Product Manual

CV-Cathepsin L Detection Kit

Instruction Manual

BML-AK127

For research use only

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Product Manual

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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.

BACKGROUND

Enzo Life Science's CV-Cathepsin L Detection Kit enables researchers to quantitate and monitor intracellular cathepsin L activity over time *in vitro*. The CV-(FR)2 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 one of a number of pathological conditions, including: Alzheimer's disease, cancer, autoimmune diseases and osteoporosis[4,7]. Up-regulated cathepsin B and L activity has been linked to cancer of the colon, pancreas, ovaries, breast, lung, and skin (melanoma)[1,6,8,9]. Up-regulation of cathepsin K has been shown in lung tumors[5] and increased cathepsin K activity has 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 family[11] and the cathepsin family name has been synonymous with lysosomal proteolytic enzymes[4]. In actuality, the cathepsin family 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[15]. Cathepsin C is the noted exception, existing as an oligomeric enzyme with a MW ~200 kDa[14]. 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].

Enzo Life Science's CV Cathepsin L detection kit utilizes the photostable fluorophore, cresyl violet. When bi-substituted via amide linkage to two cathepsin target peptide sequences, such as (Phenylalanine-Arginine)$_2$, the cresyl violet leaving group is non-fluorescent[15]. The attached PheArg groups are substrates for cathepsin L cleavage. Following enzymatic cleavage at Phe and/or Arg amide linkage sites, the mono and non-substituted cresyl violet fluorophores generate red fluorescence when excited at 550-590nm. It is important to note that cathepsin B will also hydrolyze this substrate. If both cathepsins B and L are present in the cell type being tested, both activities will contribute to the fluorescence.

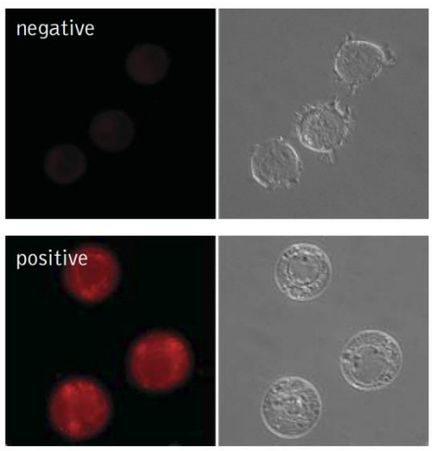
To use the CV-$(FR)_2$ reagent, add it directly to the cell culture media, incubate, and analyze. Because this reagent is cell-permeant, it easily penetrates the cell membrane and the membranes of the internal cellular organelles - no lysis or permeabilization steps are required. The CV-$(FR)_2$ reagent 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 of the 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 L activity. By varying the duration and concentration of exposure to the 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 fluorophore, cresyl violet, fluoresces red when excited at 550-590nm[15]. The red fluorescent signal can be monitored with a fluorescence microscope or plate reader. It has an optimal excitation of 592nm and emission of 628nm[2]. At these higher excitation wavelengths, the amount of cell structure derived auto-fluorescence is minimal[15]. Fortunately, the excitation peak is rather broad allowing good excitation efficiency at 540-560nm. The unsubstituted red fluorescent 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 the CV-$(FR)_2$ reagent.

Hoechst stain is included with the kit to concurrently label nuclei after labeling with the CV-$(FR)_2$ reagent (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 organelles[13].

FIGURE 1: NEGATIVE VS. POSITIVE CELLS

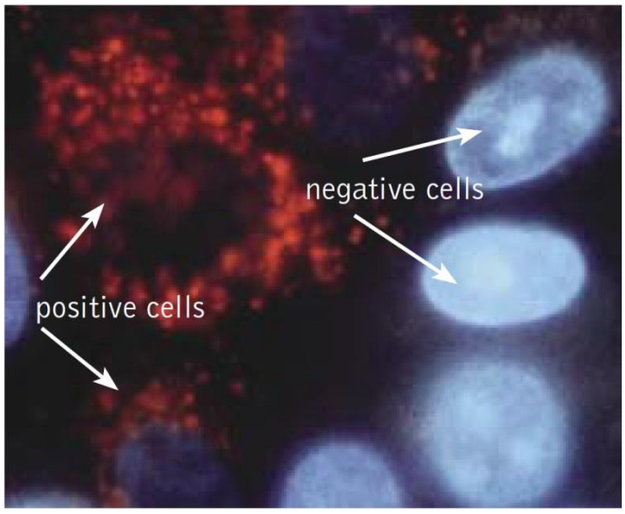


Using Enzo Life Science's CV Cathepsin assay kits to detect enzymatic activity in suspension cells, there is a clear differential between negative (top) and positive (bottom) cells. Suspension cells were incubated with a control (DMSO, top) or a stimulant (bottom) for 3 hours at 37°C to induce enzymatic activity. Cell cultures were subsequently stained with CV-$(FR)_2$ for 1 hour at 37°C. Left panels contain fluorescence images obtained using a Nikon Eclipse E800 photomicroscope equipped with a 100 W mercury lamp and excitation (510-560nm) and emission (570-620nm) filter pairings. Right panels contain the corresponding differential-interferencecontrast (DIC) image[15].

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 Science's CV-$(FR)_2$ substrate 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.

FIGURE 3: DUAL STAINING WITH HOECHST



Cells were dually stained using Enzo Life Science's CV-$(FR)_2$ substrate and Hoechst 33342 nuclear stain. Experimental cells were stained with the CV-$(FR)_2$ substrate for 30 minutes at 37°C, washed twice in PBS, and supravitally 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-$(FR)_2$ substrate. 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, NY[15].

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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 FOR ASSISTANCE IF NECESSARY.

MATERIALS SUPPLIED

NOTE ON STORAGE AND SHELF LIFE:

Store the unopened kit and each unopened component at 2-8°C until the expiration date. Once reconstituted with DMSO, use the CV-$(FR)_2$ reagent immediately, or store at ≤-20°C up to 6 months, protected from light and thawed no more than twice during that time.

Kit Components

BML-KI248 CV-$(FR)_2$ reagent, Lyophilized

BML-KI223 Acridine Orange (AO), 1 mM (0.5ml)

BML-KI189 Hoechst 33342 stain, 200µg/ml (1 ml)

Protect all kit components from light!

OTHER MATERIALS NEEDED

Depending on experimental protocol used, the following materials may be needed.

- Reagents to induce apoptosis or other experimental condition
- 15ml polystyrene centrifuge tube (1 per sample)
- Amber vials or polypropylene tubes for storage of 250X CV-$(FR)_2$ concentrate at -20°C, if aliquoted
- Slides and coverslips
- Hemocytometer
- Clinical centrifuge
- Pipette(s) capable of dispensing at 10, 50, 200, 300µl, 1ml
- Deionized water (diH_2O)
- Dimethyl sulfoxide (DMSO), 50 or 200µl needed
- Phosphate Buffered Saline (PBS) pH 7.4, 100ml
- Trypsin
- Ice or 4°C refrigerator to store cells
- 37°C CO_2 incubator

DETECTION EQUIPMENT

The CV-$(FR)_2$ 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 (Refer to Hoechst Stain section). AO excites at 480nm and emits >540nm (Refer to Acridine Orange section). Select a filter combination that best approximates these settings.

- 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-$(FR)_2$ 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-$(FR)_2$ 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).
- Fluorescence plate reader: the CV-$(FR)_2$ 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.

SAFETY INFORMATION

Use gloves while handling the CV-$(FR)_2$ reagent, AO, and Hoechst stain.

Dispose of all liquid components down the sink and flush with copious amounts of water. Solid components may be discarded in standard trash bins.

PROTOCOL

Experimental Preparation

Staining cells with the CV-$(FR)_2$ reagent can be completed within a few hours. However, CV-$(FR)_2$ 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-$(FR)_2$ 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-$(FR)_2$ reagent staining solution is 10-20µl per 300µl of cells at 10^6 cells/ml, but the ideal amount may vary based on the experimental conditions and method of analysis. Culture cells to a density optimal for the specific experimental protocol. Cell density should not exceed 10^6 cells/ml as cells cultivated in excess of this concentration may begin to naturally enter apoptosis due to nutrient deprivation or the accumulation of cell degradation products in the media.

Cells with active cathepsin enzymes will generate a stronger red fluorescence with the CV-$(FR)_2$ 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 substrate used to stain cells and the incubation time.

Hoechst stain can be used with the CV-$(FR)_2$ reagent to label nuclei (Figure 3). Because of the overlap in emissions, dual staining of cells with both the CV-$(FR)_2$ reagent and AO will yield confusing results and is not recommended; these dyes should be used separately. Do not use the CV-$(FR)_2$ reagent with paraffin-embedded tissues as the chemicals used for paraffin-embedding may denature and inactivate the substrate.

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). 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-$(FR)_2$ reagent, Hoechst stain, and Acridine Orange, make 10 control populations:

- Unlabeled, stimulated and non-stimulated populations.
- CV-$(FR)_2$-labeled, stimulated and non-stimulated populations.
- CV-$(FR)_2$- and Hoechst-labeled, stimulated and nonstimulated populations.
- Hoechst-labeled, stimulated and non-stimulated populations.
- AO-labeled, stimulated and non-stimulated populations.

Cathepsin Induction

The CV-$(FR)_2$ reagent works with your existing protocols – stimulate cathepsin enzymatic activity as you normally would, then label cells with CV-$(FR)_2$.

Cathepsin L Substrate

The 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 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. Prepare the 260X stock solution by reconstituting CV-$(FR)_2$ 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 450µl diH2O to dilute the vial. The vial contains 50µl of the stock (1); this yields 500µl of the staining solution. Use immediately.

b. For other amounts, dilute the stock 1:10 in diH2O. For example, add 10µl stock to 90µl diH2O; this yields 100µl of the staining solution. Use immediately.

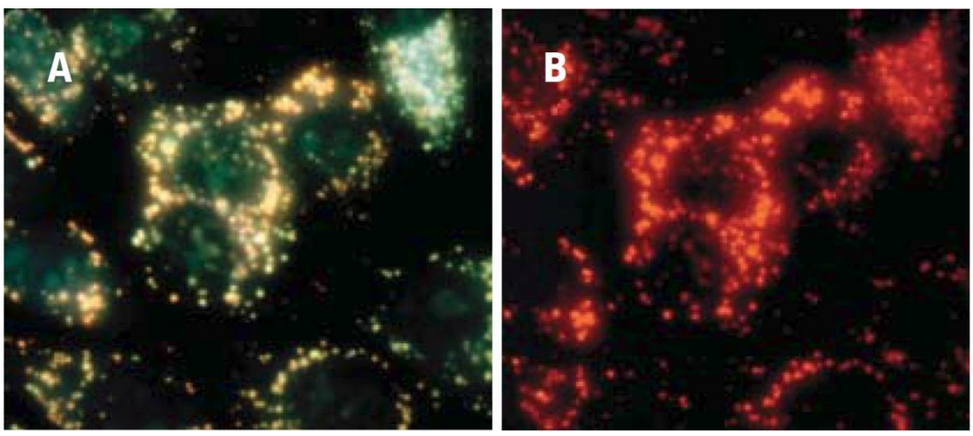
c. Mix by inverting or vortexing the vial at RT.

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 200µg/ml. Hoechst Stain can be used concurrently with the CV-$(FR)_2$ substrate 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-$(FR)_2$ 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].

Acridine Orange

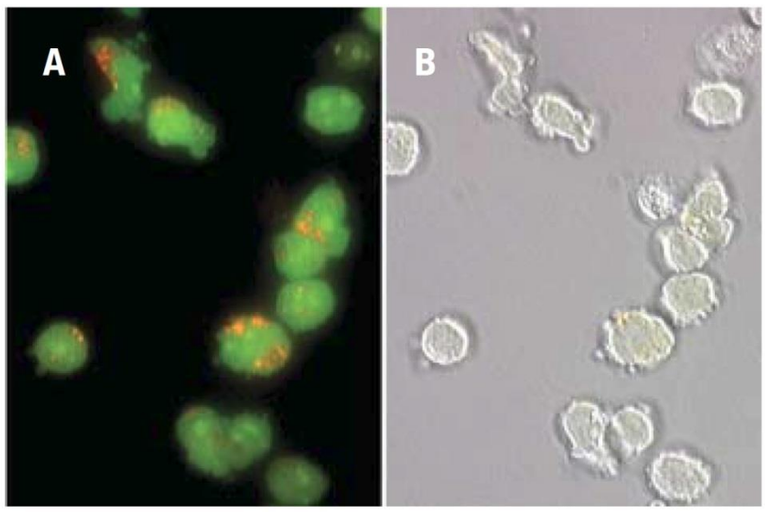
Acridine Orange (AO) is a DNA chelating dye and can be used to reveal lysosomes, nuclei, and nucleoli (Figures 4 and 5). 0.5 mL of AO is provided at 1mM. AO may be used neat or diluted in diH2O or media prior to pipetting into the cell suspension. Always protect AO from bright light. Lysosomal structures 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-$(FR)_2$ 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-$(FR)_2$ 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.

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 fi lter set at 300X. AO-stained lysosomes appear in photo A; photo B shows the corresponding DIC image of the cells (compare with Figure 4).

Microscopy Analysis of Suspension Cells

1. Prepare cell populations. Initial cell concentrations should be 3-5 x 10^5 cells/ml and should not exceed 7 x 10^5 cells/ml, as cells cultivated in excess of this concentration may begin to naturally enter apoptosis.
2. Expose cells to the experimental conditions and create positive and negative controls (Refer to Controls section) or induce cells to stimulate cathepsin activity.
3. When ready to label with the staining solution, cell concentrations should be 0.5 - 2 x 10^6 cells/ml for best viewing. Density can be determined by counting cell populations on a hemocytometer. If necessary, concentrate cells by gentle centrifugation at 200 x g for 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-$(FR)_2$ staining solution may be required.
5. Reconstitute the CV-$(FR)_2$ reagent to form the 260X stock solution (Refer to Cathepsin L Substrate section):
    a. Use 50µl DMSO to reconstitute the vial vial.

6. When ready to stain cells, dilute the stock 1:10 in diH2O to form the CV-$(FR)_2$ staining solution (Refer to Cathepsin L Substrate section):
   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-$(FR)_2$ staining solution at a ratio of approximately 1:26. For example, add 40µl CV-$(FR)_2$ staining solution to 1,000µl of cell suspension forming a final volume of 1,040µl. Do not add the CV-$(FR)_2$ 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% CO_2 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 substrate. 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-$(FR)_2$ 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-$(FR)_2$ (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-$(FR)_2$ 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-$(FR)_2$ 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.

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 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 (Refer to Controls section) or stimulate cells to trigger cathepsin enzymatic activity.
4. Reconstitute the CV-$(FR)_2$ reagent to form the 260X stock solution (Refer to Cathepsin L Substrate section):
    a. Reconstitute the vial with 50µl DMSO.
5. When ready to stain cells, dilute the stock 1:10 in diH2O to form the CV-$(FR)_2$ staining solution (Refer to Cathepsin L Substrate section):
    a. Add 450µl diH2O to dilute the vial.
6. Add the CV-$(FR)_2$ staining solution at approximately 1:26 and gently mix to ensure an even distribution of the CV-$(FR)_2$. For example, add 12µl staining solution to 300µl cells forming a final volume of 312µl. Do not add the CV-$(FR)_2$ solution 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-$(FR)_2$ solution 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 CV-$(FR)_2$ and AO is not recommended; the dyes should be used separately. To stain cells that have not been exposed to the CV-$(FR)_2$ 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-$(FR)_2$ reagent will appear red with more brightly stained vacuoles and lysosomes.

    If samples were stained with both the CV-$(FR)_2$ 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-$(FR)_2$ 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.

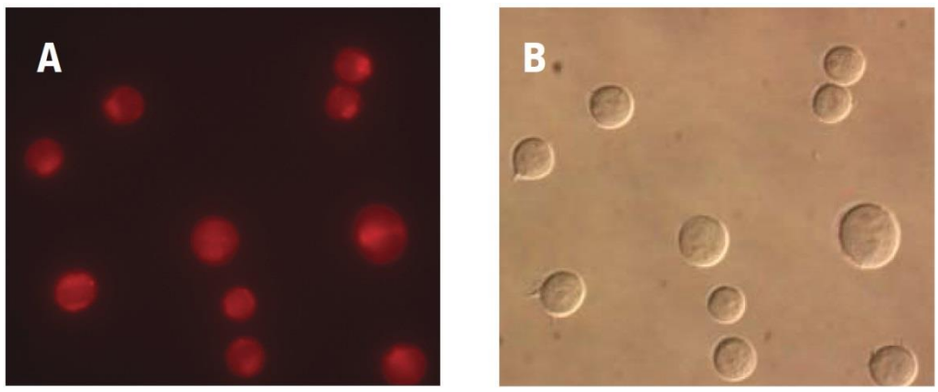
    When illuminating with a blue light (480nm) excitation filter, a green light (540-550nm) emission/barrier filter combination works well. Lysosomes will appear yellowish green instead of red.

Fluorescence Plate Reader Staining Protocol

1. Prepare cell populations. Cell concentrations should be 2-8 x 10^6 cells/ml. If this is too dense for the cell line, stimulate cathepsin activity first, then concentrate the cells and stain with the CV-$(FR)_2$ 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 (Refer to Controls section) or stimulate cells to trigger cathepsin activity.
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-$(FR)_2$ substrate will be required per sample.

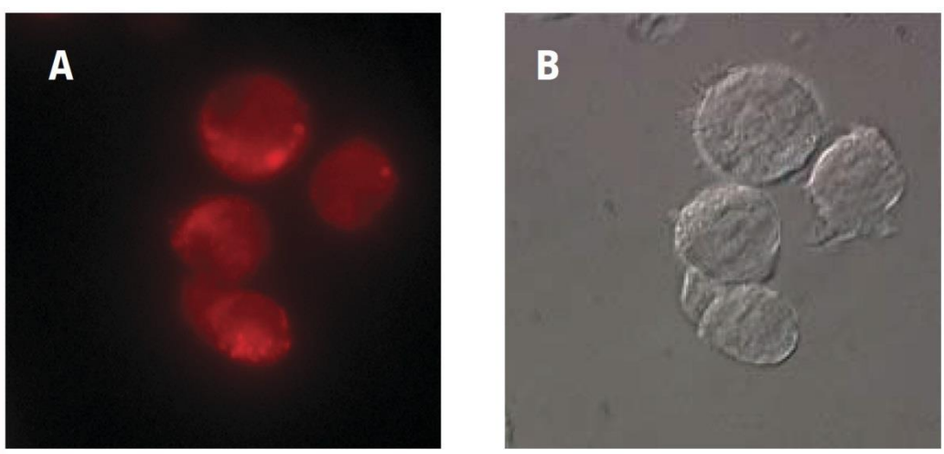
4. When ready to label with the CV-$(FR)_2$ staining solution, cells should be at least 2 x 10^5 cells/100µl aliquot (equal to 2 x 10^6 cells/ml) for each microtiter plate well.
5. Reconstitute the CV-$(FR)_2$ to form the stock solution (Refer to Cathepsin L Substrate section):
   a. Reconstitute the vial with 50µl DMSO.
6. When ready to stain cells, dilute the stock 1:10 in diH2O to form the CV-$(FR)_2$ staining solution (Refer to Cathepsin L Substrate section):
   a. Add 450µl diH2O to dilute the vial.
7. Add 20µl of the CV-$(FR)_2$ staining solution directly to 300µl cell sample. If different cell volumes are used, add the CV-$(FR)_2$ staining solution at approximately 1:15. Due to sensitivity limitations, plate readers require a higher concentration of the CV-$(FR)_2$ solution 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-$(FR)_2$ is evenly dispersed among all cells.
10. Read the 300µl sample as one sample or split it into 3 wells of 100µl each. If cells were stained in a tube, transfer 100-300µl 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-$(FR)_2$ 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.

FIGURE 6: CATHEPSIN L IN JURKAT CELLS



Intracellular cathepsin L activity was detected in Jurkat cells using the CV-$(FR)_2$ cathepsin L fluorogenic substrate. Intracellular localization of the hydrolyzed (fluorescent) CV-$(FR)_2$ product was detected using a Nikon Eclipse E800 photomicroscope equipped with a 510 – 560nm excitation filter and a 570 – 620nm emission/barrier filter at 400X (A). Photo at right (B) shows the corresponding DIC image of the cells.

FIGURE 7: CATHEPSIN L IN THP-1 CELLS



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

NOTES

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