MAGIC RED® CATHEPSIN Assays

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Not for use in diagnostic procedures.

1. INTRODUCTION

ICT's Magic Red® Cathepsin assay kits enable researchers to quantitate and monitor intracellular cathepsin-B, -K, or -L activity over time *in vitro*. The Magic Red (MR) reagent is a non-cytotoxic substrate that fluoresces red upon cleavage by active cathepsin enzymes.

Elevated cathepsin enzyme activity in serum or the extracellular matrix often signifies a number of gross pathological conditions. Cathepsin-mediated diseases include: Alzheimer's; numerous types of cancer; autoimmune related diseases like arthritis; and the accelerated breakdown of bone structure seen with osteoporosis[1,2]. Up-regulated cathepsin-B and -L activity has been linked to several types of cancer. These include cancer of the colon, pancreas, ovaries, breast, lung, and skin (melanoma)[3-6]. Upregulation of cathepsin-K has been shown in lung tumors[7]. Increased cathepsin-K activity has also been linked to degenerative bone diseases including osteopetrosis and post-menopausal osteoporosis[1, 8].

Assess cathepsin activity in whole cells in vitro using Magic Red®

Cathepsins are usually characterized as members of the lysosomal cysteine protease (active site) family[9] and the cathepsin family name has been synonymous with lysosomal proteolytic enzymes[1]. In actuality, 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[10]. Cathepsin-C is the noted exception, existing as an oligomeric enzyme with a MW ~200 kDa[11]. Initially synthesized as inactive zymogens, cathepsins are post-translationally processed into their active configurations after passing through the endoplasmic reticulum and subsequent incorporation into the acidic environment of the lysosomes[1, 11].

Magic Red detection substrates utilize the photostable red fluorophore, cresyl violet. When bi-substituted via amide linkage to two cathepsin target peptide sequences, such as $(leucine\text{-}arginine)_2$, the bi-substituted cresyl violet is nonfluorescent[10]. Following enzymatic cleavage at one or both arginine (R) amide linkage sites, the mono and non-substituted cresyl violet fluorophores generate red fluorescence when excited at 550-590 nm. ICT's Magic Red cathepsin-B substrate, MR-$(RR)_2$, is comprised of cresyl violet coupled to two pairs of the amino acid sequence, arginine-arginine (RR), which is the preferential target sequence for cathepsin-B. In ICT's cathepsin-K substrate, MR-$(LR)_2$, cresyl violet is coupled to two pairs of leucine-arginine (LR). ICT's MR cathepsin-L substrate, MR-$(FR)_2$, contains two pairs of phenylalanine-arginine (FR) coupled to cresyl violet. Cathepsins, like most other crucial cell survival enzymes, are somewhat permissive in the target amino acid sequence they will recognize and cleave. Although Magic Red substrates contain the amino acid target sequence preferred by a particular cathepsin enzyme, they can also recognize other active cathepsins or proteases when they are present. ICT encourages validation of cathepsin activity by an orthogonal technique.

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 or permeabilization steps are required. If cathepsin enzymes are active, they will cleave off the two dipeptide cathepsin targeting sequences and allow the cresyl violet fluorophore to become fluorescent upon excitation. The red fluorescent product will stay inside the cell and will often aggregate inside lysosomes (cathepsins are lysosomal) and other areas of low pH, such as inside the mitochondria. As protease activity progresses and more MR substrate is cleaved, the signal will intensify as the red fluorescent product accumulates within various organelles, enabling researchers to watch the color develop over time (Figure 4) and quantify cathepsin-B, -K, or -L activity. By varying the duration and concentration of exposure to the MR substrate, a picture can be obtained of the relative abundance of cathepsin enzymatic activity. Positive cells will fluoresce red and have pronounced red lysosomes and mitochondria. Negative cells will exhibit very low levels of background red fluorescence evenly distributed throughout the cell. This background level of substrate activity could be the result of constitutively synthesized serine proteases that target analogous amino acid sequences for hydrolysis. Please note that Magic Red substrates can undergo spontaneous hydrolysis over time, resulting in increased background fluorescence. Appropriate controls are necessary for accurate interpretation of the results. There is no interference from pro-cathepsins forms of the enzymes. If the treatment or experimental condition stimulates cathepsin activity, cells containing elevated levels of cathepsin activity will appear brighter red than cells with lower levels of cathepsin activity.

The MR fluorophore, cresyl violet, fluoresces red when excited at 550-590 nm[10]. The red fluorescent signal can be monitored with a fluorescence microscope or plate reader. It has an optimal excitation of 592 nm and emission of 628 nm[12]. Hoechst 33342 is included with the kit to concurrently label nuclei after labeling with MR (Figure 3). It is revealed under a microscope using a UV-filter with excitation at 365 nm and emission at 480 nm. Acridine orange (AO) is also included in the kit to identify lysosomes and other intracellular organelles (Figures 5 and 6). It is revealed under a microscope using excitation at 480 nm and emission at >540 nm, or alternatively with excitation at 550 nm and emission at >610 nm. Magic Red® is for research use only. Not for use in diagnostic procedures.

2. KIT CONTENTS

Trial size kits #937, 939, 941 contain:

- 1 vial of Magic Red Substrate, small, 25 Tests:
  Kit #937 contains Cathepsin-B Substrate (MR-RR_2) 25 Tests, #6133
  Kit #939 contains Cathepsin-K Substrate (MR-LR_2) 25 Tests, #6135
  Kit #941 contains Cathepsin-L Substrate (MR-FR_2) 25 Tests, #6137
- 1 vial of Hoechst 33342, 200 µg/mL (1 mL), #639
- 1 vial of Acridine Orange, 266 µg/mL, 1 mM (0.5 mL), #6130

Standard size kits #938, 940, 942 contain:

- 1 vial of Magic Red Substrate, large, 100 Tests:
  Kit #938 contains Cathepsin-B Substrate (MR-RR_2) 100 Tests, #6134
  Kit #940 contains Cathepsin-K Substrate (MR-LR_2) 100 Tests, #6136
  Kit #942 contains Cathepsin-L Substrate (MR-FR_2) 100 Tests, #6138
- 1 vial of Hoechst 33342, 200 µg/mL (1 mL), #639
- 1 vial of Acridine Orange, 266 µg/mL, 1 mM (0.5 mL), #6130

3. STORAGE

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

4. SAFETY DATA SHEETS (SDS)

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

5. RECOMMENDED MATERIALS

- DMSO, 50-200 µL to reconstitute Magic Red
- DiH_2O, 450-1600 µL to dilute Magic Red
- Phosphate buffered saline (PBS) pH 7.4, 100 mL

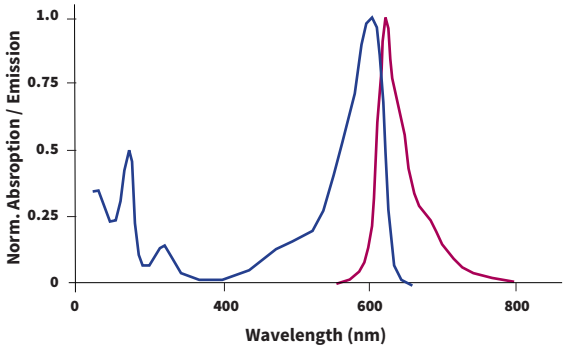
FIGURE 1: CRESYL VIOLET PERCHLORATE EXCITATION AND EMISSION SPECTRA IN ETOH

Absorption λ_{max}: 603 nm, 320 nm

Emission λ_{max}: 622 nm

Solvent: EtOH

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



- Cultured cells treated with the experimental conditions ready for staining
- Reagents to induce cathepsin activity and create controls
- Hemocytometer
- Centrifuge at 200 x g
- 15 mL polypropylene centrifuge tubes (1 per sample)
- 12 x 75 mm glass or polypropylene tubes
- 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
- Ice or refrigerator

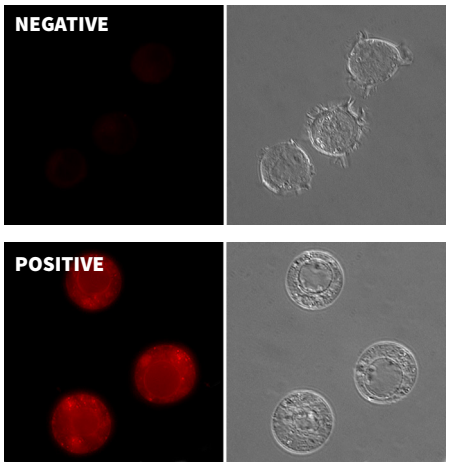
6. DETECTION EQUIPMENT

The assay can be analyzed with a:
- Fluorescence microscope
- Fluorescence plate reader

Use filter pairings that best approximate these settings:
- Magic Red excites at 550-590 nm and emits at >610 nm. It has an optimal excitation and emission wavelength tandem of 592 nm and 628 nm, respectively.
- Hoechst 33342 can be seen using a UV-filter with excitation at 365 nm and emission at 480 nm (Section 11).

FIGURE 2: NEGATIVE VS. POSITIVE CELLS

Using ICT's Magic Red substrate to detect enzymatic activity in suspension cells, there is a clear differential between negative (top) and positive (bottom) cells. Suspension cells were incubated with a control (DMSO, top) or a stimulant (bottom) for 3 hours at 37°C to induce enzymatic activity. Cell cultures were subsequently stained with Magic Red 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. Data courtesy of Dr. Brian Lee, ICT 091902.



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- Acridine Orange (AO) exhibits a very broad emission range; one of several filter pairings may be used. The same excitation/emission filter pairings used to view Magic Red 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).

7. EXPERIMENTAL PREPARATION

Staining 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.

As Magic Red detects cathepsin enzymes, plan the experiment so that the substrate will be diluted and administered at the time when the target cathepsins are expected to be activated in the cells.

The recommended volume of the Magic Red staining solution is 10-20 µL per 300-500 µL of cells at 10^6 cells/mL; the ideal amount may vary based on the experimental conditions and method of analysis. Each investigator should adjust the amount of Magic Red to accommodate the particular cell line and research conditions.

Culture cells to a density optimal for the specific experimental conditions or cathepsin activation 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. An initial experiment may be necessary to determine when and how much Magic Red to use as the resulting positive signal is a direct measurement of cathepsin activity occurring during the incubation period.

Cells with active cathepsin enzymes will generate a stronger red fluorescence with Magic Red than negative cells of the same lineage. To optimize this assay, determine the greatest difference in the fluorescent signal between positive and negative cell populations. Adjust the amount of Magic Red substrate used to stain cells and the incubation time.

Hoechst 33342 can be used with Magic Red to label nuclei (Figure 3). Because of the overlap in emissions, dual staining of cells with both Magic Red and AO will yield confusing results and is not recommended; these dyes should be used separately. Do not use Magic Red with paraffin-embedded tissues as the chemicals used for paraffin-embedding may denature and inactivate the substrate.

8. CONTROLS

It is highly recommended that two sets of controls be run: one positive control population of cells that was activated to stimulate cathepsin activity (Section 9); and a placebo population of cells that received just the vehicle used to deliver the stimulating agent. Create negative controls by culturing an equal volume of non-activated cells for every labeling condition. The negative control and activated positive control populations should contain similar quantities of cells. For example, if labeling with Magic Red, Hoechst 33342, and Acridine Orange, make 10 control populations:

1&2. Unlabeled, stimulated and non-stimulated populations.

3&4. Magic Red-labeled, stimulated and non-stimulated populations.

5&6. Magic Red- and Hoechst-labeled, stimulated and non-stimulated populations.

7&8. Hoechst-labeled, stimulated and non-stimulated populations.

FIGURE 3: DUAL STAINING WITH HOECHST

Cells were dually stained using ICT's Magic Red substrate and Hoechst 33342 nuclear stain. Experimental cells were stained with Magic Red for 30 minutes at 37°C, rinsed briefly with PBS, and supravitally stained with 1 µg/mL Hoechst 33342 for 10 minutes. A Nikon Microphot FXA system with multi-wavelength filter pairs was used: UV for Hoechst 33342; and green light for Magic Red. Positive cells bearing orange-red lysosomal bodies with less intense blue nuclei are intermixed with negative cells bearing bright blue nuclei and absent or reduced orange-red lysosomal staining. In this particular experiment, the treatment is killing the positive cells. Photo provided by Dr. Zbigniew Darzynkiewicz at Brander Cancer Research Center Institute, New York City, NY.

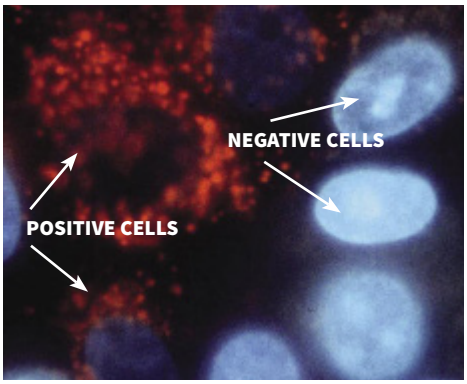
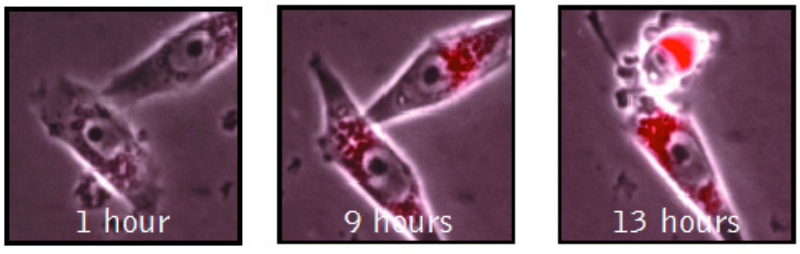


FIGURE 4: WATCH ENZYMATIC ACTIVITY IN REAL TIME

Adherent cells were seeded in a 12-well plate and exposed to the experimental treatment the following day. ICT's Magic Red substrate 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 enzymatic activity progressed over time. Data courtesy of Dr. Martin Purschke, Massachusetts General Hospital.



9&10. AO-labeled, stimulated and non-stimulated populations.

9. CATHEPSIN INDUCTION

Magic Red works with your existing protocols - stimulate cathepsin enzymatic activity as you normally would, then label cells with Magic Red.

10. PREPARATION OF MAGIC RED

Each Magic Red cathepsin-B, -K, or -L substrate is supplied as a highly concentrated lyophilized powder that may be slightly visible as an iridescent sheen inside the vial. It must first be reconstituted with DMSO, forming the stock concentrate, and then diluted 1:10 with diH_2O to form the final staining solution. The staining solution is typically used to stain cells at approximately 1:26 for microscopy analysis (Sections 13 and 14) or 1:16 for plate reader analysis (Section 15). 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. It is vialed in 2 sizes: small (approximately 25 Tests); and large (approximately 100 Tests). Trial size kits contain the small vial; standard size kits contain the large vial. The reconstitution volume will vary based on the vial size:
   - Reconstitute the small (trial size) vial #6133 (B), 6135 (K), or 6137 (L) with 50 µL DMSO.
   - Reconstitute the large (standard size) vial #6134 (B), 6136 (K), or 6138 (L) with 200 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 for 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 1:10 with diH_2O to form the staining solution. Use the staining solution within 15 minutes of dilution to prevent substrate hydrolysis.
   - The small vial (#6133, 6135, or 6137) contains 50 µL of the stock concentrate in DMSO. Add 450 µL diH_2O to it. This yields 500 µL of the staining solution.
   - The large vial (#6134, 6136, or 6138) contains 200 µL of the stock concentrate in DMSO. Add 1,800 µL diH_2O to it. This yields 2 mL of the staining solution.
   - For other amounts, dilute the stock concentrate 1:10 in diH_2O. For example, add 10 µL stock to 90 µL diH_2O; this yields 100 µL of the staining solution.
4. Mix by inverting or vortexing the vial at RT.
5. Use immediately.

11. HOECHST 33342

Hoechst 33342 (catalog #639) 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 is provided ready-to-use at 200 µg/mL. It can be used with Magic Red to label nuclei (Figure 3).

When bound to nucleic acids, it has a maximum absorbance at 350 nm and a maximum emission at 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 contains a low concentration of Bis benzimide H 33342 trihydrochloride (CAS 23491-52-3) which is below the threshold for reporting. Hoechst is a suspected mu-

FIGURE 5: ACRIDINE ORANGE STAINING

MCF-7 cells were stained with acridine orange (AO) in PBS for 30 minutes, then washed twice in PBS (cells were not stained with Magic Red). Cells were photographed with a Nikon Microphot-FXA epifluorescence microscope at 400X using either a blue light excitation (492 nm) with a 540-550 nm emission filter (A, lysosomes appear yellowish green), or green light excitation (540 nm) with a long pass >640 nm barrier filter (B, lysosomes appear red; compare with Figure 6). Experiment performed in the laboratory of Dr. Zbigniew Darzynkiewicz (Brander Cancer Research Center Institute, New York City, NY).

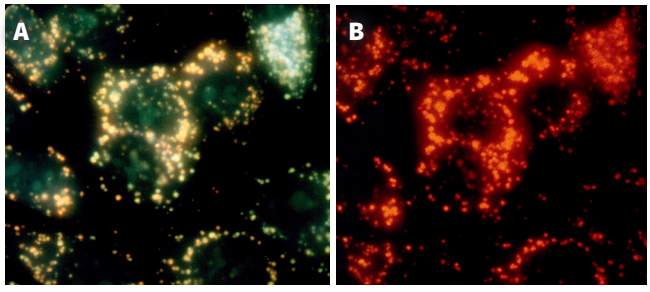
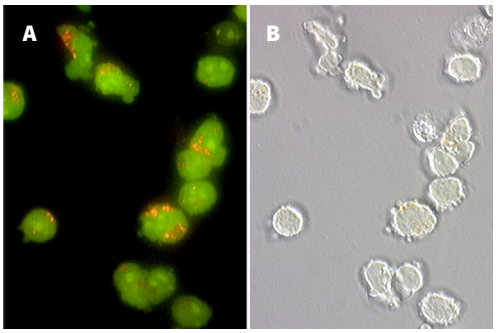


FIGURE 6: ACRIDINE ORANGE STAINING

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).



tagen at high concentrations. Prolonged skin contact may cause redness and irritation. Because of the small quantity of product, the health hazard is small. See SDS for further information.

12. ACRIDINE ORANGE

Acridine orange (AO) is a chelating dye and can be used to reveal lysosomes, nuclei, and nucleoli (Figures 5 and 6). The acidic pH of the lysosome results in the concentration and aggregation of AO. It is provided ready-to-use at 1 mM (#6130). AO 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 diH_2O; e.g., put 10 µL AO into 990 µL diH_2O. 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 pairings used to view Magic Red can be used: an excitation filter of 550 nm (540-560 nm) and a long pass >610 nm emission/barrier filter pair. With this pairing, the lysosomes appear red.

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 Magic Red and AO will yield confusing results. Therefore, these dyes should be used separately.

- **Warning:** Acridine Orange contains a concentration of 3,6-Acridinediamine, N,N,N',N'-Tetramethyl-, monohydrochloride (CAS 65-61-2, or CAS 494-38-2 free base) at less than 0.1% which is below the threshold for reporting. This product may be a potent mutagen at high concentrations and probable carcinogen. Because of the small quantity of product, the health hazard is small. 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 10^6 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 8-9).
3. When ready to label with the staining solution, cell concentrations should be 2-5 x 10^6 cells/mL for best viewing. Fluorescence microscopy requires an excess of 2 x 10^6 cells/mL to obtain 5-20 cells per image field. 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 at room temperature (RT). Remove the supernatant and resuspend with cell culture media or PBS.
4. Transfer 500 µL cell suspension into 12 x 75 mm glass or polypropylene tubes. If desired, larger cell volumes can be used, but additional Magic Red staining solution may be required.
5. Reconstitute Magic Red (Section 10) to form the concentrated stock solution at 260X:
   - Add 50 µL DMSO to the small (trial size) vial #6133 (B), 6135 (K), or 6137 (L).
   - Add 200 µL DMSO to the large (standard size) vial #6134 (B), 6136 (K), or 6138 (L).
6. When ready to stain cells, dilute the 260X stock concentrate 1:10 in diH_2O to form the staining solution at 26X:
   - Add 450 µL diH_2O to the small vial.
   - Add 1,800 µL diH_2O to the large vial.
7. Add 20 µL of the staining solution to each 500 µL cell suspension and mix thoroughly. If different cell volumes are used, add the Magic Red staining solution at a dilution of approximately 1:26. For example, add 40 µL Magic Red staining solution to 1,000 µL

FIGURE 7: CATHEPSIN-B IN JURKAT CELLS

Intracellular cathepsin-B activity was detected in Jurkat cells using ICT's MR-$(RR)_2$ cathepsin-B fluorogenic substrate. Intracellular localization of the hydrolyzed (fluorescent) Magic Red product was detected using a Nikon Eclipse E800 photomicroscope equipped with a 510 – 560 nm excitation filter and a 570 – 620 nm emission/barrier filter at 500X (A). Photo at right (B) shows the corresponding DIC image of the cells. Data courtesy of Dr. Brian Lee, ICT, 061202.

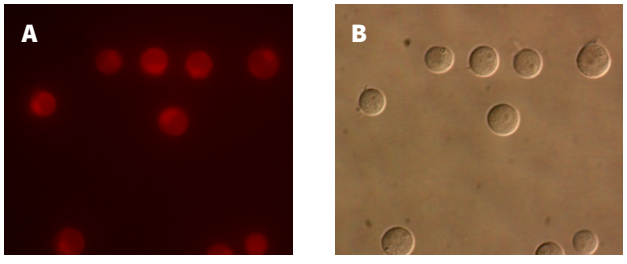
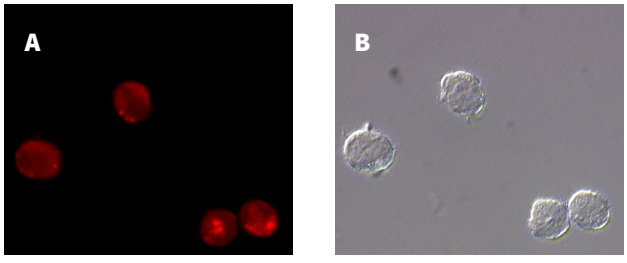


FIGURE 8: CATHEPSIN-B IN THP-1 CELLS

Intracellular cathepsin-B activity was detected in THP-1 cells using ICT's MR-$(RR)_2$ cathepsin-B fluorogenic substrate. Intracellular localization of the hydrolyzed (fluorescent) Magic Red product was detected using a Nikon Eclipse E800 photomicroscope equipped with a 510 – 560 nm excitation filter and a 570 – 620 nm emission/barrier filter at 400X (A). Photo at right (B) shows the corresponding DIC image of the cells. Data courtesy of Dr. Brian Lee, ICT, 061202.



of cell suspension forming a final volume of 1,040 µL.

- Do not add Magic Red to cells that are to be labeled with AO; add a placebo instead, such as diH_2O (Step 10).

8. Incubate cells for 30-60 minutes at 37°C protected from light. Cells may settle on the bottom of the tubes; gently resuspend by swirling cells every 20 minutes during the incubation to ensure even distribution of Magic Red substrate. After the incubation, cells can be stained with Hoechst 33342 (Section 11), or unstained cells may be labeled with AO (Section 12).
9. If cells are to be labeled with Hoechst 33342, add it at approximately 0.5% v/v. For example, if the cell suspension is 520 µL, add 2.5 µL Hoechst 33342. Incubate 5-10 minutes at 37°C. Go to Step 11.
10. Because of the overlap in emissions, dual staining of cells with both Magic Red and AO is not recommended; the dyes should be used separately. To stain cells with AO:
    a. Dilute AO to 1:2,000-1:200 (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 diH_2O e.g., put 10 µL AO into 990 µL diH_2O. Pipette the diluted AO into the cell suspension at 1:10; e.g., add 55 µL to 500 µL cell suspension.
    b. Incubate 30 minutes at 37°C.
    c. If viewing under the same filters used for Magic Red (excitation at 550-590 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 500 µL or a similar volume of PBS in which the cells were originally suspended.
11. Place 15-20 µL of cell suspension onto a microscope slide and cover with a coverslip.
12. Observe cells using a fluorescence microscope equipped with an excitation filter of 550 nm (540-560 nm) and a long pass >610 nm emission/barrier filter pairing. Select a filter combination that best approximates these settings. Using these filters, positive cells will appear red with brightly stained vacuoles and lysosomes.

    If the samples were stained with both Magic Red and Hoechst 33342, and if a multi-wavelength filter option is available on the fluorescence microscope, the dual staining properties can be examined. Hoechst 33342 can be seen using a UV-filter with excitation at 365 nm and emission at 480 nm.

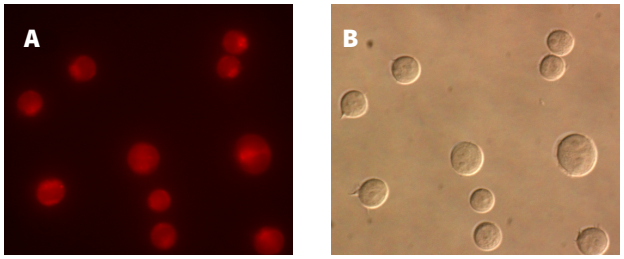
    As AO exhibits a very broad emission range, one of several filter pairings can be used. The same excitation/emission pairing filters used to view Magic Red can be used: a 550 nm (540-560 nm) excitation and long pass >610 nm emission/barrier filter pairing. With this pairing, the lysosomes appear red.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

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 4).
2. Grow cells until 80-90% confluent. This usually takes about 24 hours but will vary with each cell line. Please note that some cell lines will not tolerate confluency levels >60%; adjust as necessary for the particular cells being used.
3. Expose cells to the experimental conditions and create positive and negative controls (Sections 8 and 9).
4. Reconstitute Magic Red (Section 10) to form the concentrated stock solution at 260X:
    - Add 50 µL DMSO to the small (trial size) vial #6133 (B), 6135 (K), or 6137 (L).
    - Add 200 µL DMSO to the large (standard size) vial #6134 (B), 6136 (K), or 6138 (L).
5. When ready to stain cells, dilute the 260X stock concentrate 1:10 in diH_2O to form the staining solution at 26X:
    - Add 450 µL diH_2O to the small vial.
    - Add 1,800 µL diH_2O to the large vial.
6. Add Magic Red staining solution at approximately 1:26 and gently mix to ensure an even distribution of Magic Red. For example, add 12 µL staining solution to 300 µL cells forming a final volume of 312 µL.
    - Do not add Magic Red 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 protected from light.
8. Remove the media from the cell monolayer surface and rinse twice with PBS, 1 minute per rinse. At this point, cells can be analyzed (Step 12) or stained with Hoechst 33342 (Step 9). Unstained cells can be labeled with AO (Step 10).
9. If cells are to be labeled with Hoechst 33342, add it at approxi-

FIGURE 9: CATHEPSIN-L IN JURKAT CELLS

Intracellular cathepsin-L activity was detected in Jurkat cells using ICT's MR-$(FR)_2$ cathepsin-L fluorogenic substrate. Intracellular localization of the hydrolyzed (fluorescent) Magic Red product was detected using a Nikon Eclipse E800 photomicroscope equipped with a 510 – 560 nm excitation filter and a 570 – 620 nm emission/barrier filter at 400X (A). Photo at right (B) shows the corresponding DIC image of the cells. Data courtesy of Dr. Brian Lee, ICT, 061202.



mately 0.5% v/v. For example, add 1.6 µL Hoechst 33342 to 312 µL cells labeled with Magic Red and control samples. Incubate 5-10 minutes at 37°C. Go to Step 11.

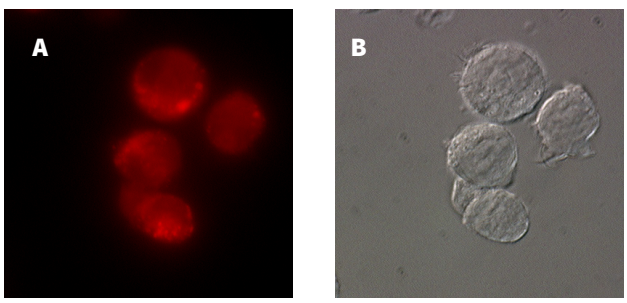
10. Because of the overlap in emissions, dual staining of cells with both Magic Red and AO is not recommended; the dyes should be used separately. To stain cells that have not been exposed to Magic Red:
    a. Dilute AO at 1:2,000-1:200 (which is 0.05-0.5% v/v) into the final cell volume. For example, if using AO at 1.0 µM in the final cell volume, it must be diluted 1:1,000. First dilute it 1:100 in diH_2O; e.g., add 10 µL AO to 990 µL diH_2O. Pipette the diluted AO to the cells at 1:10; e.g., add 30 µL diluted AO to 270 µL cell media (or 33 µL diluted AO to 300 µL cell media).
    b. Incubate 30 minutes at 37°C.
    c. Remove the media from the cell monolayer surface. Rinse twice with PBS, 1 minute per rinse.
11. Mount the coverslip with cells facing down onto a drop of PBS. If a chamber-slide was used, pull off the plastic frame and add a drop of PBS to the cell surface and cover with a coverslip.
12. Observe Magic Red-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 Magic Red will appear red with more brightly stained vacuoles and lysosomes.
13. If samples were stained with both Magic Red and Hoechst 33342, and if a multi-wavelength filter option is available on the fluorescence microscope, the dual staining properties of the sample can be examined. Hoechst 33342 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 can be used. The same excitation/emission pairing filters used to view Magic Red can 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 10: CATHEPSIN-L IN THP-1 CELLS

Intracellular cathepsin-L activity was detected in THP-1 cells using ICT's MR-$(FR)_2$ cathepsin-L fluorogenic substrate. Intracellular localization of the hydrolyzed (fluorescent) Magic Red product was detected using a Nikon Eclipse E800 photomicroscope equipped with a 510 – 560 nm excitation filter and a 570 – 620 nm emission/barrier filter at 700X (A). Photo at right (B) shows the corresponding DIC image of the cells. . Data courtesy of Dr. Brian Lee, ICT, 061202.



15. FLUORESCENCE PLATE READER ANALYSIS

1. Prepare cell populations. Cell concentrations should be >3 x 10^6 cells/mL. If this is too dense for the cell line, stimulate cathepsin activity first, then concentrate the cells and stain with Magic Red. Adherent cells should be cultured to ~80-90% confluency. Please note that some cell lines will not tolerate confluency levels >60%; adjust as necessary for the particular cells being used.
2. Expose cells to the experimental conditions and create positive and negative controls (Sections 8 and 9).
3. If using suspension cells, transfer 300 µL cell suspension into 12 x 75 mm glass or polypropylene tubes or a black microtiter plate. 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 Magic Red substrate will be required per sample.
4. When ready to label with the Magic Red staining solution, cells should be at least 3 x 10^5 cells/100 µL aliquot (equal to 3 x 10^6 cells/mL) for each microtiter plate well.
5. Reconstitute Magic Red (Section 10) to form the concentrated stock solution at 160X:
    - Add 50 µL DMSO to the small (trial size) vial #6133 (B), 6135 (K), or 6137 (L).
    - Add 200 µL DMSO to the large (standard size) vial #6134 (B), 6136 (K), or 6138 (L).
6. When ready to stain cells, dilute the 160X stock concentrate 1:10 in diH_2O to form the staining solution at 16X:
    - Add 450 µL diH_2O to the small vial.
    - Add 1,800 µL diH_2O to the large vial.
7. Add 20 µL Magic Red staining solution directly to 300 µL cell sample and gently mix. If different cell volumes are used, add Magic Red staining solution at approximately 1:16. Due to sensitivity limitations, plate readers require a higher concentration of Magic Red for detection compared to microscopes.
8. Incubate cells for 30-60 minutes at 37°C protected from light. As cells settle to the bottom, gently resuspend them approximately every 10-20 minutes to ensure Magic Red is evenly dispersed among all cells.
9. Read the 300 µL sample as one sample or split it into 3 wells of 100 µL each. If cells were stained in a tube, transfer to a black microtiter plate.
10. 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 pairings that best approximate these settings. If available, use a cut-off filter at 630 nm to filter out shorter wavelength excitation interference.

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Immunochemistry Technologies, LLC
9401 James Ave. S. #155, Bloomington, MN 55431 USA 800-829-3194
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