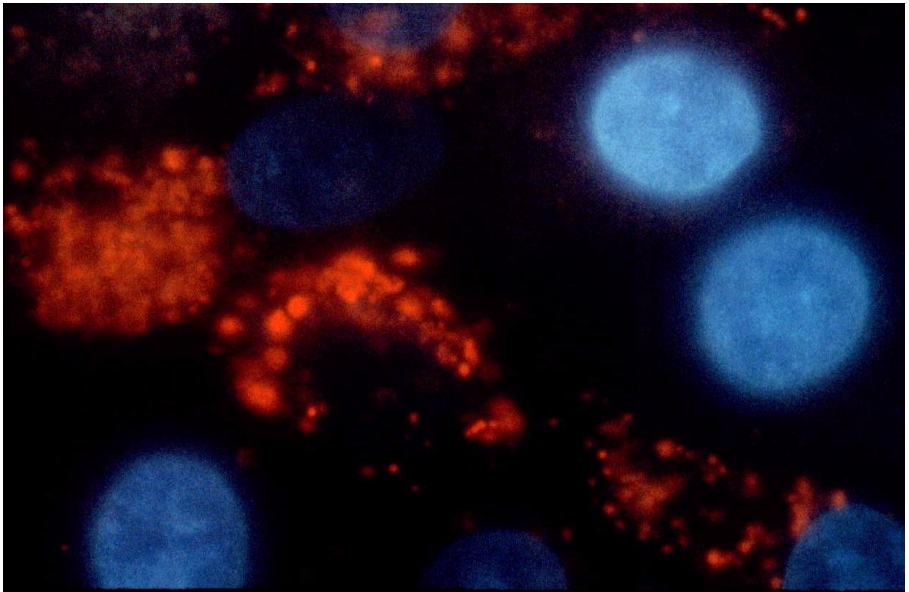


Caspases 3&7-Catalog #s: KF17306 and KF17307

Magic Red™ Caspase Detection Kit

For Research Use Only.



Dual staining of MCF-7 cells with Hoechst 33342 stain and **MR-(DEVD)₂** following 24 hour exposure to 0.15 μ M Camptothecin at 37°C.

For technical questions, please contact us at:

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Magic Red™ Apoptosis Detection Kit Ordering Information

target enzyme	peptide	25-test kit	100-test kit
Caspases 3&7	MR-(DEVD) ₂	KF17306	KF17307
Cathepsin B	MR-(RR) ₂	KF17308	KF17309
Cathepsin K	MR-(LR) ₂	KF17310	KF17311
Cathepsin L	MR-(FR) ₂	KF17312	KF17313

Picture on cover: Dual staining of MCF-7 cells with Hoechst 33342 stain and MR-(DEVD)₂ following 24 hour exposure to 0.15 μM Camptothecin at 37°C. Apoptotic cells bearing orange lysosomal bodies with less intense blue nuclei can be seen intermixed with non-apoptotic cells bearing bright blue nuclei and absent or reduced lysosomal staining. Photo was kindly provided by Dr. Zbigniew Darzynkiewicz (Brander Cancer Research Center Institute, New York, NY); see figure 6.

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1. Introduction

Up-regulation and initiation of the caspase enzyme cascade is the central driving force behind apoptosis (2). Although a number of other intracellular enzyme families, including the cathepsins, calpains, and granzymes, participate in the cell break-down mechanism, the caspase enzyme cascade seems to occupy the central effector role in the cell suicide process (3-6). Caspase 3 is the predominant effector caspase in apoptosis (with few exceptions such as in the MCF-7 caspase-3-deficient cell line).

Caspase enzymes are classified as cysteine proteases based on the mechanism of substrate hydrolysis at their active site. They also require an aspartic acid residue at the C-terminal end (P1 site) of the 4-5 amino acid target sequence (7). Like other intracellular protease systems, caspases are initially synthesized as inactive zymogen precursors which can be rapidly activated upon auto and heterologous enzymatic processing at specific sites containing an aspartic acid (8).

Caspase activity can be detected within whole living cells using Neuromics' Magic Red™ substrate-based MR-Caspase assay kit. Designated as the MR-Caspase product line, this user-friendly kit enables researchers to quickly visualize intracellular caspase activity within their particular experimental cell line.

Neuromics' Magic Red™ detection kit utilizes the fluorophore, cresyl violet. As the 4 amino acid sequence, aspartylglutamylalanylaspatic acid (DEVD) is the optimal sequence for caspase 3 as well as 7 (11), it was coupled to cresyl violet to create the caspase 3/7 substrate, MR-(DEVD)₂ (synthesized by Enzyme Systems Products). When bi-substituted via amide linkage to two target caspase sequence groups {(DEVD)₂}, cresyl violet does not fluoresce (9). Following enzymatic hydrolysis at one or both of the aspartic acid amide linkage sites, the mono and non-substituted cresyl violet fluorophores fluoresce red when excited at 550-590 nm (10).

This MR-Caspase photostable fluorogenic substrate easily penetrates the cell membrane and the membranes of the internal cellular organelles, entering the cell in the non-fluorescent state. In the presence of caspase 3 and 7 enzymes (DEVDases), the 4 amino acid (DEVD) caspase target sequences are cleaved off yielding a red fluorescent product. DEVDase-mediated production of the red fluorophore signals apoptotic activity within that particular cell.

The red fluorescence signal can be monitored using fluorescence microscopy or 96-well microtiter plate fluorometry. The unsubstituted, red fluorescent MR product has an optimal excitation and emission of 592 nm

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and 628 nm respectively (9). At these higher excitation wavelengths, the amount of cell-mediated auto-fluorescence is minimal (11). Fortunately, the excitation peak of this fluorophore is rather broad allowing good excitation efficiency at 540-560 nm. The typical mercury lamp used in fluorescence microscopy has a maximum light output at 542 nm which is quite compatible with the **Magic Red™** substrate. Excitation and emission filter pairs should be selected which best approximate these optimal settings. Good fluorescence photographs can be obtained at 510 – 560 nm excitation and > 610 nm emission. Optimal assay performance was obtained using 590 nm excitation and 640 nm emission filters in the 96-well fluorometer.

Viewing cells through a fluorescence microscope, apoptotic cells will fluoresce red and have pronounced red lysosomes and mitochondria (see figures 1, 3, and 4). Non-apoptotic cells will exhibit very low levels of red fluorescence and it will appear evenly distributed throughout the cell (see figure 2). Cells in more advanced stages of apoptosis, containing peak levels of DEVDase activity, will appear brighter red than cells in earlier stages of apoptosis. Non-apoptotic cell populations will exhibit background levels of red fluorescence.

Using a fluorescence plate reader (with **black** microtiter plates), apoptosis induction can be quantitated as the amount of red fluorescence generated in induced versus non-induced cell populations. Cell populations in more advanced stages of apoptosis will generate a higher RFU intensity than cell populations in earlier stages of apoptosis (see figure 8). A variable base-level of DEVDase activity is present in all cell lines to a varying degree and will be seen in non-apoptotic cell populations. This baseline substrate processing activity could be the result of constitutively synthesized serine proteases which target analogous aspartic acid sequences for hydrolysis.

Hoechst stain is included and can be used to label the cell nuclei after labeling with the **MR-Caspase** reagent. It is revealed under a microscope using a UV-filter with excitation at 365 nm and emission at 480 nm (see figure 6).

In addition, acridine orange (AO) is included in the kit to help identify lysosomes and other intracellular organelles. The acidic pH of the lysosome results in the concentration and aggregation of the AO molecules. Aggregated AO molecules fluoresce orange rather than green thus clearly differentiating the lysosomes from other organelles (see figure 7) (13).

NEUROMICS is initially offering 1 **Magic Red™** Caspase product, the **MR-(DEVD)₂** kit to detect Caspases 3 and 7. (NEUROMICS also offers 3

different Magic Red™ Cathepsin assay kits: MR-(RR)₂ to detect Cathepsin B; MR-(LR)₂ to detect Cathepsin K; and MR-(FR)₂ to detect Cathepsin L.)

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2. Contents of the MR-Caspase Apoptosis Detection Kit:

- MR-(DEVD)₂ reagent, lyophilized, part# 6131 for the 25-test size, #6132 for the 100-test size
- Hoechst 33342 stain, 1 mL, part# 639
- Acridine orange (AO), 0.5 mL, part# 6130
- Assay manual
- MSDS sheets

3. Recommended Materials and Equipment (not all are required):

- Cultured cells with media
- Reagents to induce apoptosis
- 15 mL polypropylene centrifuge tube (1 per sample)
- Sterile black 96-well microtiter tissue culture plates, round or flat
- Amber vials or polypropylene tubes for storage of 155X concentrate at -20°C, if aliquoted
- Slides and coverslips
- Hemocytometer
- Clinical centrifuge at 200 X g
- 37°C CO₂ incubator
- Pipette(s) capable of dispensing at 10, 50, 200, or 300µL, 1mL
- diH₂O, up to 2 mL needed
- Phosphate buffered saline (PBS) pH 7.4, up to 100 mL needed
- Trypsin-versine
- Dimethyl sulfoxide (DMSO), 50 or 200µL needed
- Ice or 4°C refrigerator to store cells

4. Instrumentation (not all are required):

- Fluorescence microscope with appropriate filters: excitation 550 nm, emission >610 nm for MR-(DEVD)₂; excitation at 480 nm and emission at >540 nm for AO; and if Hoechst is used, a UV-filter with excitation at 365 nm, emission at 480 nm.
- 96-well fluorescence plate reader with excitation at 590 nm, and emission at 630 – 640 nm filters, and black round or flat sterile 96-well tissue culture plates.

5. Storage and Shelf-Life

- Store the unopened kit (and each unopened component) at 2°C to 8°C until the expiration date.
- Protect the **MR-(DEVD)₂** reagent from light at all times.
- Once reconstituted, the 155X **MR-(DEVD)₂** stock should be stored at or below -20°C protected from light. This reagent is stable for 6 months and may be thawed twice during that time.
- Replacement components can be ordered by calling NEUROMICS at 1-866-350-1500 or 952-374-6161

6. Safety Information

- Use gloves while handling the **MR-(DEVD)₂** reagent, AO, and Hoechst stain.
- Dispose of all liquid components down the sink and flush with copious amounts of water. Solid components may be tossed in standard trash bins.

7. Overview of the **MR-Caspase Protocol**

Staining apoptotic cells with the MR-Caspase kit can be completed within a few hours. However, the MR-Caspase kit 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 induction process (which typically requires a 2-5 hour incubation at 37°C). Therefore, as the 31X **MR-(DEVD)₂** solution must be used immediately, the **MR-(DEVD)₂** reagents should not be prepared until the end of the apoptosis induction process, just prior to staining. The following is a quick overview of the MR-Caspase protocol:

1. Culture cells to the density optimal for your specific apoptosis induction protocol, but not to exceed 10⁶ cells/mL.
2. At the same time, culture a non-induced negative control cell population at the same density as the induced population for every labeling condition. For example, if labeling with **MR-(DEVD)₂**, Hoechst stain, and AO make 10 populations:
 - a. Unlabeled, induced and non-induced populations.
 - b. **MR-(DEVD)₂** labeled, induced and non-induced populations.
 - c. **MR-(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.

3. Induce apoptosis following your protocol (4 sample protocols are mentioned in Section 8).
4. Reconstitute the vial of lyophilized MR-(DEVD)₂ with DMSO to form the 155X MR-(DEVD)₂ stock concentrate (Section 11).
5. Dilute the 155X MR-(DEVD)₂ stock to the 31X working solution (Section 12 or 14).
6. Stain cells by adding the 31X MR-(DEVD)₂ solution.
7. Incubate cells for > 1 hour at 37°C.
8. If desired, label cells with Hoechst stain (Section 9).
9. If desired, label cells that have **not** been exposed to MR-(DEVD)₂ with AO (Section 10).
10. Analyze data via fluorescence microscopy (Section 15 or 16) or microtiter plate fluorometry (Section 18).

8. Induction of Apoptosis

The MR-Caspase kit works with your current apoptosis protocols - induce apoptosis as you normally would, then label the cells with MR-(DEVD)₂. Four quick examples of protocols to induce apoptosis in suspension culture are:

- 1) treating Jurkat cells with 2 µg/ml camptothecin for 3 hours.
- 2) treating Jurkat cells with 1 µM staurosporine for 3 hours.
- 3) treating HL-60 cells with 4 µg/ml camptothecin for 4 hours.
- 4) treating HL-60 cells with 1 µM staurosporine for 4 hours.

9. Hoechst 33342 Stain

Hoechst stain can be used to label the nuclei of apoptotic cells after labeling with the MR-(DEVD)₂ reagent. It can be observed under a microscope equipped with a UV-filter with excitation at 365 nm and an emission at 480 nm. Hoechst stain is provided ready-to-use at 200 µg/mL.

Warning: Hoechst stain is a potential mutagen. Use of gloves, protective clothing, and eyewear are strongly recommended. When disposing, flush sink with copious amounts of water; see MSDS for further information.

10. Acridine Orange

Acridine orange (AO) is a chelating dye and can be used to reveal lysosomes, nuclei, and nucleoli. 0.5 mL of AO is provided at 1 mM. 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 using AO concentrations ranging from 0.5 to 5.0 µM. This concentration range can be obtained by diluting the AO reagent stock 1:2000 to 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 the AO 1:100 in diH_2O : put 10 μL AO into 990 μL diH_2O . Then pipette the AO into the cell suspension at 1:10: put 55.5 μL diluted AO into 500 μL cell suspension.

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 pairing filters used to view the **MR-(DEVD)₂** can also be used: a 550 nm (540-560 nm) excitation and long pass >610 nm emission/barrier filter pairing. In this case, 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. Cells read at this combination may need to be washed prior to viewing to remove any excess AO.

- **Because of the emissions overlap, dual staining of cells with both **MR-(DEVD)₂** and AO will yield confusing results. Therefore these dyes should not be used to stain the same cells.**

Warning: AO is a potent mutagen and probable carcinogen. Use of gloves, protective clothing, and eyewear are strongly recommended. When disposing, flush sink with copious amounts of water; see MSDS for further information.

11. Reconstitution of the 155X **MR-(DEVD)₂** Stock

The **MR-(DEVD)₂** reagent is a highly concentrated lyophilized powder. It must first be reconstituted in DMSO, forming a 155X stock concentrate, and then diluted 1:5 in diH_2O to form a final 31X working solution. For best results, the 31X working solution should be prepared immediately prior to use; however, the reconstituted 155X stock concentrate can be stored at or below -20°C for future use.

- **The newly reconstituted 155X **MR-(DEVD)₂** stock must be used or frozen immediately after it is prepared.**
- **Protect the **MR-(DEVD)₂** stock from light during handling.**

1. Reconstitute the lyophilized **MR-(DEVD)₂** with DMSO to yield a 155X concentrate:

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- a. For the 25-test vial, add 50 μL .
- b. For the 100-test vial, add 200 μL DMSO.
2. Mix by swirling or tilting the vial, allowing the DMSO to travel around the base of the vial until completely dissolved. At room temperature (RT), the reagent should be dissolved within a few minutes.
3. If immediately using this solution, dilute it to 31X (Section 12).
4. Or, if using later, aliquot and store it at or below -20°C (Section 13).

12. Preparation of 31X MR-(DEVD)₂ Solution for Immediate Use

Using the freshly reconstituted 155X MR-(DEVD)₂ stock, prepare the 31X working-strength MR-(DEVD)₂ solution by diluting the stock 1:5 in diH₂O. Following the suggested protocols here, each sample to be tested requires only 10 μL of 31X MR-(DEVD)₂ solution (or 2 μL of the 155X MR-(DEVD)₂ stock).

1. For the 25-test vial, if using the entire vial, add 200 μL diH₂O (the vial contains 50 μL of the 155X stock; this yields 250 μL of a 31X solution).
2. For the 100-test vial, if using the entire vial, add 800 μL diH₂O (the vial contains 200 μL of the 155X stock; this yields 1 mL of a 31X solution).
3. If not using the entire vial, dilute the 155X stock 1:5 in diH₂O. For example, add 10 μL of the 155X stock to 40 μL diH₂O (this yields 50 μL of a 31X solution). Store the unused 155X stock at or below -20°C (Section 13).
4. Mix by inverting or vortexing the vial at RT.

● **The 31X working strength MR-(DEVD)₂ solution must be used the same day that it is prepared.**

13. Storage of 155X MR-(DEVD)₂ Stock for Future Use

If not all of the 155X MR-(DEVD)₂ stock will be used the same time it is reconstituted, the unused portion may be stored at or below -20°C for 6 months. During that time, the 155X MR-(DEVD)₂ stock may be thawed and used twice. After the second thaw, discard any remaining 155X MR-(DEVD)₂ stock. If you anticipate using it more than twice, make small aliquots in amber vials or polypropylene tubes and store at or below -20°C protected from light. When ready to use, follow Section 14 below.

14. Preparation of 31X MR-(DEVD)₂ Solution from a Frozen Aliquot

If some of the 155X MR-(DEVD)₂ reagent was previously reconstituted and then stored at or below -20°C, it may be used 2 more times within 6 months.

1. Thaw the 155X MR-(DEVD)₂ stock and protect from light.
2. Once the aliquot has become liquid, dilute the 155X stock solution 1:5 in diH₂O and vortex. For example, mix 10 µL of 155X MR-(DEVD)₂ reagent with 40 µL diH₂O.
3. If the 155X MR-(DEVD)₂ stock was frozen immediately after reconstitution and was never thawed, return it to the freezer. If the stock was thawed once before, discard it.
4. Proceed to the labeling protocol (Section 15, 16, or 18).

15. Fluorescence Microscopy Staining Protocol for Suspension Cells

1. Culture cells to a density optimal for apoptosis induction according to your specific induction protocol (as discussed in Section 8).
2. Cultivate or concentrate cells to a density of at least 5×10^5 cells/mL.

- **Cell density in the cell culture flasks should not exceed 10^6 cells/mL. 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. Optimal cell concentration will vary depending on the cell line used.**
3. Induce cells to undergo apoptosis and take samples according to your specific protocol (as mentioned in Section 8).
 4. At the same time, culture an equal volume of non-induced cells for a negative control cell population. Make sure that both the negative control and induced positive cell population tubes contain similar quantities of cells.
- **When ready to label with the 31X MR-(DEVD)₂ solution, cells should be between 5×10^5 – 2×10^6 cells/mL for best viewing. Density can be determined by counting cell populations on a hemocytometer.**
5. If necessary, concentrate cells by gentle centrifugation at 200 X g for 3 – 8 minutes.
 6. Prepare the 155X stock by reconstituting the vial of lyophilized MR-(DEVD)₂ with 50 µL (25-test vials) or 200 µL DMSO (100-test vials) (see Section 11 for details).

7. Prepare the 31X stock by diluting the reconstituted vial with 200 μL (25-test vials) or 800 μL diH₂O (100-test vials) (see Section 12 for details).
8. Transfer 300 μL of each induced and negative control cell populations into fresh 12 x 75 mm glass or polypropylene tubes. Or, if desired, larger cell volumes can be used, however more of the 31X MR-(DEVD)₂ solution may be required. Larger volume cell suspensions label nicely using 25 cm² tissue culture flasks (laid flat) as incubator vessels.
9. Add 10 μL of the 31X working dilution MR-(DEVD)₂ solution directly to each 300 μL cell suspension forming a final volume of 310 μL .
10. Or, if a different volume was used, add the 31X MR-(DEVD)₂ solution at a 1:31 ratio of the final volume. For example, if 1,000 μL of cell suspension was used, add 33 μL of the 31X MR-(DEVD)₂ solution forming a final volume of 1,033 μL . (To optimize this assay to your specific research conditions, adjust the amount of 31X MR-(DEVD)₂ used to determine the greatest difference in the fluorescence signal between induced and non-induced cell populations.)

● **Each investigator should titrate the amount of MR-(DEVD)₂ used to accommodate their particular cell line and research conditions.**

11. Mix the cells thoroughly.
12. Incubate cells for 1 hour at 37°C under 5% CO₂, protecting the tubes from light. As cells may settle on the bottom of the tubes, gently resuspend them by swirling the tubes every 20 minutes during this incubation time. This will ensure an even distribution of the MR-(DEVD)₂ reagent among all cells. (To optimize this assay to your specific research conditions, adjust this incubation time to determine the greatest difference in the fluorescence signal between induced and non-induced cell populations.)

● **Each investigator should adjust the incubation time to accommodate their particular cell line and research conditions.**

13. At this point, the cells may be stained with Hoechst stain (Section 9), and cells unexposed to MR-(DEVD)₂ can be stained with AO (Section 10).
14. If cells are to be monitored using Hoechst stain (Section 9):
 - a. Add 1.6 μL Hoechst stain (0.5% v/v) to the 310 μL cell suspension labeled with MR-(DEVD)₂.
 - b. Or, add 1.5 μL Hoechst stain (0.5% v/v) to a 300 μL cell suspension that was not labeled.
 - c. Incubate for an additional 5 – 10 minutes at 37°C under 5% CO₂.
 - d. Go to Step 16.

15. If cells are to be monitored using AO (Section 10):
 - a. Pipette the AO reagent stock 1:2000 to 1:200 (0.05 – 0.5% v/v) into the final cell suspension. Because of the emissions overlap, dual staining of cells with both MR-(DEVD)₂ and AO will yield confusing results. Therefore, the dyes should be used separately.
 - b. For example, if using AO at 1.0 μM in the final cell suspension, first dilute the AO 1:100 in PBS: put 10 μL AO into 990 μL PBS. Then pipette the diluted AO into the cell suspension at 1:10: put 55.5 μL diluted AO into 500 μL cell suspension.
 - c. Incubate for an additional 30 minutes at 37°C under 5% CO₂.
 - d. If viewing under the same filters used for the MR-(DEVD)₂ staining (excitation at 550 nm, emission >610 nm), cells may be viewed immediately after staining, without a wash step - go to Step 16.
 - e. If viewing under blue (480 nm) excitation and green (540-550 nm) emission wavelengths, cells may need to be washed with PBS to remove any excess AO as the cells may appear too bright. Brightness will depend on the type of microscope used, and the type of cell line. To wash the cells:
 - i) Gently pellet cells at 200 X g for 3-8 minutes at RT.
 - ii) Remove and discard supernatant.
 - iii) Resuspend cells in a similar volume of PBS.
 - iv) Go to Step 16.
16. Place 15 – 20 μL of the cell suspension onto a microscope slide and cover with a coverslip.
17. 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. (If these filters are not available, select a filter combination that best approximates these settings.) Using this excitation/emission filter pairing, cells should stain red with more brightly stained vacuoles and lysosomes.

18. If the same sample was 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. If these exact filter pairings are not available, select a filter combination that best approximates these settings.
19. 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 the MR-(DEVD)₂ may be used: a 550 nm (540-560 nm) excitation and long pass >610 nm emission/barrier filter pairing. In this case, 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. Because of the emissions overlap, dual staining of cells with both MR-(DEVD)₂ and AO will yield confusing results. Therefore, these dyes should be used separately.
20. Depending upon your cell line and method of apoptosis induction, cells undergoing apoptosis will generate a stronger red fluorescence with MR-(DEVD)₂ than non-apoptotic cells of the same lineage.
21. To optimize this assay for your specific research conditions, vary the amount of 31X MR-(DEVD)₂ used (Step 8), and the incubation time (Step 12) to determine the greatest difference in the fluorescence signal between induced and non-induced cell populations.

16. Fluorescence Microscopy Staining Protocol for Adherent Cells

1. Select the cell culture flask containing the cells that will be studied and dislodge the attached cells.
 - a. Aseptically remove the media from the flask.
 - b. Depending on the size of your culture flask, aseptically add 5 - 25 mL sterile PBS or saline to the flask.
 - c. Aseptically remove this solution and discard.
 - d. Add trypsin-versine to the flask, varying the amount depending on the flask surface area. For example, a 25 cm² flask should receive approximately 1 mL of the trypsin reagent; a 75 cm² flask should receive approximately 3 mL.
 - e. Incubate the flask for 1-2 minutes, rocking the flask gently back and forth to dislodge the attached cells.
 - f. To neutralize the trypsin activity and count the cells, dilute the contents of the flask 1:20 into culture media. For example, take 1 mL of suspension and add to a sterile culture tube containing 19 mL of the complete cell culture media (with serum)
 - g. Count the cells using a hemocytometer.

- Seed about $10^4 - 10^5$ cells onto a sterile coverslip in a 35 mm petri dish or onto chamber slides.
- Grow the cells using your culture media formulation until about 80% confluent. This usually takes about 24 hours, but will vary with your cell line.
- Induce cells to undergo apoptosis (Section 8).
- Remove 300 μL cell overlay media samples at time points according to your specific protocol. Or, if desired, larger cell volumes can be used, however more of the 31X MR-(DEVD)₂ solution may be required.
- Add 10 μL of the 31X MR-(DEVD)₂ solution directly to each 300 μL cell overlay media forming a final volume of 310 μL .
- Or, if a different volume was used, add the 31X MR-(DEVD)₂ solution at 1:31. For example, if 1,000 μL of cell media was used, add 33 μL of the 31X MR-(DEVD)₂ solution forming a final volume of 1,033 μL . (To optimize this assay to your specific research conditions, adjust the amount of 31X MR-(DEVD)₂ used to determine the greatest difference in the fluorescence signal between induced and non-induced cell populations.)

● **Each investigator should titrate the amount of MR-(DEVD)₂ used to accommodate their particular cell line and research conditions.**

- Gently mix the cell overlay media to ensure an even exposure to the MR-(DEVD)₂.
- Incubate cells for 30 - 60 minutes at 37°C in a CO₂ incubator.
- Remove the media.
- Rinse twice with PBS, 1 minute per rinse.
- At this point, labeled and unlabeled cells can be stained with Hoechst stain (Section 9), and unlabeled cells can be stained with AO (Section 10).
- If cells are to be monitored using Hoechst stain (Section 9):
 - Add 1.6 μL Hoechst stain (0.5% v/v) to the 310 μL of cell overlay material labeled with MR-(DEVD)₂.
 - Or, add 1.5 μL Hoechst stain (0.5% v/v) to 300 μL cell overlay material that was not labeled.
 - Incubate for an additional 5 – 10 minutes at 37°C under 5% CO₂.
 - Go to Step 15.
- If cells are to be monitored using AO (Section 10):
 - Pipette the AO reagent stock 1:2000 to 1:200 (0.05 – 0.5% v/v) into the final cell overlay media. Because of the emissions overlap, dual staining of cells with both MR-(DEVD)₂ and AO will yield confusing results. Therefore, the dyes should be used separately.

- b. For example, if using AO at 1.0 μM in the final cell media, first dilute the AO 1:100 in PBS: put 10 μL AO into 990 μL PBS. Then pipette the diluted AO into the cell overlay media at 1:10: put 33 μL diluted AO into 300 μL cell overlay media forming a final volume of 333 μL .
 - c. Incubate for an additional 30 minutes at 37°C under 5% CO_2 .
 - d. Remove the media from the cell mono-layer surface.
 - e. Rinse twice with PBS, 1 minute per rinse.
 - f. Go to Steps 15 and 18.
15. Mount the coverslip with cells facing down onto a drop of PBS. If a chamberslide was used, pull off the plastic frame and add a drop of PBS to the cell surface and cover with a coverslip.
 16. 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. (If these filters are not available, select a filter combination that best approximates these settings.) Using this excitation/emission filter pairing, cells should stain red with more brightly stained vacuole and lysosomal internal structures.
 17. If the same sample was stained with both **MR-(DEVD)₂** and Hoechst, and 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. If these exact filter pairings are not available, select a filter combination that best approximates these settings.
 18. As AO exhibits a very broad emission range, one of several filter pairings on the fluorescence microscope may be used. The same excitation/emission pairing filters used to view the **MR-(DEVD)₂** may also be used: a 550 nm (540-560 nm) excitation and long pass >610 nm emission/barrier filter pairing. Lysosomes will appear red. When illuminating with a blue light (480 nm) excitation filter, a green light (540-550 nm) emission/barrier filter combination works well. In this case, lysosomes will appear yellowish green instead of red. Because of the emissions overlap, dual staining of cells with both **MR-(DEVD)₂** and AO will yield confusing results. Therefore, these dyes should be used separately.
 19. Depending upon your cell line and method of apoptosis induction, cells undergoing apoptosis will generate a stronger red fluorescence with **MR-(DEVD)₂** than non-apoptotic cells of the same lineage.
 20. To optimize this assay for your specific research conditions, vary the amount of 31X **MR-(DEVD)₂** used (Step 6), and the incubation time (Step 9) to determine the greatest difference in the fluorescence signal between induced and non-induced cell populations.

17. Fluorescence Microscopy Sample Data

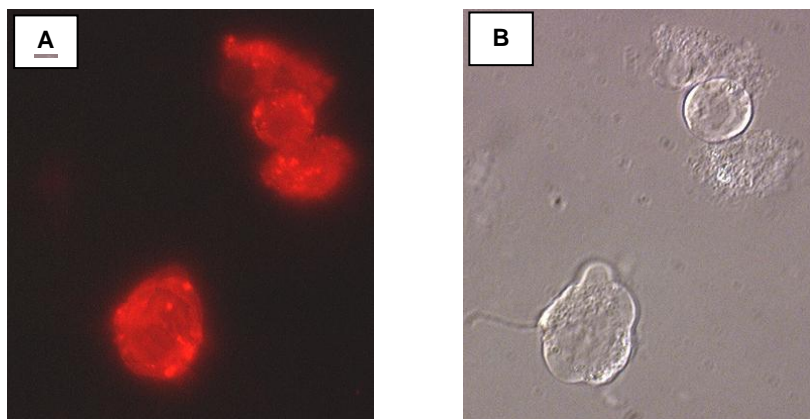


Figure 1. Detection of DEVDase activity in staurosporine-induced THP-1 cells using (z-DEVD)₂-Magic Red™ fluorogenic substrate. Induced THP-1 cells were stained with 20 μM MR-(DEVD)₂ for 60 minutes at 37°C. Photomicrographs were taken using a Nikon Eclipse E 800 photomicroscope with a 510-560 nm excitation filter and a 570-620 nm emission/barrier filter at 500X. Photo A shows a high level of substrate hydrolysis in the induced cells (bright red with organelle fluorescence). Photo B shows the corresponding differential interference contrast (DIC) of the cells.

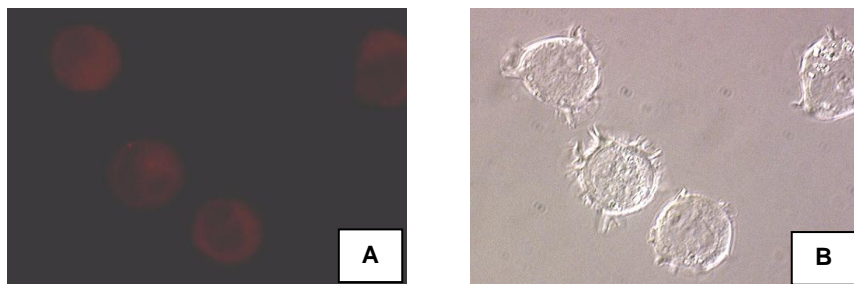


Figure 2. No DEVDase activity in non-induced (DMSO control) THP-1 cells using (z-DEVD)₂-Magic Red™ fluorogenic substrate. Non-induced THP-1 cells were stained and photographed in the same manner as Figure 1. Photo A shows a low level of substrate hydrolysis in the non-induced cells (very faint red with no organelle fluorescence). Photo B shows the corresponding DIC.

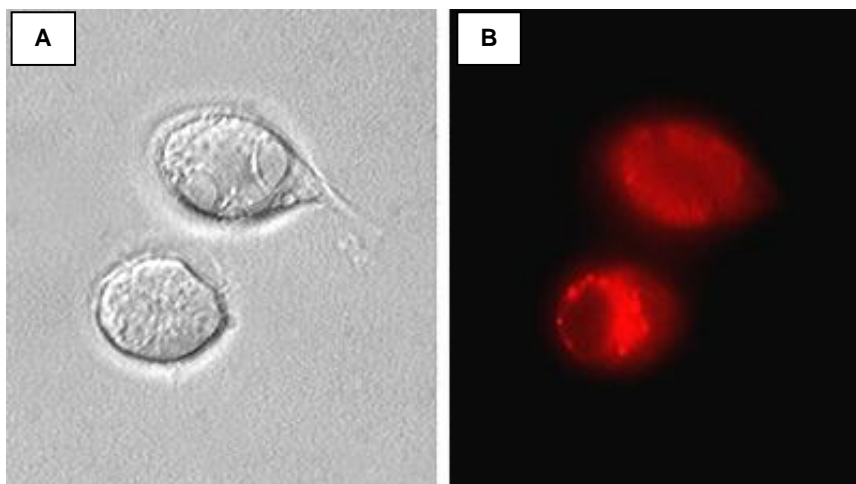


Figure 3. (z-DEVD)₂-Magic Red™ fluorogenic substrate staining of staurosporine-induced Jurkat cells. Staurosporine-induced Jurkat cells were stained with 20 μM MR-(DEVD)₂ for 60 minutes at 37°C. Intracellular structures were detected on a Nikon Eclipse E 800 photomicroscope using a 510-560 nm excitation filter and a 570-620 nm emission/barrier filter at 700X. Photo A shows the corresponding DIC image of the cells.

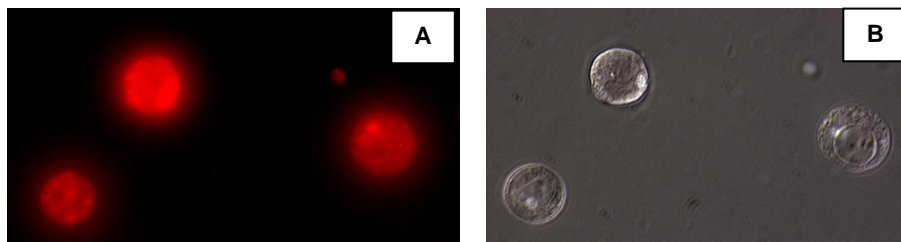


Figure 4. (z-DEVD)₂-Magic Red™ fluorogenic substrate staining of staurosporine-induced THP-1 cells. Staurosporine-induced THP-1 cells were stained with 20 μM MR-(DEVD)₂ for 60 minutes at 37°C. Intracellular structures are detected on a Nikon Eclipse E 800 photomicroscope using a 510 – 560 nm excitation filter and a 570 – 620 nm emission/barrier filter set at 400X. Photo B shows the corresponding DIC image of the cells.

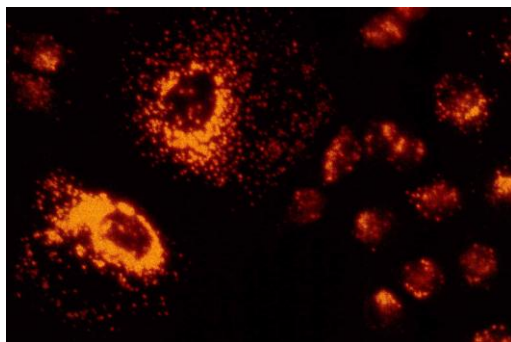


Figure 5. Detection of DEVDase activity in camptothecin-induced MCF-7 cells using (z-DEVD)₂-Magic Red™ fluorogenic substrate. MCF-7 cells were induced for 24 hours with 0.15 μM camptothecin at 37°C, then exposed to 10 μM MR-(DEVD)₂ for 60 minutes at 37°C. DEVDase activity is demonstrated by the appearance of orange-red lysosomal bodies within the cytoplasm of the cell. The photograph was taken on a Nikon Microphot FXA system at 541– 551 nm excitation with a long pass >640 nm barrier filter in the laboratory of Dr. Zbigniew Darzynkiewicz (Brander Cancer Research Center Institute, New York, NY).

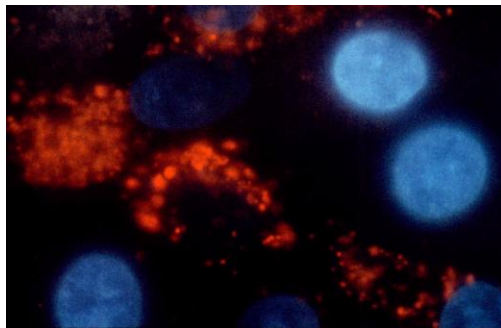


Figure 6. Dual staining of camptothecin-induced MCF-7 cells using (z-DEVD)₂-Magic Red™ fluorogenic substrate and Hoechst 33342 stain.

MCF-7 cells were exposed to 0.15 μ M camptothecin at 37°C for 24 hours, then stained for 30 minutes with 10 μ M MR-(DEVD)₂ at 37°C, washed twice in PBS, and supravivally stained with 1 μ g/mL of Hoechst stain (>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 lysosomal bodies with less intense blue nuclei can be seen intermixed with non-apoptotic cells bearing bright blue nuclei and absent or reduced lysosomal staining. Photo provided by Dr. Zbigniew Darzynkiewicz (Brander Cancer Research Center Institute, New York, NY)

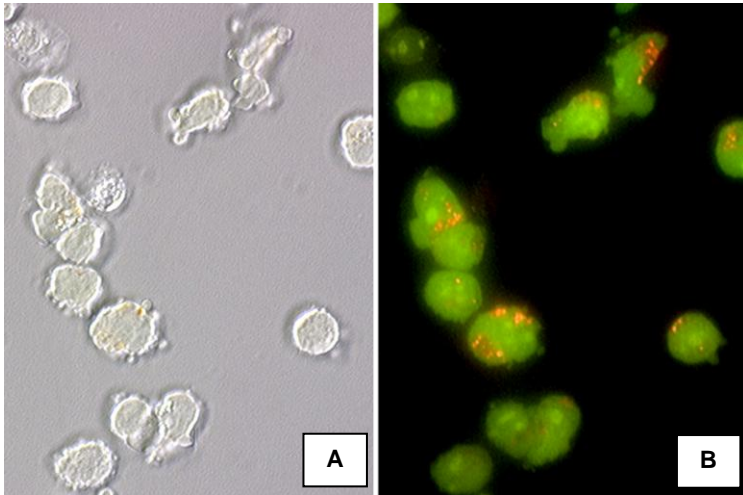


Figure 7. Acridine orange staining of normal Jurkat cells showing orange lysosomal staining. Jurkat cells were stained with 5 μ M AO in PBS for 60 minutes at 37°C. Photomicrographs were taken using a Nikon Eclipse E800 photomicroscope using a 460-500nm excitation filter and a 505-560 nm emission / barrier filter set at 300X. Photo A shows the corresponding DIC image of the cells (AO appears faintly).

18. 96-Well Fluorescence Plate Reader Staining Protocol

Following this fluorescence plate reader protocol, each sample requires 10 μL of 31X MR-(DEVD)₂ substrate (equal to 2 μL of the 155X MR-(DEVD)₂ stock).

1. Culture cells to a density optimal for apoptosis induction according to your specific induction protocol (as discussed in Section 8).
 - Cell density in the cell culture flasks should not exceed 10^6 cells/mL. 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. Optimal cell concentration will vary depending on the cell line used.**
 2. Concentrate cells, if necessary, to achieve a $2 - 8 \times 10^6$ cells/mL density. Cell concentration can be achieved by low speed centrifugation ($<400 \times g$ at RT) for 5 minutes. (If $2 - 8 \times 10^6$ cells/mL is too dense for your cell line, induce apoptosis first, then concentrate the cells and add the MR-(DEVD)₂.)
 3. Induce apoptosis following your experimental protocol (Section 8). At the same time, culture an equal volume of non-induced cells for use as a negative control cell population.
 4. Once induction is completed, transfer 300 μL of each cell suspension to sterile tubes or a black microtiter plate. Larger cell volumes can also be used as determined by each investigator, however, more of the MR-(DEVD)₂ substrate will be required per sample. Small (25 cm^2) tissue culture flasks (laid flat) as the incubation vessel work well for this purpose.
 - When ready to label with the 31X MR-(DEVD)₂ solution, cells should be at least 2×10^5 cells/100 μL aliquot for each microtiter plate well.**
 5. Add 10 μL 31X MR-(DEVD)₂ solution directly to the 300 μL cell suspension.
 6. Or, if a different cell volume was used, add the 31X MR-(DEVD)₂ solution at a 1:31 v/v ratio. For example, if 3 mL of cell suspension was used, add 100 μL of the 31X MR-(DEVD)₂ substrate solution (forming a final volume of 3.1 mL).
- Note: Each investigator should adjust the amount of MR-(DEVD)₂ reagent used to accommodate their particular cell line and research conditions.**

7. Gently mix the cells. If in a tube, gently flick the tubes. For cells in a microtiter plate, mix by gently aspirating and expelling the cells with a pipette (to minimize cell shearing, cut back the tip of the pipette to enlarge the hole of the tip).
8. Incubate the cells for at least 60 minutes at 37° C under 5% CO₂, protecting them from light. As cells settle to the bottom, gently resuspend them approximately every 20 minutes to ensure the MR-(DEVD)₂ is evenly dispersed among all cells.
9. If the cells are in tubes, pipette 100 µL of the cell suspensions into black microtiter plate wells. If the cells are in a microtiter plate, either split the 300 µL sample well contents into 3 wells of 100 µL each, or read the 300 µL volume sample as one sample. Do not use clear plates. Avoid bubbles.
10. Measure fluorescence intensity of the red fluorescent (mono and non-substituted) Magic Red™ fluorophore.
 - a. Set the plate reader to perform an endpoint read.
 - b. Magic Red™ has an optimal excitation and emission wavelength tandem of 592 nm and 628 nm respectively. Select the filter pairings on your instrument that best approximate these settings. For some instruments, the closest wavelenths are 590 nm for excitation and 640 nm for emission. On Molecular Devices fluorometers, an additional cutoff filter option is available to filters out shorter wavelength excitation interference - set this at 630 nm.
 - c. Read the samples.

19. 96-Well Fluorescence Plate Reader Sample Data

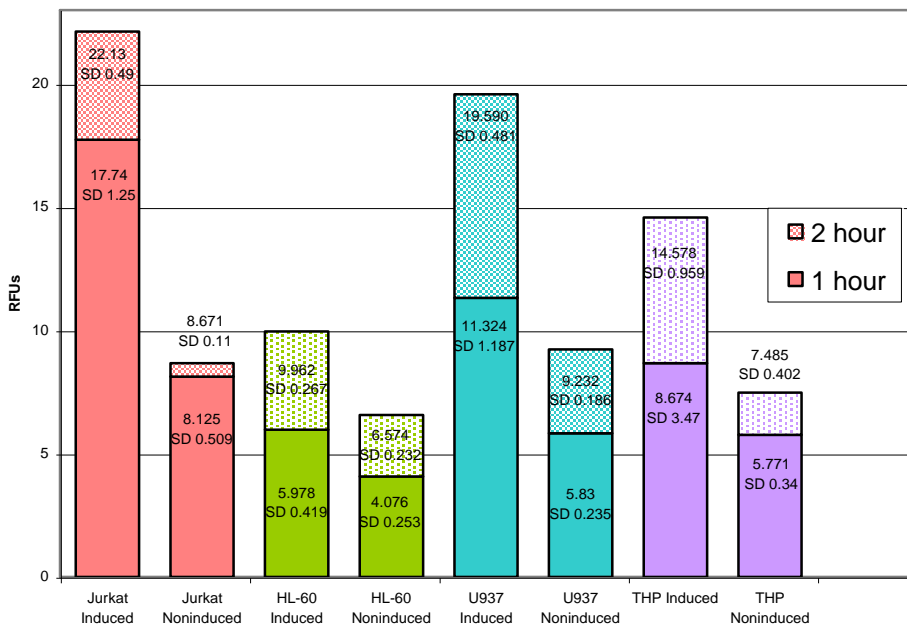


Figure 8. Fluorometric detection of DEVDase activity in Jurkat, HL-60, U937, and THP-1 cells using MR-DEVD fluorogenic substrate. Cells were incubated with 1 μ M staurosporine or DMSO control for 3 hours (Jurkat cells) or 4 hours (HL-60, U937, THP-1 cells) at 37° C. MR-DEVD substrate, at a 20 μ M concentration, was added to the cell cultures to reveal the intracellular, apoptosis-associated, increase in DEVDase activity. DEVDase activity in Jurkat cells, HL-60 cells, U937 cells and THP-1 cells, is shown after 1 hour (solid bars) and 2 hour (spotted) exposures to the fluorogenic substrate. Cells were analyzed using a Molecular Devices Gemini XS fluorometric plate reader set at 590 nm excitation, 640 nm emission, and using a 630 nm cut-off filter function.

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