

## Cathepsin B Detection Assay

Product Number **CS0370**

Storage Temperature 2-8 °C

## Product Information

### Technical Bulletin

#### Product Description

Cathepsin B detection assay kit detects protease activity within whole living cells as a marker of intracellular cathepsins activity. Researchers can quickly visualize intracellular cathepsin B activity within their particular experimental cell line.

Magic Red<sup>™</sup> (MR) detection kits utilize the fluorophore, cresyl violet. When bi-substituted via amide linkage to two cathepsin target sequence peptides {such as (Arginine-Arginine)<sub>2</sub>}, the cresyl violet leaving group is non-fluorescent.<sup>1</sup> Following enzymatic cleavage at one or both arginine (R) cresyl violet amide linkage sites, the mono and non-substituted cresyl violet fluorophores generate red fluorescence when excited at 550-590 nm.

In the cathepsin B detection assay (z-Arginine-Arginine)<sub>2</sub> (z-RR)<sub>2</sub>, derivative of the cresyl violet fluorophore, is incorporated into a user-friendly assay format. MR-Cathepsin B photostable fluorogenic substrate easily penetrates the cell membrane and the membranes of the internal cellular organelles enabling us to detect cathepsin B activity within whole living cells.

Intracellular substrate hydrolysis can be monitored by the accumulation of red fluorescent product within various organelles. By varying the duration and concentration of exposure to MR-Cathepsin B substrate, a picture can be obtained of the intracellular location of cathepsin enzyme activity. This activity can be monitored using a fluorescence microscope or multiwell plate fluorometer.<sup>2</sup>

The unsubstituted red fluorescent MR-Cathepsin B product has an optimal excitation and emission wavelength of 592 nm and 628 nm, respectively. At these higher excitation wavelengths, the amount of cell-mediated auto-fluorescence is minimal.<sup>1</sup> 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 MR<sup>™</sup> substrate.

Hoechst stain can be used to label the cell nuclei after labeling with the MR-Cathepsin B substrate. It is revealed under a microscope in a UV-filter with excitation at 365 nm and emission at 480 nm. Acridine orange (AO) helps identify lysosomes and other intracellular organelles. In the acidic pH of the lysosome AO molecules aggregate. Aggregated AO fluorescence's orange rather than green thus clearly differentiating the lysosomes from the other organelles.<sup>3</sup>

Cathepsins are members of the lysosomal cysteine protease (active site) family<sup>4</sup> and the cathepsin family name has been synonymous with lysosomal proteolytic enzymes.<sup>5</sup> However, the cathepsin family also contains members of the serine protease (cathepsin A,G) and aspartic protease (cathepsin D,E) families as well. These enzymes exist in their processed form as disulfide-linked heavy and light chain subunits with molecular weights ranging from 20-35 kDa.<sup>6</sup> Cathepsin C is the exception with a MW ~200 kDa,<sup>6</sup> Initially synthesized as inactive zymogens, they are post-translationally processed into their active forms after passing through the endoplasmic reticulum and subsequent incorporation into the acidic environment of the lysosomes.<sup>5-7</sup>

Cathepsin B (amyloid precursor protein secretase) is generated from pre-procathepsin B in hepatoma and kidney. Three forms of cathepsin B were identified from normal human liver and several human tumor tissues, 2 forms of 25 and 26 kD that appeared as a doublet and a third form of about 30 kD. Cathepsin B is over expressed in tumors of the lung, prostate, colon, breast, and stomach.<sup>8-9</sup>

## Reagents

This kit is sufficient for 100 assays.

- **MR-(RR)<sub>2</sub> substrate, 1 vial, Product No. M 2318** - Cathepsin B (z-Arginine-Arginine)<sub>2</sub>-Magic Red™, photostable fluorogenic substrate, lyophilized orange-red powder.
- **Hoechst 33342 stain, 1 mL, Product No. H 2413**-solution of 200 µg/mL in DI H<sub>2</sub>O.
- **Acridine orange stain (AOS), 0.5 mL, Product No. A 5728**-contains Acridine Orange Reagent (AOR) at 302 µg/mL (1 mM).

## Reagents and equipment required but not provided

- 15 mL polypropylene centrifuge tube
- Microcentrifuge polypropylene tubes
- Slides and coverslips
- Hemocytometer
- Centrifuge at 200 X g
- Incubator at 37 °C, CO<sub>2</sub>
- Calibrated adjustable precision pipettes for volumes between 5 µl and 1,000 µL.
- Deionized or distilled water
- Phosphate buffered saline (PBS) pH 7.4
- Dimethyl sulfoxide (DMSO)
- Trypsin versene (EDTA) (Product No. T 3924)
- Fluorescence microscope or multiwell plate reader with the following filters:
  - o for MR-(z-RR)<sub>2</sub> - excitation filter 550-590 nm, emission filter >610 nm
  - o for AO - excitation filter at 480 nm and emission at >540 nm
  - o for Hoechst - a UV-filter with excitation at 365 nm, emission at 480 nm.

## Precautions and Disclaimer

The kit is for R&D use only, not for drug, household or other uses. Please consult the Material Safety Data Sheet for information regarding hazards and safe handling practices.

## Preparation Instructions

### Sample Preparation

- Sample of choice - cultured cells of chosen cell line
- Culture the cells in media of choice to a density optimal for the cell line being studied.
- Optimal cell concentration will vary depending on the cell line used and experimental conditions.

- However, cell density in the cell culture flasks should not exceed 10<sup>6</sup> cells/mL.
- Cells cultivated in excess of 10<sup>6</sup> cells/mL may begin to naturally enter apoptosis due to nutrient deprivation or the accumulation of cell degradation products in the media.
- Immediately prior to staining, gently centrifuge the cells at 200 X g for 3 –8 minutes to concentrate them to an optimal density for the staining ranging from 5 X 10<sup>5</sup> to 1 X 10<sup>6</sup> cells/mL.
- Prepare a non-induced negative control cell population at the same density as the induced population for every labeling condition.
- Prepare the following experimental cells:
  1. MR- Cathepsin B labeled cells.
  2. MR- Cathepsin B and Hoechst labeled cells.
  3. Hoechst labeled cells.
  4. AO labeled cells.

### Reagent Preparation

#### MR-Cathepsin B (z-RR)<sub>2</sub> substrate

1. Each 0.5 mL cell sample to be tested requires 20 µL of 26X working concentrate of MR-Cathepsin B
2. For 100 assays prepare 2 mL MR-Cathepsin B
3. Reconstitute one vial of MR-Cathepsin B to 260X concentrate as follows:
  - a. Add 200 µL DMSO to the supplied vial
  - b. Mix by swirling or tilting the vial, allowing the DMSO to travel around the base of the vial until completely dissolved.
  - c. At room temperature (RT), the reagent should be dissolved within a few minutes.
4. Prepare working 26X concentrate solution of MR Cathepsin B as follows:
  - a. Add 1.8 mL PBS to 200 µL reconstituted substrate
  - b. Use immediately – do not store.
  - c. The 26X MR- Cathepsin B working solution must be used only on the day of preparation and prepared just before addition to cells.
5. Store unused 260X concentrate in aliquots in polypropylene microcentrifuge tubes or amber vials at or below -20°C protected from light for up to 6 months.
6. Thaw only 2 times
7. Prepare working 26X solution from frozen 260X aliquots as follows:
  - a. Thaw the 260X MR- Cathepsin B stock and protect from light.
  - b. Once the aliquot has become liquid, dilute the 260X stock solution 1:10 in PBS and vortex. For example, mix 10 µL of 260X MR-Cathepsin B reagent with 90 µL PBS.

- c. If the 260X MR-Cathepsin B stock was frozen immediately after reconstitution and was never thawed, return it to the freezer. If the stock was thawed once before, discard it.

#### Hoechst 33342 Stain

1. Use Hoechst stain to label the cell nuclei after labeling with the MR- Cathepsin B reagent.
2. Hoechst stain is provided ready-to-use at 200 µg/mL.
3. It is revealed under a microscope using a UV-filter with excitation at 365 nm and emission at 480 nm.

#### Acridine orange (AO)

1. Acridine orange (AO) is a chelating reagent and can be used to reveal lysosomes, nuclei, and nucleoli.
2. 0.5 mL of AO is provided at 1 mM.
3. Use AO neat or diluted in diH<sub>2</sub>O or media prior to pipetting into the cell suspension.
4. Always protect AO from bright light.
5. To visualize lysosomes use from 0.5 to 5.0 µM; prepare 1:200 or 1:2000 (0.5 – 0.05% v/v) AO dilution into the final cell suspension.

#### Storage/Stability

All components of this kit are stable at 2 to 8 °C for 6 months from the date of purchase.

MR-Cathepsin B substrate should be stored protected from light.

Reconstituted 260X 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

Refer to the Certificate of Analysis for kit shelf date.

To obtain C of A go to [www.sigma-aldrich.com](http://www.sigma-aldrich.com)

#### Procedure.

##### Staining cell suspension with MR-Cathepsin B

1. Design the experimental protocol and determine optimal incubation times, culture conditions and stimulatory procedure for cell line employed.
2. Prior to the staining determine the optimal amount of MR- Cathepsin B and incubation times for the experimental cell line.
3. Run negative control cells as a reference population.
4. Make sure that both the experimental (positive) and the control (negative) samples contain similar quantities of cells.
5. Adjust the incubation time to create the best conditions for each experimental cell line and to achieve the greatest possible difference in the

fluorescence signal between positive and negative cell populations.

6. Transfer 500 µL of each cell sample (controls and experimental) into fresh 12 x 75 mm glass or polypropylene tubes.
7. Add 20 µL of the 26X MR- Cathepsin B solution directly to each 500 µL cell suspension, for a final volume of 520 µL.

Component	Volume, Conditions
Cells (5X10 <sup>5</sup> -1X10 <sup>6</sup> /mL)	500 µL
Add MR-Cathepsin B	20 µL
Mix	Swirl gently
Incubate	15-60 min, 37°C, 5% CO <sub>2</sub> protect from light
Add Hoechst stain	2.5 µL
Incubate	5-10 min, 37°C, 5% CO <sub>2</sub>
Put cell suspension under microscope slide	15-20 µL

8. For larger volume cell suspensions use 25 cm<sup>2</sup> tissue culture flasks (laid flat) as incubator vessels.
9. Mix the cells thoroughly.
10. Incubate 15 to 60 minutes at 37°C under 5% CO<sub>2</sub>, protecting from light.
11. Every 20 minutes during this incubation, swirl the cells gently to ensure an even distribution of the MR- Cathepsin B substrate among all cells.
12. Place 15–20 µL of the cell suspension onto a microscope slide and cover with a coverslip.
13. Visualize MR Cathepsin B 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.
14. Using this excitation/emission filter pairing, cells should stain red with more brightly stained vacuoles and lysosomes.
15. If these filters are not available, select a filter combination that best approximates these settings.
16. At this point, the cells may be counter-stained with Hoechst stain, if so desired, to view nuclear morphology. Cells stained with MR-Cathepsin reagent cannot be counter-stained with AO due to overlapping excitation and emission spectra.

##### Staining cell suspension with Hoechst stain

1. Hoechst stain is provided ready-to-use at 200 µg/mL
2. Add 2.5 µL Hoechst stain (0.5% v/v) to all the cells suspensions labeled with MR- Cathepsin B (520 µL) and to the non-labeled controls (500 µL).
3. Incubate 5 – 10 minutes at 37°C under 5% CO<sub>2</sub>.

4. Place 15–20  $\mu\text{L}$  of the cell suspension onto a microscope slide and cover with a coverslip.
5. Observe the dual staining of MR-Cathepsin B and Hoechst in the same sample using a multi-wavelength filter option.
6. Hoechst stain can be seen using a UV-filter with excitation at 365 nm and emission at 480 nm.
7. If these exact filter pairings are not available, select a filter combination that best approximates these settings.

#### Staining cell suspension with Acridine Orange (AO)

1. Acridine orange (AO) is used to reveal lysosomes, nuclei, and nucleoli.
2. Because of the emissions overlap, dual staining of cells with MR-Cathepsin B and AO yields confusing results.
3. Therefore, the dyes should not be used on the same cells but separately.
4. Use AO on the non-stained cells.
5. Dilute 1 mM stock AO as follows:
  - a. 10  $\mu\text{L}$  stock AO + 990  $\mu\text{L}$  distilled water (1)
  - b. 55  $\mu\text{L}$  (1) +500  $\mu\text{L}$  cell suspension
6. Incubate 30 min at 37 °C under 5%  $\text{CO}_2$
7. View the stained lysosomes immediately after incubation using the same filters as those used for the MR-Cathepsin B staining (excitation at 550 nm, emission >610 nm).
8. Use of a 480 nm excitation/540 nm emission lengths requires a wash step with PBS to remove any excess AO to reduce excessive brightness.
9. Wash procedure:
  - Gently pellet cells at 200 X g for 3-8 minutes at RT.
  - Remove and discard supernatant.
  - Resuspend cells in a similar volume of PBS.
10. Place 15–20  $\mu\text{L}$  of the cell suspension onto a microscope slide and cover with a coverslip.
11. Visualize using blue (480 nm) excitation filter and a green (540-550 nm) emission filter pairing.
12. This filter combination produces yellowish green staining of the lysosomes, instead of red.
13. Brightness will depend on the type of microscope used, and the type of cell line.

**Note:** To optimize this assay for your specific research conditions, vary the amount of 26X MR-Cathepsin B and the incubation time to determine the greatest difference in the fluorescence signal between positive and negative cell populations.

#### Staining of adherent cells with MR-Cathepsin B

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 the culture flask, aseptically add 5-25 mL sterile PBS or saline to the flask.
  - c. Aseptically remove this solution and discard.
  - d. Add Trypsin-Versane to the flask, varying the amount depending on the flask surface area. For example, a 25  $\text{cm}^2$  flask should receive approximately 1 mL of the trypsin reagent; a 75  $\text{cm}^2$  flask should receive approximately 3 mL.
  - e. Incubate 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.
2. Seed about  $10^4 - 10^5$  cells onto a sterile coverslip in a 35 mm petri dish or onto chamber slides.
  3. 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.
  4. Expose cells to your experimental conditions.
  5. Remove samples of the cell overlay media at time points according to your specific protocol. The precise volume used should be optimized to your specific conditions. For example, if working in a multiwell plate, 300  $\mu\text{L}$  samples may be appropriate, in a chamber slide -1 mL or more of the sample may be necessary.
  6. Add the 26X MR-Cathepsin B solution directly to each sample at a ratio of 1:26. For example, if 300  $\mu\text{L}$  was used, add 12  $\mu\text{L}$  of the 26X MR-Cathepsin B solution forming a final volume of 312  $\mu\text{L}$ . If 1 mL sample was used, add 40  $\mu\text{L}$  of the 26X MR-Cathepsin B solution to a final volume of 1,040  $\mu\text{L}$ .
1. Gently mix the cell overlay media to ensure even exposure to the MR-Cathepsin B.
  2. Incubate cells for 30-60 minutes at 37 °C in a  $\text{CO}_2$  incubator.
  3. Remove the media.
  4. Rinse cells twice with PBS, 1 minute per rinse.
  5. At this point labeled and unlabeled cells can be stained with Hoechst stain and unlabeled cells can be stained with AO.
  6. To visualize MR-Cathepsin B stained cells 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.

7. Observe MR-Cathepsin B 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.
8. If these filters are not available, select a filter combination that best approximates these settings.
9. Using this excitation/emission filter pairing, cells stain red with more brightly stained vacuoles and lysosomes.

**Note:** Researchers should optimize this assay to fit their specific research conditions by titrating the volume of sample and the amount of 26X MR-Cathepsin B in order to establish the conditions for the greatest difference in the fluorescence signal between positive and negative cell populations

#### Staining of adherent cells with Hoechst stain

1. Add 5.2  $\mu$ L Hoechst stain (0.5% v/v) to the 1040  $\mu$ L of cell overlay material labeled with MR-Cathepsin B or to the unlabeled cell suspension.
2. Incubate additional 5 – 10 minutes at 37°C under 5% CO<sub>2</sub>.
3. Follow the visualization procedure as described above for MR-Cathepsin B (see points 13-15)

#### Staining of adherent cells with Acridine orange (AO)

1. Because of the emissions overlap, dual staining of cells with MR-Cathepsin B and AO yields confusing results.
2. Therefore, the dyes should not be used on the same cells but separately.
3. Use AO on the non-stained cells.
4. Dilute 1 mM stock AO as follows:
  - a. 10  $\mu$ L stock AO + 990  $\mu$ L distilled water (1)
  - b. 111  $\mu$ L (1) + 1000  $\mu$ L cell overlay media - a final volume of 1111  $\mu$ L.
5. Incubate an additional 30 minutes at 37°C under 5% CO<sub>2</sub>.
6. Remove the media from the cell mono-layer surface.
7. Rinse twice with PBS, 1 minute per rinse.
8. 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.
9. View the stained lysosomes using the same filters as those used for the MR-Cathepsin B staining (excitation at 550 nm, emission >610 nm..
10. Lysosomes will stain orange to red depending on the specific properties of the lysosomes.

11. View the stained lysosomes using blue (480 nm) excitation filter and a green to orange (530-580 nm) emission filter.
12. In this filter combination lysosomes will appear orange, while other cell structures appear various shades of green.

**Note:** To optimize this assay for your specific research conditions, vary the amount of 26X MR- Cathepsin B and the incubation time to establish the conditions for achieving the greatest difference in the fluorescence signal between positive and negative cell populations.

## Results

### Fluorescence Microscopy Data

#### Detection of intracellular Cathepsin B activity in Jurkat (Photos 1 & 2)

1. Cathepsin B Intracellular activity was visualized by localized hydrolyzed (fluorescent) Magic Red™ product (Photo 1) in Nikon Eclipse E800 photomicroscope using a 510 – 560 nm excitation filter and a 570 – 620 nm emission/barrier filter at 500X.
2. Bottom photo (Photo 2) shows the corresponding DIC (differential interference contrast) image of the cells

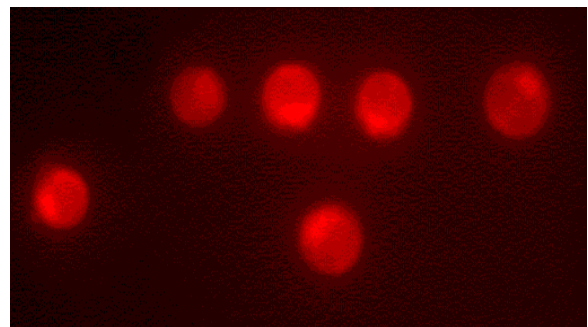


Photo 1

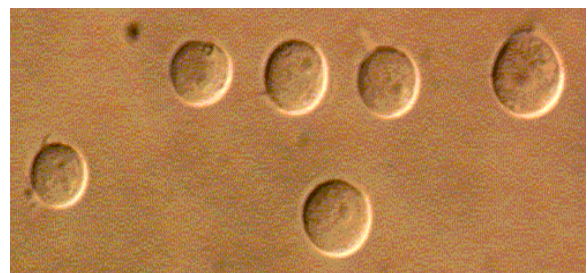
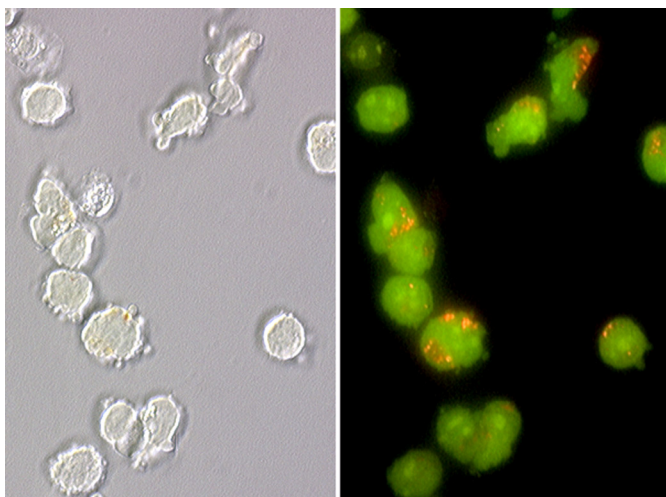


Photo 2

### Acridine orange staining of normal Jurkat cells



**Photo A**

**Photo B**

1. 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.
2. Photo A shows the corresponding DIC image of the cells (AO appears faintly).
3. Photo B shows intracellular staining of lysosomes in Jurkat cells using 5  $\mu$ M AO in PBS for 60 minutes at 37°C.

### References

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