5).

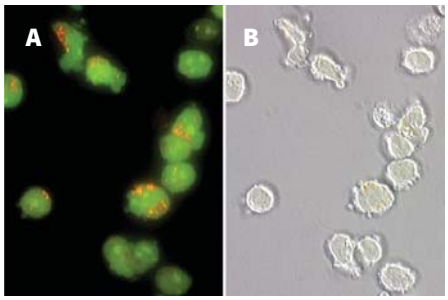
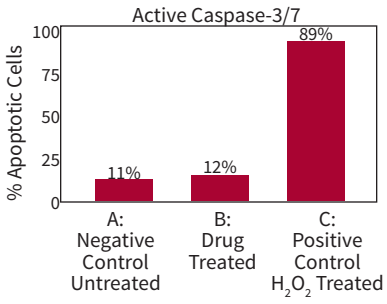
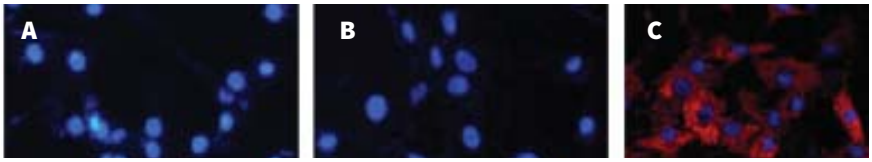


Figure 7: Quantifying the Apoptotic Effects of a Drug on Pulmonary Artery Smooth Muscle Cells

ICT's Magic Red® caspase-3/7 fluorogenic substrate, MR-$(DEVD)_2$ (catalog #936), was used to quantify apoptosis via caspase-3/7 activity in human pulmonary artery smooth muscle cells (PASMC). Cells were treated with: (A) a negative control condition; (B) a drug that inhibits proliferation of PASMCs; or (C) 1 mM H_2O_2 as a positive control to induce apoptosis. Cells were then labeled with MR-$(DEVD)_2$ to detect active caspase-3/7 and with Hoechst to stain nuclei blue. Cells in red (MR-$(DEVD)_2$) contain active caspase-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 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., Université Paris-Sud 11; INSERM U764, Clamart; INSERM U841, Hôpital Henri-Mondor, Créteil, France[13].





A: Negative Control; 11% Apoptotic

B: Drug Treatment to Inhibit Proliferation of PASMC; 12% Apoptotic

C: Positive Control using H_2O_2 to Induce Apoptosis; 89% Apoptotic

14. MICROSCOPY ANALYSIS OF ADHERENT CELLS

1. Seed 10^4-10^5 cells onto a sterile coverslip in a 35 mm petri dish or onto chamber slides, or grow in a plate (Figure 3).
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 (Sections 8 and 9).
4. Reconstitute MR-$(DEVD)_2$ with DMSO to form the concentrated stock solution (Section 10):
   - Reconstitute the trial size vial (#6131) with 100 µL DMSO.
   - Reconstitute the standard size vial (#6132) with 400 µL DMSO.
5. When ready to stain cells, dilute the stock 1:5 in diH_2O to form the MR staining solution (Section 10):
   - Dilute the trial size vial (#6131) by adding 400 µL diH_2O.
   - Dilute the standard size vial (#6132) by adding 1,600 µL diH_2O.
6. Add MR staining solution at approximately 1:30 and gently mix to ensure an even distribution of MR-$(DEVD)_2$. For example, add 10 µL staining solution to 300 µL cells forming a final volume of 310 µL. Do not add MR-$(DEVD)_2$ to cells that will be stained with AO: add a placebo instead, such as diH_2O (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), stained with Hoechst (Step 9), or unstained cells can be labeled with AO (Step 10).
9. If cells are to be labeled with Hoechst, add it at approximately 0.5% v/v. Add 1.55 µL Hoechst to 310 µL cells labeled with MR-$(DEVD)_2$ 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 MR-$(DEVD)_2$ and AO is not recommended; the dyes should be used separately. To stain cells that have not been exposed to MR-$(DEVD)_2$:
    a. Dilute the 1 mM AO reagent 1:2,000-1:200 (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 990 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.
    d. Rinse twice with PBS, 1 minute per rinse.
11. Mount the coverslip with cells facing down onto a drop of PBS on a slide. 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 MR-$(DEVD)_2$-stained 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 MR-$(DEVD)_2$ will appear red with more brightly stained vacuoles and lysosomes.

    If samples were stained with both MR-$(DEVD)_2$ 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 filters used to view MR-$(DEVD)_2$ 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: Negative vs. Apoptotic THP-1 Cells

Using ICT's Magic Red® caspase-3/7 fluorogenic substrate (catalog #936) to detect DEVDase activity in THP-1 cells, there is a clear differential between non-apoptotic (negative, top) and apoptotic (positive, bottom) cells. THP-1 cells were incubated with DMSO (top) or 1 µM staurosporine (catalog #6212; bottom) for 3 hours at 37°C to induce DEVDase activity. Cell cultures were subsequently stained with MR-$(DEVD)_2$ 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 9).

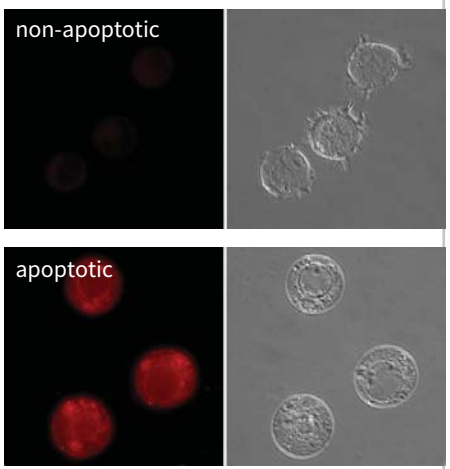
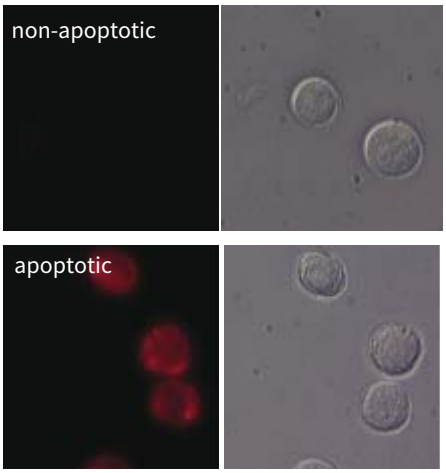


Figure 9: DEVDase Activity in Jurkat Cells

Following the same protocol outlined in Figure 8, Jurkat cells were analyzed to detect DEVDase activity. Negative cells do not fluoresce (top), while apoptotic cells fluoresce red using MR (bottom). Left panels contain the fluorescence image; right panels contain the DIC image (compare with THP-1 cells in Figure 8).



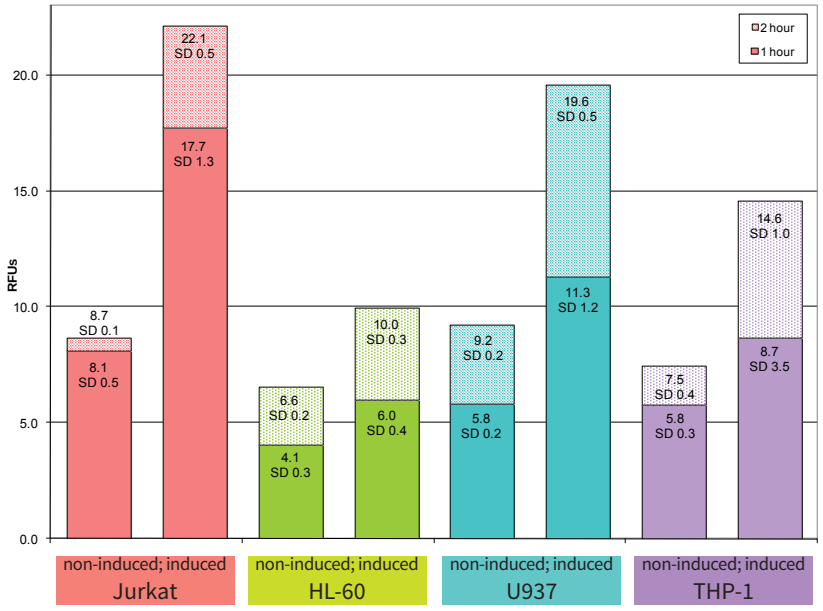
15. FLUORESCENCE PLATE READER STAINING PROTOCOL

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 MR-$(DEVD)_2$. Cell concentration can be achieved by low speed centrifugation (<200 x g at RT) for 5 - 10 minutes.
2. Expose cells to the experimental conditions and create positive and negative controls (Sections 8 and 9).
3. Transfer 300 µL cell suspension into 12 x 75 mm glass or polypropylene tubes or a black microtiter plate (catalog #266). If using a bottom reading instrument, use a plate with black walls and a clear bottom. Avoid bubbles. Larger cell volumes may also be used, but additional MR-$(DEVD)_2$ substrate will be required per sample.
4. When ready to label with the MR staining solution, cells should be at least 2 x 10^5 cells/100 µL aliquot (equal to 2 x 10^6 cells/mL) for each microtiter plate well.
5. Reconstitute MR-$(DEVD)_2$ with DMSO to form the concentrated stock solution (Section 10):
   - Reconstitute the trial size vial (#6131) with 100 µL DMSO.
   - Reconstitute the standard size vial (#6132) with 400 µL DMSO.
6. When ready to stain cells, dilute the stock 1:5 in diH_2O to form the MR-$(DEVD)_2$ staining solution (Section 10):
   - Dilute the trial size vial (#6131) by adding 400 µL diH_2O.
   - Dilute the standard size vial (#6132) by adding 1,600 µL diH_2O.
7. Add 20 µL MR-$(DEVD)_2$ staining solution directly to 300 µL cell sample. If different cell volumes are used, add MR-$(DEVD)_2$ staining solution at approximately 1:15. Due to sensitivity limitations, plate readers require a higher concentration of MR-$(DEVD)_2$ 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 MR-$(DEVD)_2$ is evenly dispersed among the cells.
10. Read the 300 µL sample as one sample or split into 3 wells of 100 µL each. If cells were stained in a tube, transfer to a black microtiter plate.
11. Measure the fluorescence intensity of the red fluorescent Magic Red cresyl violet fluorophore. Set the plate reader to perform an endpoint read. Magic Red has an optimal excitation and emission wavelength tandem of 592 nm and 628 nm, respectively. Select the filter pairing that best approximates these settings. If available, use a cut-off filter at 630 nm to filter out shorter wavelength interference (Figure 10).

Figure 10: Quantification of Caspase Activity in 4 Cell Lines with a Fluorescence Plate Reader

DEVDase activity in Jurkat, HL-60, U937, and THP-1 cells was quantified using ICT's Magic Red® caspase-3/7 fluorogenic substrate, MR-$(DEVD)_2$ (catalog #936), and analyzed with a fluorescence plate reader. Cells were incubated with 1 µM staurosporine (catalog #6212) or DMSO control for 3 hours (Jurkat cells) or 4 hours (HL-60, U937, THP-1 cells) at 37°C to induce caspase activity. Cells were incubated with MR-$(DEVD)_2$ 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 MR fluorogenic caspase-3/7 substrate. 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. Data courtesy of Dr. Brian Lee, ICT.



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16. SINGLE COLOR FLOW CYTOMETRY ANALYSIS OF SUSPENSION CELLS

1. Expose cells to the experimental conditions and prepare control cell populations; also prepare an unstained control for gating (Sections 8 and 9).
2. Initial cell concentration should be 1 x 10^5 - 1 x 10^6 cells/mL. The optimal cell concentration for staining will vary based on the experimental conditions. In general, flow cytometric analysis has lower cell density requirements than analyzing with a fluorescence plate reader or microscope.
3. Transfer 300 µL cell suspension per sample into FACS tubes. Different sample volumes may be used; however, this will change the amount of Magic Red needed for optimal staining and alter the number of tests per vial.
4. Reconstitute MR-$(DEVD)_2$ with DMSO to form the concentrated stock solution (Section 10):
   - Reconstitute the trial size vial (#6131) with 100 µL DMSO.
   - Reconstitute the standard size vial (#6132) with 400 µL DMSO.
5. When ready to stain cells, dilute the stock 1:5 in diH_2O to form the MR-$(DEVD)_2$ staining solution (Section 10):
   - Dilute the trial size vial (#6131) by adding 400 µL diH_2O.
   - Dilute the standard size vial (#6132) by adding 1,600 µL diH_2O.
6. Add MR-$(DEVD)_2$ staining solution at approximately 1:30 and gently mix to ensure an even distribution of MR-$(DEVD)_2$. For example, add 10 µL staining solution to 300 µL cells forming a final volume of 310 µL.
7. Incubate 30-60 minutes at 37°C protected from light. Gently resuspend cells approximately every 20 minutes throughout the staining process.
8. For single-color analysis, a 640 nm red excitation laser or comparable can be used with an emission filter pairing that best approximates 675/25 (often FL4). If available, use laser filter pairings that more closely resemble the excitation and emission optima of Magic Red (592 nm and 628 nm, respectively).
9. Run the unstained control and generate an FSC-A vs. SSC-A plot to gate whole cells from cellular debris. Generate a histogram displaying log fluorescence on the channel being used to detect Magic Red® (X-axis) versus the number of cells (Y-axis). If possible, adjust the voltage to place the unstained sample in the first decade of the log scale. Voltage adjustment is not possible on some instruments, such as the Accuri C6 (Figures 11 and 12).
10. Run the experimental samples and observe data on the above histogram. Caspase negative/MR-$(DEVD)_2$ (-) cells will fall within the lower log fluorescence output decades of the X-axis, whereas caspase positive/MR-$DEVD)_2$ (+) cells will appear as a shoulder or as a separate peak on the right side of the negative peak histogram (Figure 11).

17. DUAL COLOR FLOW CYTOMETRY ANALYSIS OF SUSPENSION CELLS

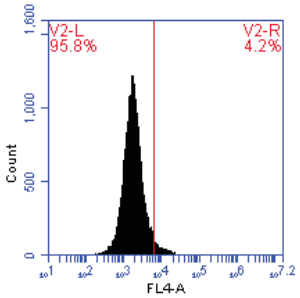
Because of minimal spectral overlap with the green/FITC channel (often FL1), Magic Red can be easily combined with green fluorochromes for dual color analysis studies. When MR-$(DEVD)_2$ is used in combination with ICT's Green Live/Dead Stain (catalog #6342), four populations can be identified (Figure 12):

- **Early Apoptosis:**
  MR-$(DEVD)_2$ (+) / Green Live/Dead Stain (-)
  Early to mid-stage apoptotic cells
  Intact cell membranes
  Active caspase-3/7 enzymes
  Figure 12, Lower Right (LR)
- **Late Apoptosis:**
  MR-$(DEVD)_2$ (+)/ Green Live/Dead Stain (+)
  Late stage apoptotic cells
  Compromised cell membranes
  Active caspase-3/7 enzymes
  Figure 12, Upper Right (UR)
- **Necrosis:**
  MR-$(DEVD)_2$ (-) / Green Live/Dead Stain (+)
  Dead cells
  Compromised cell membranes
  Figure 12, Upper Left (UL)

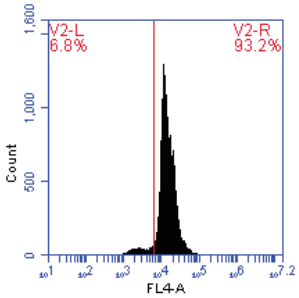
Figure 11: Single Color Analysis Using Flow Cytometry

Jurkat cells were treated with a negative control (left) or staurosporine, an apoptosis-inducing agent (right), for 4 hours, then labeled with ICT's Magic Red® caspase-3/7 substrate, MR-(DEVD)2 (kit #936), for 1 hour. Cells were read on an Accuri C6 flow cytometer using red laser excitation at 640 nm and a 675/25 emission filter pairing (FL4).

Treatment with the negative control induced caspase activity in only 4.2% of the cell population (left, V2-R), whereas treatment with staurosporine induced caspase activity in 93.2% of the experimental cells (right, V2-R). This is a ratio of 22:1. Data courtesy of Ms. Tracy Hanson, ICT, 082313.



Negative Control, Non-Induced



Positive Control, Apoptosis-Induced

	V2-L MR-$(DEVD)_2$ Negative	V2-R MR-$(DEVD)_2$ Positive
Negative, Non-Induced (left)	95.8%	4.2%
Positive, Induced (right)	6.8%	93.2%
Ratio		22:1

- **Live:**
  MR-$(DEVD)_2$ (-) / Green Live/Dead Stain (-)
  Live, healthy cells
  Figure 12, Lower Left (LL)

For meaningful dual color data analysis, fluorescence emission values must be corrected for spectral overlap through compensation techniques. The goal of compensation is to correctly quantify each dye with which a cell is labeled. This is done by subtracting a portion of one detector's signal from another, leaving only the desired signal. When the data has been correctly compensated, the median fluorescence intensity (MFI) values in non-primary detectors of any single-stained control sample should be the same as an unstained control sample. Depending on the instrument and software, compensation may be set either in the instrument hardware before samples are run or within the software after data collection.

Follow Section 16, Steps 1-8; include single-color compensation controls.

9. Perform the staining procedure for the second fluorochrome according to the manufacturer's protocol.
10. Run the unstained control and generate an FSC-A vs. SSC-A plot to gate whole cells from cellular debris. Generate two histograms displaying:
    a. Log fluorescence on the channel being used to detect Magic Red® (X-axis) versus the number of cells (Y-axis).
    b. Log fluorescence on the FL channel being used for the second fluorochrome (X-axis) versus the number of cells (Y-axis).

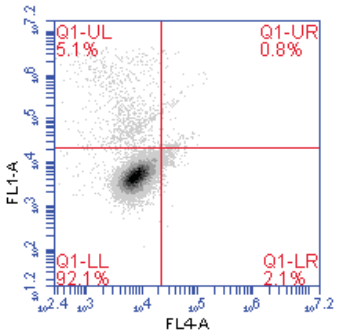
If possible, adjust the voltages to place the unstained sample in the first decade of the log scale on both histograms. The voltages are not changed after this step or the compensation would be made invalid.

11. Run single color controls. While monitoring dual color density plots, adjust compensation to remove spectral overlap from interfering FL channels. A red 640 nm excitation laser or comparable can be used with an emission filter pairing that best approximates 675/25 (often FL4) to measure Magic Red. If available, use laser filter pairings that more closely resemble the excitation and emission optima, e.g. 592 nm and 628 nm, respectively. Read the single color control for the second dye using laser/filter pairings recommended by the manufacturer.
12. Run dual color experimental samples and analyze.

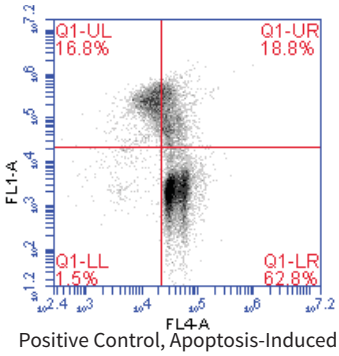
Figure 12: Dual Color Analysis Using Flow Cytometry

Jurkat cells were treated with a negative control (left) or staurosporine, an apoptosis-inducing agent (right), for 4 hours, then labeled with Magic Red® caspase-3/7 substrate, MR-$(DEVD)_2$ (kit #936), for 1 hour, followed by Green Live/Dead Stain (catalog #6342) for 10 minutes.
Cells were read on an Accuri C6 flow cytometer.

Treatment with the negative control induced Magic Red labeling in only 2.9% of the cell population (UR + LR, left density plot), whereas treatment with staurosporine induced Magic Red labeling in 81.6% of the experimental cells (UR + LR, right density plot). Treatment with staurosporine increased the proportion of necrotic or late stage apoptotic from 5.9% (UL + UR, left) to 35.6% (UL + UR, right). Data courtesy of Ms. Tracy Hanson, ICT, 092614.



Negative Control, Non-Induced



Positive Control, Apoptosis-Induced

	Negative Control			Positive, Induced		
Status Quadrant	**Necrosis UL, 5.1%**	**Late Apoptosis UR, 0.8%**	**Necrotic and late apoptotic**	**Necrosis UL, 16.8%**	**Late Apoptosis UR, 18.8%**	**Necrotic and late apoptotic**
Red MR-$(DEVD)_2$	-	+		-	+	
Live/Dead Stain	+	+	**5.9%**	+	+	**35.6%**
Status Quadrant	**Live LL, 92.1%**	**Early Apoptosis LR, 2.1%**		**Live LL, 1.5%**	**Early Apoptosis LR, 62.8%**	
Red MR-$(DEVD)_2$	-	+		-	+	
Live/Dead Stain	-	-		-	-	
Apoptosis Caspase-3/7 Positive		**Apoptotic 2.9%**			**Apoptotic 81.6%**	

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immunochemistry.com