# Cathepsin L Assay Kit

**Catalog No.:** abx299031

**Size:** 25 tests

**Storage:** Store all components at 2-8°C. Once reconstituted with DMSO, use Cathepsin-X Substrate immediately, or store at -20°C for up to 6 months in the dark. Avoid freeze-thaw cycles.

| Catalogue Number | Cathepsin | Substrate Dye |
|---|---|---|
| abx299029 | B | Cathepsin-B |
| abx299030 | K | Cathepsin-K |
| abx299031 | L | Cathepsin-L |

**Application:** For detection and quantification of Cathepsin activity in cells.



## Introduction

Cathepsin is a type of protease enzyme which is secreted by osteoclasts. Elevated cathepsin enzyme activity in serum or the extracellular matrix usually indicates a number of pathological conditions. The primary function of Cathepsin is to break down the organic phase of bone during bone reabsorption.

Abbexa's Cathepsin Assay Kit is a quick, convenient, and sensitive method for measuring and calculating Cathepsin activity. The Cathepsin-X Substrate uses the red fluorophore, cresyl violet. When bi-substituted via amide linkage to two cathepsin target peptide sequences, the cresyl violet is non-fluorescent. Cathepsin cleaves one or both arginine amide linkage sites, causing the cresyl violet fluorophores to generate a red fluorescence when excited at 550-590 nm, with an optimal excitation of 592 nm and optimal emission wavelength of 628 nm. The intensity of the color is proportional to the Cathepsin activity, which can then be calculated.

### Kit components

1. Cathepsin-X Substrate: 1 vial
2. Hoechst 33342: 1 ml
3. Acridine Orange: 0.5 ml

### Materials required but not provided

1. DMSO: 50 - 200 µl
2. Deionized water: 450 – 1600 µl
3. PBS (pH 7.4): 100 ml
4. Cultured cells treated with the experimental conditions ready for staining
5. Reagents to induce cathepsin activity and set up controls
6. Hemocytometer
7. Centrifuge (200 x g)
8. 15 ml polypropylene centrifuge tubes (1 per sample)
9. Glass or polypropylene tubes: 12 x 75 mm
10. Black 96-well microtiter plate, flat bottomed
11. Slides and coverslips.
12. Ice or refrigerator.
13. Fluorescence plate reader or fluorescence microscope.

## Protocol

### A. Preparation of samples and reagents

#### 1. Samples

The following sample preparation methods are intended as a guide and may be adjusted as required depending on the specific samples used.

- **Cell samples:** Carefully cultivate cells several days prior to the testing and ensure they do not exceed a cell density of $10^6$ cells/ml. Once a sufficient number of cells has been cultivated, the Cathepsin-X Substrate can be added to the cells. The recommended volume of the Cathepsin-X Substrate to be added is 10-20 µl per 300-500 µl of cells at $10^6$ cells/ml, but this may vary depending on experimental conditions and method of analysis. Then, culture the cells to an optimal density for the cathepsin activation protocol or specific experimental conditions, ensuring it does not exceed a cell density of $10^6$ cells/ml. A preliminary experiment may be required to deduce the correct volume of Cathepsin-X Substrate to use, and the optimal timings to use the Cathepsin-X Substrate.

**Note:**

- Fresh samples or recently obtained samples are recommended to prevent degradation and denaturalization that may lead to erroneous results.
- Since the Cathepsin-X Substrate detects cathepsin enzymes, ensure that the substrate is diluted and administered when the target cathepsins are expected to be activated in the cells.

## 2. Reagents

- **Controls:** It is highly recommended to run two sets of controls. The positive control, which should consist of a population of cells activated to stimulate cathepsin activity. The negative control should consist of a placebo population of cells which are not activated. Negative controls can be set up by culturing an equal volume of non-activated cells for each labelling condition. Ensure the cells are labelled with Cathepsin-X Substrate after the cathepsin enzyme activity has been stimulated. The activated positive control and negative control populations should be of a similar number of cells. If labeling with Cathepsin-X Substrate, Hoechst 33342, and Acridine Orange (AO), make 10 control populations as exemplified in the table below:

| **Control Population Numbers** | 1&2 | 3&4 | 5&6 | 7&8 | 9&10 |
|---|---|---|---|---|---|
| **Population Label Examples** | Unlabeled, stimulated and non-stimulated populations. | Cathepsin-X Substrate-labeled, stimulated and non-stimulated populations. | Cathepsin-X Substrate- and Hoechst-labeled, stimulated and non-stimulated populations. | Hoechst-labeled, stimulated and non-stimulated populations. | AO-labeled, stimulated and non-stimulated populations. |

- **Cathepsin-X Substrate:** First, reconstitute the Cathepsin-X Substrate with 50 µl of DMSO to form the stock concentrate. Gently vortex or swirl the vial to allow the DMSO to travel around the base of the vial until it is completely dissolved to produce a red solution, which should only take a few minutes at room temperature. Once reconstituted, the solution can be stored at -20°C for up to 6 months in the dark. Avoid multiple freeze-thaw cycles. If not diluting within one hour after forming this solution, aliquot and freeze. Immediately prior to staining the samples, dilute the stock solution in a 1:10 ratio with deionized water to form the final Staining Solution, which must be used within 15 minutes of dilution to prevent substrate hydrolysis. Mix by inverting the vial or by vortex mixing the vial at room temperature and use immediately.

- **Hoechst 33342:** Provided ready-to-use at 200 µg/ml and can be used with Cathepsin-X Substrate to label nuclei. When bound to nucleic acids, Hoechst 33342 has a maximum absorbance at 350 nm and maximum emission at 480 nm. It can be viewed using a microscope with a UV-filter with an excitation at 365 nm and an emission at 480 nm.

- **Acridine Orange:** Provided ready-to-use at 1 mM and can be used undiluted or diluted in deionized water prior to pipetting into the cell suspension. Protect from light. Since Acridine Orange has a wide emission range, the same excitation and emission filter pairings used to view Cathepsin-X Substrate can be used to view Acridine Orange; excitation of 540-560 nm excitation filter or > 610 nm emission filter.

**B. Microscopy Analysis of Suspension Cells**

1. Prepare cell populations with an initial concentration of 3-5 x $10^5$ cells/ml. Do not exceed $10^6$ cells/ml to prevent the cell population from undergoing apoptosis.
2. Prepare the experimental conditions.
3. Set up controls as specified in '**2. Reagents**' section.
4. Before labelling with the Staining Solution, ensure that the cell concentration is 2-5 × $10^6$ cells/ml for optimal viewing. Density can be determined by counting cell populations on a hemocytometer. Cells can be further concentrated by centrifuging at 200 × g for 5-10 minutes at room temperature, before removing the supernatant and resuspending with cell culture media or PBS.
5. Transfer 500 µl of cell suspension into 12 x 75 mm glass or polypropylene tubes. If larger cell volumes are used, additional Cathepsin-X Substrate Staining Solution may be required.
6. Reconstitute Cathepsin-X Substrate, as specified in '**2. Reagents**' section, to form the concentrated stock solution at 260 X.
7. Just before staining the cells, dilute the 260 X stock concentrate in a 1:10 ratio in deionized water to form the Staining Solution at 26 X.
8. Add 20 µl of the Staining Solution to each 500 µl cell suspension and mix thoroughly. If using different cell volumes, ensure that the Cathepsin-X Substrate Staining Solution is added in a dilution ratio of approximately 1:26 to the cell suspension. For any cells labelled with AO, add a placebo such as deionized water.
9. Incubate the cells for 30-60 minutes at 37°C in the dark. If the cells settle on the bottom of their tube during the incubation, gently resuspend them by swirling the cells in 20 minute intervals.
10. After the incubation, the cells can be stained with Hoechst 33342 as appropriate. Go to step 15.
11. It is not recommended to perform dual staining of cells with both Cathepsin-X Substrate and AO. To stain cells with AO, dilute by serial dilution in deionized water. Pipette the diluted AO into the cell suspension at a 1:10 ratio.
12. Incubate for 30 minutes at 37°C.
13. If viewing under the same filters used for Cathepsin-X Substrate, the cells can be viewed immediately after staining without washing - go to step 15.
14. To view under blue excitation (480 nm) and green emission (540-550 nm) wavelengths, excess AO may have to be washed to avoid oversaturation. To wash the cells:

    **14 i)** Gently pellet cells at 200 × g for 5-10 minutes at room temperature.

    **14 ii)** Remove and discard the supernatant.

    **14 iii)** Resuspend the cells in 500 µl of PBS, or a similar volume of PBS in which the cells were originally suspended.
15. Place 15-20 µl of cell suspension onto a microscope slide and cover it with a coverslip.
16. To view the cells, use a fluorescence microscope with an excitation filter of 550 nm (540-560 nm) and an emission filter of >610 nm. A red colour will be seen in positive cells, with strongly stained lysosomes and vacuoles. If samples are stained with Hoechst 33342 in addition to the Cathepsin-X Substrate, dual staining properties can be seen by using a UV-filter with emission at 480 nm and excitation at 365 nm to observe the Hoechst 33342.
17. Many different filter pairings can be used with AO stained cells, due to its wide emission range. The same filter pairings for Cathepsin-X Substrate can be used for this, a 550 nm (540-560 nm) excitation filter and a >610 nm emission filter, which results in the lysosomes appearing red. A yellowish-green colour will instead be seen in the lysosomes if a blue light 480 nm excitation filter and a green light 540-550 nm emission filter are used as a filter pair.

### C. Microscopy Analysis of Adherent Cells

1. Seed $10^4$-$10^5$ cells onto a sterile coverslip in a 35 mm petri dish, or seed onto chamber slides, or grow in a plate. Grow cells until they are about 80-90% confluent. Please note that some cell lines do not tolerate confluency levels over 60%; adjust accordingly for the particular cells being used.
2. Expose cells to the experimental conditions.
3. Set up controls as specified in '**2. Reagents**' section.
4. Reconstitute Cathepsin-X Substrate, as specified in '**2. Reagents**' section, to form the concentrated stock solution at 260 X.
5. Just before staining the cells, dilute the 260 X stock concentrate in a 1:10 ratio in deionized water to form the Staining Solution at 26 X.
6. Add 12 µl of Staining Solution to 300 µl of cells to form a final volume of 312 µl. If using different cell volumes, ensure that the Cathepsin-X Substrate Staining Solution is added in a dilution ratio of approximately 1:26 to the cell suspension. For any cells labelled with AO, add a placebo such as deionized water.
7. Incubate for 30-60 minutes at 37°C in the dark.
8. Remove the media from the cell monolayer surface before rinsing twice with PBS, for 1 minute per rinse.
9. The cells can now be observed (step 14). Alternatively, the cells can be stained with Hoechst 33342 (step 10). Unstained cells can be labelled with AO (step 12).
10. If labelling cells with Hoechst 33342, add it at approximately 0.5% v/v. For example, add 1.6 µl of Hoechst 33342 to 312 µl of cells labelled with Cathepsin-X Substrate and control samples.
11. Incubate for 5-10 minutes at 37°C. Go to step 13.
12. To stain cells that have not been exposed to Cathepsin-X Substrate:

    a) Dilute by serial dilution in deionized water, and then pipette the diluted AO to the cell media at a 1:10 ratio.

    b) Incubate for 30 minutes at 37°C.

    c) Remove the media from the cell monolayer surface and rinse twice with PBS for 1 minute per rinse.
13. Mount the coverslip with cells facing down onto a drop of PBS.
14. Use a fluorescence microscope with a 550 nm (540-560 nm) excitation filter and a >610 nm emission filter to view Cathepsin-X Substrate-stained cells. A red colour will be seen in positive cells, with strongly stained lysosomes and vacuoles.
15. To observe cells stained with both Hoechst 33342 and Cathepsin-X Substrate, the Hoechst 33342 can be viewed by using a UV-filter with 365 nm excitation filter and a 480 nm emission filter. Many different filter pairings can be used with AO stained cells, due to its wide emission range. The same filter pairings for Cathepsin-X Substrate can be used for this, a 550 nm (540-560 nm) excitation filter and a >610 nm emission filter, which results in the lysosomes appearing red. A yellowish-green colour will instead be seen in the lysosomes if a blue light 480 nm excitation filter and a green light 540-550 nm emission filter are used as a filter pair.

### D. Fluorescence Plate Reader Analysis

1. When preparing the cell populations, ensure that the concentration of the cells is at least 3 × $10^6$ cells/ml. If the concentration of 3 × $10^6$ cells/ml is excessively dense for the cell line, initiate cathepsin activity before concentrating the cells and staining with Cathepsin-X Substrate. Ensure that the adherent cells reach 80-90% confluency. Please note some cell lines do not tolerate over 60% confluency, so adjust accordingly for the cells in use.
2. Expose the cells to the experimental conditions.
3. Set up controls as specified in '**2. Reagents**' section.
4. If using suspension cells, transfer 300 µl of cell suspension into 12 × 75 mm glass or polypropylene tubes or a black microtiter plate.
5. Reconstitute Cathepsin-X Substrate, as specified in '**2. Reagents**' section, to form the concentrated stock solution at 160 X.
6. Just before staining the cells, dilute the 160 X stock concentrate in a 1:10 ratio in deionized water to form the Staining Solution at 16 X. Ensure that the cell concentration has reached 3 × $10^6$ cells/ml prior to staining with the Cathepsin-X Substrate Staining Solution.
7. For each 300 µl of cell sample, add 20 µl of Cathepsin-X Substrate Staining Solution and mix thoroughly. If using different cell volumes, ensure Cathepsin-X Substrate Staining Solution is added to the cell samples at a ratio of approximately 1:16. Please note, plate readers require higher concentrations of Cathepsin-X Substrate than microscopes for detection.
8. Incubate the cells at 37°C in the dark for 30-60 minutes. If the cells settle on the bottom of their tube during incubation, gently resuspend them in 10-20 minute intervals.
9. Analyse one full sample as 300 µl of cells, or split the sample into thirds by transferring 100 µl of sample into 3 separate wells.
10. Perform an endpoint read with a fluorescence plate reader to measure the fluorescence intensity of the red Cathepsin-X Substrate fluorophore. Use filter pairings which can measure the optimal wavelengths of Cathepsin-X Substrate – 592 nm excitation wavelength and the 628 nm emission wavelength. To prevent shorter wavelength interference, a cut-off filter at 630 nm can be used.



## Technical Support

For troubleshooting and technical assistance, please contact us at support@abbexa.com.