



Product Manual

CV-Cathepsin K Detection Kit

**Instruction Manual
BML-AK126**

For research use only

USE FOR RESEARCH PURPOSES ONLY

Unless otherwise specified expressly on the packaging, all products sold hereunder are intended for and may be used for research purposes only and may not be used for food, drug, cosmetic or household use or for the diagnosis or treatment of human beings. Purchase does not include any right or license to use, develop or otherwise exploit these products commercially. Any commercial use, development or exploitation of these products or development using these products without the express written authorization of Enzo Life Sciences, Inc. is strictly prohibited. Buyer assumes all risk and liability for the use and/or results obtained by the use of the products covered by this invoice whether used singularly or in combination with other products.

LIMITED WARRANTY; DISCLAIMER OF WARRANTIES

These products are offered under a limited warranty. The products are guaranteed to meet all appropriate specifications described in the package insert at the time of shipment. Enzo Life Sciences' sole obligation is to replace the product to the extent of the purchasing price. All claims must be made to Enzo Life Sciences, Inc., within five (5) days of receipt of order. THIS WARRANTY IS EXPRESSLY IN LIEU OF ANY OTHER WARRANTIES OR LIABILITIES, EXPRESS OR IMPLIED, INCLUDING WARRANTIES OF MERCHANTABILITY, FITNESS FOR A PARTICULAR PURPOSE, AND NON- INFRINGEMENT OF THE PATENT OR OTHER INTELLECTUAL PROPERTY RIGHTS OF OTHERS, AND ALL SUCH WARRANTIES (AND ANY OTHER WARRANTIES IMPLIED BY LAW) ARE EXPRESSLY DISCLAIMED.

TRADEMARKS AND PATENTS

Several Enzo Life Sciences products and product applications are covered by US and foreign patents and patents pending. Enzo is a trademark of Enzo Life Sciences, Inc.

**FOR RESEARCH USE ONLY.
NOT FOR USE IN DIAGNOSTIC PROCEDURES.**



Please read entire booklet before proceeding with the assay.



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



Please contact Enzo Life Sciences Technical Support if necessary.

TABLE OF CONTENTS

Introduction.....	2
Kit Contents.....	4
Storage.....	4
MSDS.....	4
Recommended Materials & Equipment.....	4
Detection Equipment.....	5
Protocol.....	6
References.....	17
Contact Information.....	18

INTRODUCTION

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^{4,7}. 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)^{1,6 8,9}. Upregulation of cathepsin K has been shown in lung tumors⁵. Increased cathepsin K activity has also been linked to degenerative bone diseases including osteopetrosis and post-menopausal osteoporosis^{3,4}.

Cathepsins are usually characterized as members of the lysosomal cysteine protease (active site) family¹¹ and the cathepsin family name has been synonymous with lysosomal proteolytic enzymes⁴. 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 kDa¹⁵. Cathepsin C is the noted exception, existing as an oligomeric enzyme with a MW ~200 kDa¹⁴. Initially synthesized as inactive zymogens, they are post-translationally processed into their active configurations after passing through the endoplasmic reticulum and subsequent incorporation into the acidic environment of the lysosomes^{4,14}.

CV detection reagents utilize the photostable red fluorophore, cresyl violet. When bi-substituted via amide linkage to two cathepsin target peptide sequences, such as (leucine-arginine)₂, the bi-substituted cresyl violet is nonfluorescent¹⁵. 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- 590nm. In Enzo Life Sciences' cathepsin K substrate reagent, MR-(LR)₂, cresyl violet is coupled to two copies of leucine-arginine (LR).

To use CV-(LR)₂, add the reagent 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. MR will enter the cell in a non-fluorescent state. 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 (Figures 2 and 3) and other areas of low pH (cathepsins are lysosomal). 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 2) and quantify cathepsin K activity. By varying the duration and concentration of exposure to the MR substrate, a picture can be obtained of the relative abundance and intracellular location 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. 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- 590nm¹⁵. The red fluorescent signal can be monitored with a fluorescence microscope or plate reader. It has an optimal excitation of 592nm and emission of 628nm₂. At these higher excitation wavelengths, the amount of cell structure derived auto-fluorescence is minimal¹⁵. Fortunately, the excitation peak is rather broad allowing good excitation efficiency at 540-560nm. The unsubstituted red fluorescent MR product has an optimal excitation and emission wavelength pairing of 592nm and 628nm respectively. The typical mercury lamp used in fluorescence microscopy has a maximum light output at 542nm which is quite compatible with CV-Cathepsin K.

Hoechst stain 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 365nm and emission at 480nm. Acridine orange (AO) is also included in the kit to identify lysosomes and other intracellular organelles (Figures 4 and 5). 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 the other organelles¹³.

The CV-Cathepsin K detection kit is for research use only. Not for use in diagnostic procedures.

PLEASE READ ENTIRE BOOKLET BEFORE PROCEEDING WITH THE ASSAY. CAREFULLY NOTE THE HANDLING AND STORAGE CONDITIONS OF EACH KIT COMPONENT. PLEASE CONTACT ENZO LIFE SCIENCES TECHNICAL SERVICES FOR ASSISTANCE IF NECESSARY.

KIT CONTENTS

- 1 vial of CV-(LR)₂ reagent, 25 tests
- 1 vial of Hoechst 33342, 200µg/ml (1ml)
- 1 vial of Acridine Orange, 266µg/ml, 1mM (0.5ml)

STORAGE

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

MSDS

MSDS available at www.enzolifesciences.com

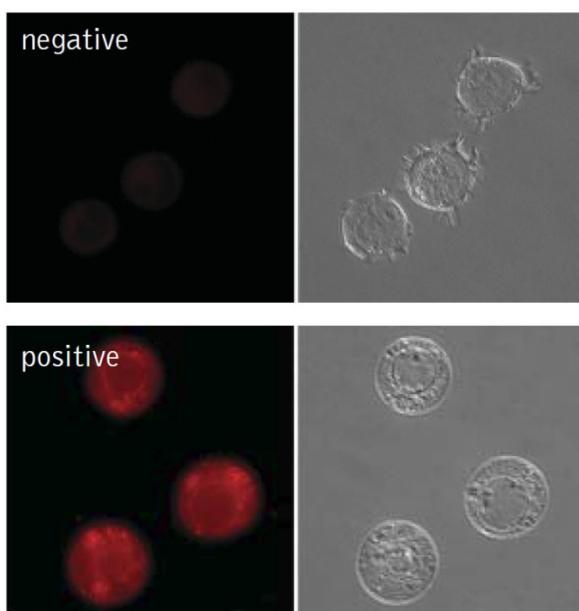
RECOMMENDED MATERIALS AND EQUIPMENT

- DMSO, 50-200µl to reconstitute the CV-(LR)₂ reagent
- diH₂O, 450-1600µl to dilute CV-(LR)₂ reagent
- Phosphate buffered saline (PBS) pH 7.4, 100ml
- Cultured cells treated with the experimental conditions ready for staining
- Reagents to induce the experimental condition and create controls
- Hemocytometer
- Centrifuge at <200g
- 15ml polystyrene centrifuge tubes
- Sterile black 96-well microtiter tissue culture plates, round or flat bottom
- Slides and coverslips
- Ice or refrigerator

DETECTION EQUIPMENT

The CV-(LR)₂ reagent excites at 550nm and emits >610nm. It has an optimal excitation and emission wavelength tandem of 592nm and 628nm, respectively. Hoechst stain can be visualized using a UV-filter with excitation at 365nm and emission at 480nm (Section 5). AO excites at 480nm and emits >540nm (Section 6). Select a filter combination that best approximates these settings.

FIGURE 1: NEGATIVE VS. POSITIVE CELLS

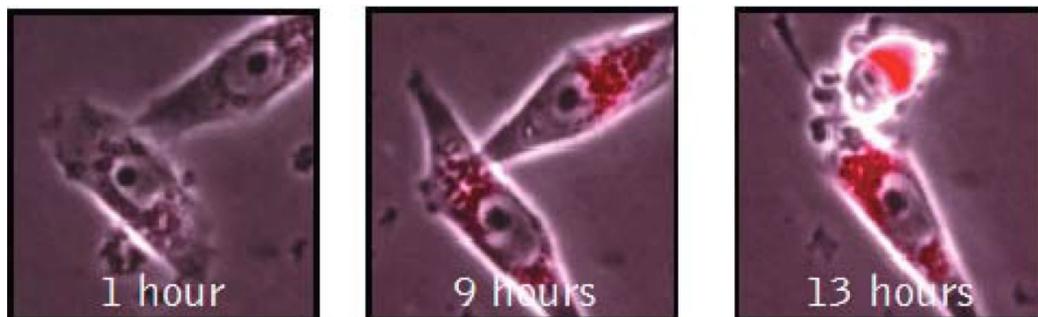


Using Enzo Life Sciences' CV-Cathepsin K detection kit to detect enzymatic activity in suspension cells, there is a clear differential between negative (top) and positive (bottom) cells. Suspension cells were incubated with a control (DMSO, top) or a stimulant (bottom) for 3 hours at 37°C to induce enzymatic activity. Cell cultures were subsequently stained with the CV-(LR)₂ reagent 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- 560nm) and emission (570-620nm) filter pairings. Right panels contain the corresponding differential-interference contrast (DIC) image¹⁵.

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

FIGURE 2: WATCH ENZYMATIC ACTIVITY IN REAL TIME



Adherent cells were seeded in a 12-well plate and exposed to the experimental treatment the following day. Enzo Life Sciences' CV-(LR)₂ reagent 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.

- Fluorescence plate reader: The CV-(LR)₂ reagent has an optimal excitation and emission wavelength tandem of 592nm and 628nm, respectively. Use a fluorescence plate reader with excitation at 590nm and emission at 630-640nm. If available, use a cutoff filter at 630nm to filter out shorter wavelength excitation interference.

PROTOCOL

1. Experimental Preparation

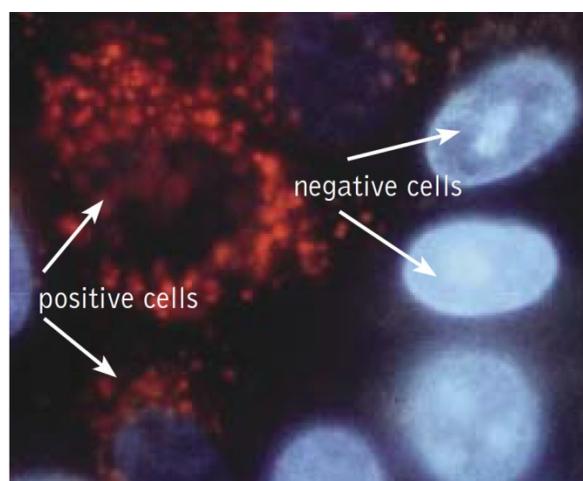
Staining cells with the CV-(LR)₂ reagent can be completed within a few hours. However, the CV-(LR)₂ reagent is used with living cells, which require periodic maintenance and cultivation several days in advance. In addition, once the proper number of cells has been cultivated, time must be allotted for the experimental procedure.

As the CV-(LR)₂ reagent 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 CV-(LR)₂ reagent staining solution is 10-20 μ l per 300 μ l of cells at 10⁶ cells/ml, but the ideal amount may vary based on the experimental conditions and method of analysis. Culture cells to a density optimal for the specific experimental protocol. Cell density should not exceed 10⁶ cells/ml as cells cultivated in excess of this concentration may begin to naturally enter apoptosis due to nutrient deprivation or the accumulation of cell degradation products in the media.

Cells with active cathepsin enzymes will generate a stronger red fluorescence with the CV-(LR)₂ reagent 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 the CV-(LR)₂ reagent used to stain cells and the incubation time.

Hoechst stain can be used with the CV-(LR)₂ reagent to label nuclei (Figure 3). Because of the overlap in emissions, dual staining of cells with both the CV-(LR)₂ reagent and AO will yield confusing results and is not recommended; these dyes should be used separately. Do not use the CV-(LR)₂ reagent with paraffin-embedded tissues as the chemicals used for paraffin-embedding may denature and inactivate the substrate.

FIGURE 3: DUAL STAINING WITH HOECHST



Cells were dually stained using Enzo Life Sciences' CV-(LR)₂ reagent and Hoechst 33342 nuclear stain. Experimental cells were stained with the CV-(LR)₂ reagent for 30 minutes at 37°C, washed twice in PBS, and supravivally stained with 1µg/ml Hoechst 33342 stain for 10 minutes. A

Nikon Microphot FXA system with multi-wavelength filter pairs was used: UV for Hoechst 33342 stain; and green light for the CV-(LR)₂ reagent. Positive cells bearing orange-red lysosomal bodies with less intense blue nuclei are intermixed with negative cells with absent or reduced orange-red lysosomal staining and bright blue nuclei. 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, NY¹⁵.

2. 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; and a placebo population of cells that received just the vehicle used to deliver the stimulating agent (Figure 1) (Section 3). 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 the CV-(LR)₂

reagent, Hoechst stain, and Acridine Orange, make 10 control populations:

- a. Unlabeled, stimulated and non-stimulated populations.
- b. CV-(LR)₂-labeled, stimulated and non-stimulated populations.
- c. CV-(LR)₂- and Hoechst-labeled, stimulated and non-stimulated populations.
- d. Hoechst-labeled, stimulated and non-stimulated populations.
- e. AO-labeled, stimulated and non-stimulated populations.

3. Cathepsin Induction

The CV-(LR)₂ reagent works with your existing protocols - stimulate cathepsin enzymatic activity as you normally would, then label cells with the CV-(LR)₂ reagent.

4. CV-Cathepsin K Substrate

The CV-(LR)₂ reagent 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 in DMSO, forming the 260X stock concentrate, and then diluted 1:10 in diH₂O to form the final staining solution at 26X. 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 260X stock solution by reconstituting the CV-(LR)₂ reagent. It is supplied as approximately 25.
 - a. Reconstitute with 50µl DMSO.
2. Gently vortex or swirl the vial, allowing the DMSO to travel around the base of the vial until completely dissolved. At room temperature (RT), this should take just a few minutes. The stock solution should appear red. Once reconstituted, it may be stored at ≤-20°C up to 6 months protected from light and thawed no more than twice during that time. If using immediately, dilute in diH₂O 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 in diH₂O to form the 26X staining

solution. Use the staining solution within 15 minutes of dilution to prevent substrate hydrolysis.

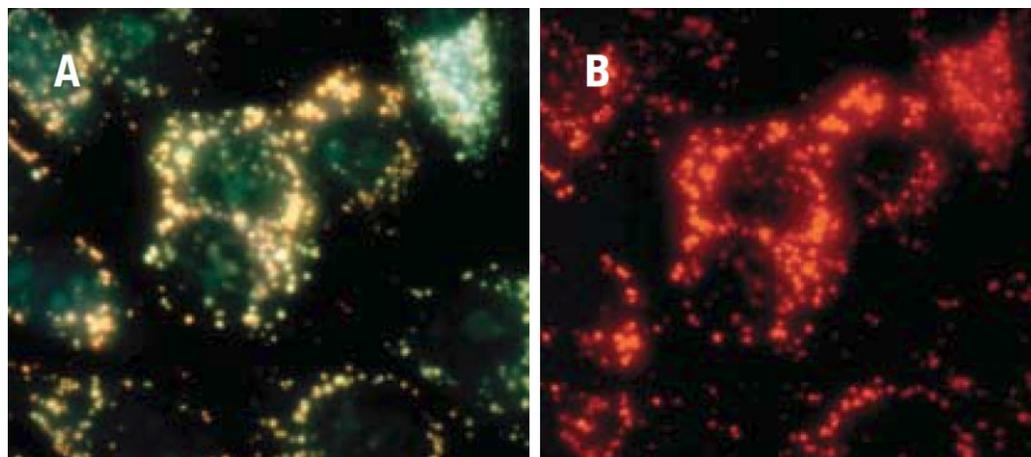
- a. Add 450 μ l diH₂O to dilute the vial. The vial contains 50 μ l of the stock (1a); this yields 500 μ l of the staining solution. Use immediately.
- b. For other amounts, dilute the stock 1:10 in diH₂O. For example, add 10 μ l stock to 90 μ l diH₂O; this yields 100 μ l of the staining solution. Use immediately.
- c. Mix by inverting or vortexing the vial at RT.

5. Hoechst Stain

Hoechst 33342 is a cell-permeant nuclear stain that emits blue fluorescence when bound to double-stranded DNA. It is used: a) to stain the nuclei of living or fixed cells; b) to distinguish condensed pyknotic nuclei in apoptotic cells; and c) for cell cycle studies. When bound to nucleic acids, the maximum absorption is 350nm and the maximum emission is 480nm. It is revealed under a microscope using a UV-filter with excitation at 365nm and emission at 480nm. Hoechst Stain is provided ready-to-use at 200 μ g/ml. Hoechst Stain can be used concurrently with the CV-(LR)₂ reagent to label nuclei (Figure 3).

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

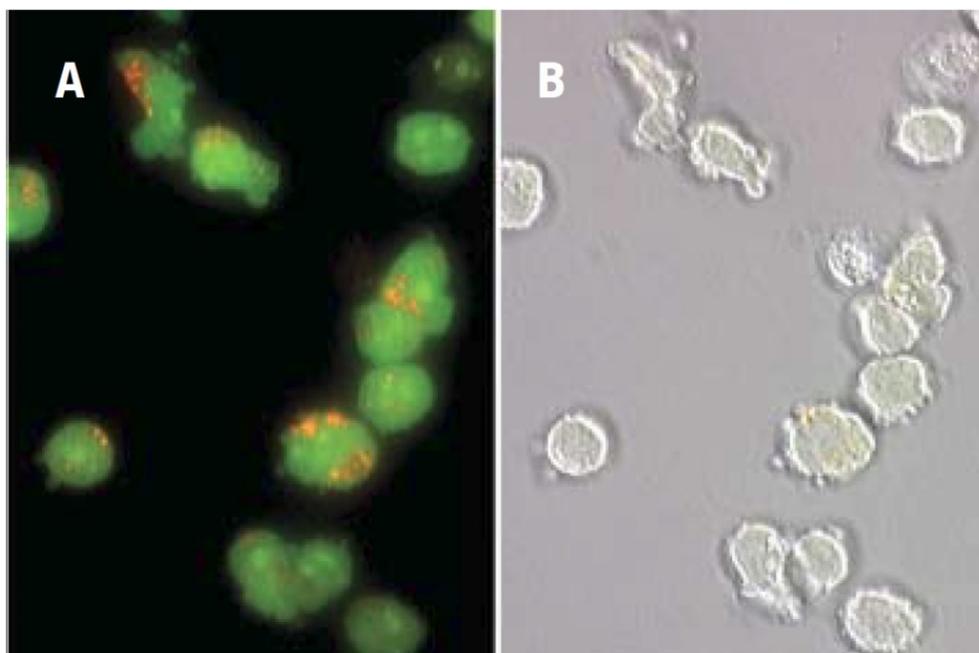
FIGURE 4: ACRIDINE ORANGE STAINING



MCF-7 cells were stained with Acridine Orange (AO) in PBS for 30 minutes, then washed twice in PBS (cells were not stained with the CV-(LR)₂ reagent). Cells were photographed with a Nikon Microphot-FXA epifluorescence microscope at 40X using either a blue light excitation (492nm) with a 540-550nm emission filter (A, lysosomes appear yellowish green), or green light excitation (540nm) with a long

pass >640nm barrier filter (B, lysosomes appear red; compare with Figure 5). Experiment performed in the laboratory of Dr. Zbigniew Darzynkiewicz (Brander Cancer Research Center Institute, New York City, NY)¹⁵.

FIGURE 5: 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 460-500nm excitation filter and a 505-560nm emission/barrier filter set at 300X. AO-stained lysosomes appear in photo A; photo B shows the corresponding DIC image of the cells (compare with Figure 4).

6. Acridine Orange

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

Lysosomal structures can be visualized by staining with AO at 0.5-5.0µM. 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 1.0µM in the final cell suspension, it must be diluted 1:1,000. First dilute it 1:100 in PBS; e.g., put 10µl AO into 990µl PBS. Pipette the diluted AO into the cell suspension at approximately 1:10; e.g., put 50µl diluted AO into 450µl 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 filter

pairings used to view the CV-(LR)₂ reagent may be used: an excitation filter of 550nm (540- 560nm) and a long pass >610nm emission/barrier filter pair. With this pairing, the lysosomes appear red instead of yellowish green.

When illuminating with a blue light (480nm) excitation filter, a green light (540-550nm) 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 the CV-(LR)₂ reagent and AO will yield confusing results. Therefore, these dyes should be used separately.

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

7. Microscopy Analysis of Suspension Cells

1. Prepare cell populations. Initial cell concentrations should be 3-5 x 10⁵ cells/ml and should not exceed 7 x 10⁵ cells/ml, as cells cultivated in excess of this concentration may begin to naturally enter apoptosis.
2. Expose cells to the experimental conditions and create positive and negative controls (Section 2) or induce cells to stimulate cathepsin activity (Section 3).
3. When ready to label with the staining solution, cell concentrations should be 0.5 - 2 x 10⁶ cells/ml for best viewing. Density can be determined by counting cell populations on a hemocytometer. If necessary, concentrate cells by gentle centrifugation at 200 x g for 3-8 minutes. Remove the supernatant and resuspend with cell culture media or PBS.
4. Transfer 500µl into 12 x 75 mm glass or polypropylene tubes. If desired, larger cell volumes can be used, but additional CV-(LR)₂ reagent staining solution may be required.
5. Reconstitute the CV-(LR)₂ reagent to form the 260X stock solution (Section 4):
 - a. Use 50µl DMSO to reconstitute the vial.

6. When ready to stain cells, dilute the stock 1:10 in diH₂O to form the CV-(LR)₂ reagent staining solution (Section 4):
 - a. Add 450µl diH₂O to dilute the vial.
7. Add 20µl of the staining solution to each 500µl cell suspension and mix thoroughly. If different cell volumes are used, add the CV-(LR)₂ reagent staining solution at a ratio of approximately 1:26. For example, add 40µl CV-(LR)₂ reagent staining solution to 1,000µl of cell suspension forming a final volume of 1,040µl. Do not add the CV-(LR)₂ reagent to cells that are to be labeled with AO; add a placebo instead, such as diH₂O (Step 10).
8. Incubate cells for 1 hour at 37°C under 5% CO₂ and protect from light. Cells may settle on the bottom of the tubes; gently resuspend them by swirling cells every 20 minutes during the incubation to ensure even distribution of the CV-(LR)₂ reagent. After the incubation, cells can be stained with Hoechst Stain (Section 9), or unstained cells may be labeled with AO (Section 10).
9. If cells are to be labeled with Hoechst Stain, add it at approximately 0.5% v/v. Add 2.5µl Hoechst to 520µl cell suspension. Incubate 5-10 minutes at 37°C. Go to Step 11.
10. Because of the overlap in emissions, dual staining of cells with both the CV-(LR)₂ reagent and AO is not recommended; the dyes should be used separately. To stain cells with AO:
 - a. Dilute AO to 1:2,000-1:200 (which is 0.05-0.5% v/v) into the final cell suspension. For example, if using AO at 1.0µM in the final cell suspension, first dilute it 1:100 in PBS; e.g., put 10µl AO into 990µl PBS. Pipette the diluted AO into the cell suspension at 1:10; e.g., add 55µl to 500µl cell suspension.
 - b. Incubate 30 minutes at 37°C.
 - c. If viewing under the same filters used for the CV-(LR)₂ reagent (excitation at 550nm; emission >610nm), cells may be viewed immediately after staining without a wash step - go to Step 11.
 - d. If viewing under blue (480nm) excitation and green (540-550nm) emission wavelengths, any excess AO

may have to be washed away as the cells may appear too bright at this range. Brightness will depend on the type of microscope used and the cell line. To wash cells:

- i. Gently pellet cells at 200 x g for 3-8 minutes at RT.
 - ii. Remove and discard supernatant.
 - iii. Resuspend cells in 300µl or a similar volume of PBS in which the cells were originally suspended.
11. Place 15-20µl 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 550nm (540-560nm) and a long pass >610nm 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 the CV-(LR)₂ reagent and Hoechst, and if a multi-wavelength filter option is available on the fluorescence microscope, the dual staining properties can be examined. Hoechst Stain can be seen using a UV-filter with excitation at 365nm and emission at 480nm.

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 CV-(LR)₂ reagent may be used: a 550nm (540-560nm) excitation and long pass >610nm emission/ barrier filter pairing. With this pairing, the lysosomes appear red. When illuminating with a blue light (480nm) excitation filter, a green light (540-550nm) emission/barrier filter combination works well. Lysosomes will appear yellowish green instead of red.

8. Microscopy Analysis of Adherent Cells

1. Seed 10⁴-10⁵ cells onto a sterile coverslip in a 35 mm petri dish or onto chamber slides, or grow in a plate (Figure 2).
2. Grow cells until 80% confluent. This usually takes about 24 hours but will vary with each cell line.

3. Expose cells to the experimental conditions and create positive and negative controls (Section 2) or stimulate cells to trigger cathepsin enzymatic activity (Section 3).
4. Reconstitute the CV-(LR)₂ reagent to form the 260X stock solution (Section 4):
 - a. Reconstitute the vial with 50µl DMSO.
5. When ready to stain cells, dilute the stock 1:10 in diH₂O to form the CV-(LR)₂ reagent staining solution (Section 4):
 - a. Add 450µl diH₂O to dilute the vial.
6. Add the CV-(LR)₂ reagent staining solution at approximately 1:26 and gently mix to ensure an even distribution of the CV-(LR)₂ reagent. For example, add 12µl staining solution to 300µl cells forming a final volume of 312µl. Do not add the CV-(LR)₂ reagent to cells that will be stained with AO: add a placebo instead, such as diH₂O (Step 10).
7. Incubate 30-60 minutes at 37°C.
8. Remove the media from the cell monolayer surface and rinse twice with PBS, 1 minute per rinse. At this point, cells can be analyzed (Step 12) or stained with Hoechst (Step 9) or unstained cells can be labeled with AO (Step 10).
9. If cells are to be labeled with Hoechst Stain, add it at approximately 0.5% v/v. Add 1.6µl Hoechst to 312µl cells labeled with the CV-(LR)₂ reagent 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 the CV-(LR)₂ reagent and AO is not recommended; the dyes should be used separately. To stain cells that have not been exposed to the CV-(LR)₂ reagent:
 - 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 1.0µM in the final cell volume, it must be diluted 1:1,000. First dilute it 1:100 in PBS; e.g., add 10µl AO to 1,000µl PBS. Pipette the diluted AO to the cells at 1:10; e.g., add 33µl diluted AO to 300µl cell media.
 - b. Incubate 30 minutes at 37°C.

- c. Remove the media from the cell monolayer surface. Rinse twice with PBS, 1 minute per rinse.
11. Mount the coverslip with cells facing down onto a drop of PBS. If a chamber-slide was used, pull off the plastic frame and add a drop of PBS to the cell surface and cover with a coverslip.
12. Observe cells using a fluorescence microscope equipped with an excitation filter of 550nm (540-560nm) and a long pass >610nm emission/barrier filter pair. Select a filter combination that best approximates these settings. Using these filters, positive cells stained with the CV-(LR)₂ reagent will appear red with more brightly stained vacuoles and lysosomes.

If samples were stained with both the CV-(LR)₂ reagent 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 365nm and emission at 480nm.

As AO exhibits a very broad emission range, one of several filter pairs may be used. The same excitation/emission pairing filters used to view the CV-(LR)₂ reagent may be used: an excitation filter of 550nm (540-560nm) and a long pass >610nm emission/barrier filter. With this pairing, the lysosomes appear red.

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

9. Fluorescence Plate Reader Staining Protocol

1. Prepare cell populations. Cell concentrations should be 2-8 x 10⁶ cells/ml. If this is too dense for the cell line, stimulate cathepsin activity first, then concentrate the cells and stain with the CV-(LR)₂ reagent. Cell concentration can be achieved by low speed centrifugation (<400 x g at RT) for 5 minutes.
2. Expose cells to the experimental conditions and create positive and negative controls (Section 2) or stimulate cells to trigger cathepsin activity (Section 3).
3. Transfer 300µl cell suspension into sterile tubes or a black microtiter plate. Do not use clear plates. Avoid

bubbles. Larger cell volumes may also be used, but additional CV-(LR)₂ reagent will be required per sample.

4. When ready to label with the CV-(LR)₂ reagent staining solution, cells should be at least 2×10^5 cells/100 μ l aliquot (equal to 2×10^6 cells/ml) for each microtiter plate well.
5. Reconstitute the CV-(LR)₂ reagent to form the stock solution (Section 4):
 - a. Reconstitute the vial with 50 μ l DMSO.
6. When ready to stain cells, dilute the stock 1:10 in diH₂O to form the CV-(LR)₂ reagent staining solution (Section 4):
 - a. Add 450 μ l diH₂O to dilute the vial.
7. Add 20 μ l of the CV-(LR)₂ reagent staining solution directly to 300 μ l cell sample. If different cell volumes are used, add the CV-(LR)₂ reagent staining solution at approximately 1:15. Due to sensitivity limitations, plate readers require a higher concentration of CV-(LR)₂ reagent for detection compared with microscopes.
8. Gently mix the cells. This can be done by gently aspirating and expelling the cells with a pipette. To minimize cell shearing, cut the tip of the pipette to enlarge the hole.
9. Incubate cells for at least 60 minutes at 37°C protected from light. As cells settle to the bottom, gently resuspend them approximately every 20 minutes to ensure the CV-(LR)₂ reagent is evenly dispersed among all cells.
10. Read the 300 μ l sample as one sample or split it into 3 wells of 100 μ l each. If cells were stained in a tube, transfer 100- 300 μ l into a well of a black microtiter plate.
11. Measure the fluorescence intensity of the red fluorescent cresyl violet fluorophore. Set the plate reader to perform an endpoint read. The CV-(LR)₂ reagent has an optimal excitation and emission wavelength tandem of 592nm and 628nm, respectively. Select the filter pairings that best approximate these settings. If available, use a cut-off filter at 630nm to filter out shorter wavelength excitation interference.

REFERENCES

1. Bank U, Kruger S, Langner J, Roessner A. Review: peptidases and peptidase inhibitors in the pathogenesis of diseases. *Adv Exp Med Biol.* 477:349-378 (2000).
2. Boonacker E, Van Noorden CJF. Enzyme cytochemical techniques for metabolic mapping in living cells, with special reference to proteolysis. *J Histochem Cytochem.* 49:1473-1486 (2001).
3. Bossard MJ, Tomaszek TA, Thompson SK, Amegadzie BY et al. Proteolytic activity of human osteoclast cathepsin K. *J Biol Chem.* 271:12517-12524 (1996).
4. Buhling F, Fengler A, Brandt W, Welte T, Ansorge S, Nagler, DK. Review: novel cysteine proteases of the papain family. *Adv Exp Med Biol.* 477:241-254 (2000).
5. Buhling F, Waldburg N, Gerber A, Hackel C, Kruger S, Reinhold D, Bromme D, Weber E, Ansorge S, Welte T. Cathepsin K expression in human lung. *Adv Exp Med Biol.* 477: 281-286 (2000).
6. Frosch BA, Berquin I, Emmert-Buck MR, Moin K, Sloane BF. Molecular regulation, membrane association and secretion of tumor cathepsin B. *APMIS.* 107:28-37 (1999).
7. Gerber A, Welte T, Ansorge S, Buhling F. Expression of cathepsin B and L in human lung epithelial cells is regulated by cytokines. *Adv Exp Med Biol.* 477:287-292 (2000).
8. Guinec N, Fumeron VD, Pagano M. In vitro study of basement membrane degradation by the cysteine proteinases, cathepsin B, B-like, and L. Digestion of collagen IV, laminin, fibronectin, and release of gelatinase activities from basement membrane fibronectin. *Biol Chem Hoppe-Seyler.* 374:1135-1146 (1993).
9. Kas J, Werle B, Lah T, Brunner N. Cysteine proteinases and their inhibitors in extracellular fluids: markers for diagnosis and prognosis in cancer. *Int J Biol Markers.* 15:84-89 (2000).
10. Lee, BW, et al. DEVDase detection in intact apoptotic cells using the cell permeant fluorogenic substrate, (z-DEVD)₂-cresyl violet. *BioTechniques.* 35(5):1080-1085 (2003).
11. Leung-Toung R, Li W, Tam TF, Kariman K. thiol-dependent enzymes and their inhibitors: a review. *Curr Med Chem.* 9:979-1002 (2002).
12. Thornberry, N. A., et al. A combinatorial approach defines specificities of members of the caspase family and granzyme. *B J Biol Chem.* 272:17907-17911 (1997).
13. Traganos F, Darzynkiewicz Z. Lysosomal proton pump activity: supravital cell staining with staining with acridine orange differentiates leukocyte subpopulations. *Methods Cell Biol.* 41:185-194 (1994).
14. Turk B, Turk D, Turk V. Lysosomal cysteine proteases: more than scavengers. *Biochim Biophys Acta.* 1477:98-111 (2000).
15. Van Noorden CJF, Boonacker E, Bissell ER, Meijer AJ, Van Marle J, Smith RE. Ala-Pro-cresyl violet, a synthetic fluorogenic substrate for the analysis of kinetic parameters of dipeptidyl peptidase IV (CD26) in individual living rat hepatocytes. *Anal Biochem.* 252:71- 77 (1997).

GLOBAL HEADQUARTERS

Enzo Life Sciences Inc.
10 Executive Boulevard
Farmingdale, NY 11735
Toll-Free: 1.800.942.0430
Phone: 631.694.7070
Fax: 631.694.7501
info-usa@enzolifesciences.com

EUROPE/ASIA

Enzo Life Sciences (ELS) AG
Industriestrasse 17
CH-4415 Lausen
Switzerland
Phone: +41/0 61 926 89 89
Fax: +41/0 61 926 89 79
info-ch@enzolifesciences.com

For local distributors and detailed product information visit us online:
www.enzolifesciences.com