Version 2, Last updated 30 April 2024

ab270772 Cathepsin B Assay Kit (Magic Red)

View ab270772

Cathepsin B Assay Kit (Magic Red) datasheet:

www.abcam.com/ab270772

(use www.abcam.cn/ab270772 for China, or www.abcam.co.jp/ab270772 for Japan)

For the detection of Cathepsin B activity in cultured cells.

This product is for research use only and is not intended for diagnostic use.

Table of Contents

1. Overview 1
2. Materials Supplied and Storage 2
3. Materials Required, Not Supplied 3
4. General guidelines, precautions, and troubleshooting 4
5. Experimental Preparation 5
6. Controls 6
7. Preparation of Magic Red 7
8. Hoechst 33342 9
9. Acridine Orange 10
10. Microscopy analysis of suspension cells 11
11. Microscopy analysis of adherent cells 13
12. Fluorescence plate reader analysis 15
13. Notes 17

1. Overview

Elevated cathepsin enzyme activity in serum or the extracellular matrix can signify a number of pathological conditions. Magic Red Assay Kits are used by researchers seeking to quantitate and monitor cathepsin activity in cultured cells and tissues. This assay kit detects cathepsin B activity.

To use Magic Red, add the substrate directly to the cell culture media, incubate, and analyze. Because it is a 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, the Magic Red substrate is cleaved and the cresyl violet fluorophore will become fluorescent upon excitation. As enzyme activity progresses and more Magic Red substrate is cleaved, the signal will intensify, allowing researchers to watch the color develop over time. Samples can be analyzed by fluorescence microscopy or with a fluorescence plate reader. Hoechst 33342 and Acridine Orange are included in the kit to detect nuclear morphology and lysosomal organelle structure, respectively.

2. Materials Supplied and Storage

Store kit at +4°C immediately on receipt.

Δ Note: 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.

Item	25 Test	100 Test	Storage temperature
Acridine Orange (1mM)	1 x 0.5 mL	1 x 0.5 mL	+4°C
Hoechst 33342, 200 µg/mL	1 x 1 mL	1 x 1 mL	+4°C
Magic Red Cathepsin B Substrate 25 tests	1 vial	n/a	+4°C
Magic Red Cathepsin B Substrate 100 tests	n/a	1 vial	+4°C

3. Materials Required, Not Supplied

These materials are not included in the kit, but will be required to successfully perform this assay:

- DMSO, 50-200 µL to reconstitute Magic Red
- DiH_2O, 450-1800 µL to dilute Magic Red
- Phosphate buffered saline (PBS) pH 7.4, 100 mL
- Cultured cells treated with the experimental conditions ready for staining
- Reagents to induce or inhibit 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. 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
- Fluorescence microscope
- Fluorescence plate reader

4. General guidelines, precautions, and troubleshooting

Please observe safe laboratory practice and consult the safety datasheet.

For general guidelines, precautions, limitations on the use of our assay kits and general assay troubleshooting tips, particularly for first time users, please consult our guide:
www.abcam.com/assaykitguidelines

For typical data produced using the assay, please see the assay kit datasheet on our website.

5. Experimental Preparation

5.1 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.

5.2 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.

5.3 The recommended volume of the Magic Red staining solution is 20 µL per 300 or 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.

5.4 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.

5.5 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.

5.6 Hoechst 33342 can be used with Magic Red to label nuclei. 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.

6. Controls

It is highly recommended that two sets of controls be run:

One positive control population of cells that was activated to elevate cathepsin activity;

And a placebo population of cells that received just the vehicle used to deliver the stimulating agent.

Δ Note: The placebo population may exhibit detectable levels of cathepsin activity, as cathepsin B is involved in normal cellular processes such as protein degradation within the lysosome. 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:

Control #	
1 and 2	Unlabeled, stimulated and non-stimulated populations.
3 and 4	Magic Red-labeled, stimulated and non-stimulated populations.
5 and 6	Magic Red- and Hoechst-labeled, stimulated and non-stimulated populations.
7 and 8	Hoechst-labeled, stimulated and non-stimulated populations.
9 and 10	AO-labeled, stimulated and non-stimulated populations.

7. Preparation of Magic Red

- Magic Red cathepsin B 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 1/25 for microscopy analysis or 1/15 for plate reader analysis.
- 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.

7.1 Create the stock solution by reconstituting Magic Red. It is vialed in 2 sizes: small (approximately 25 Tests); and large (approximately 100 Tests). The reconstitution volume will vary based on the vial size:

7.1.1 Reconstitute the 25 tests vial with 50 µL DMSO.

7.1.2 Reconstitute the 100 tests vial with 200 µL DMSO.

7.2 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.

7.3 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 mins of dilution to prevent substrate hydrolysis.

7.3.1 The 25 tests vial contains 50 µL of the stock concentrate in DMSO. Add 450 µL diH_2O to it. This yields 500 µL of the staining solution.

7.3.2 The 100 tests vial 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.

7.3.3 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.

7.4 Mix by inverting or vortexing the vial at RT.

7.5 Use immediately.

8. Hoechst 33342

Hoechst 33342 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.

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.

- 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 mutagen 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.

9. Acridine Orange

Acridine orange (AO) is a chelating dye and can be used to reveal lysosomes, nuclei, and nucleoli. The acidic pH of the lysosome results in the concentration and aggregation of AO.
It is provided ready-to-use at 1 mM. 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 to 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

- 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.

10. Microscopy analysis of suspension cells

10.1 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.

10.2 Expose cells to the experimental conditions and create positive and negative controls, see Section 6.

10.3 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 mins at room temperature (RT). Remove the supernatant and resuspend with cell culture media or PBS.

10.4 Transfer 480 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.

10.5 Reconstitute Magic Red (see Section 7) to form the concentrated stock solution at 250X.

10.6 When ready to stain cells, dilute the 250X stock concentrate 1/10 in diH_2O to form the staining solution at 25X:

10.6.1 Add 450 µL diH_2O to the 25 tests vial.

10.6.2 Add 1,800 µL diH_2O to the 100 tests vial.

10.7 Add 20 µL of the staining solution to each 480 µL cell suspension and mix thoroughly. If different cell volumes are used, add the Magic Red staining solution at a dilution of approximately 1/25. For example, add 40 µL Magic Red staining solution to 960 µL of cell suspension forming a final volume of 1,000 µL.

Δ Note: Do not add Magic Red to cells that are to be labeled with AO; add a placebo instead, such as diH_2O.

10.8 Incubate cells for 30-60 mins at 37°C protected from light. Cells may settle on the bottom of the tubes; gently resuspend by swirling cells every 20 mins during the incubation to ensure even distribution of Magic Red substrate. After the incubation, cells can be stained with Hoechst 33342 (Section 8), or unstained cells may be labeled with AO (Section 9).

10.9 If cells are to be labeled with Hoechst 33342, add it at approximately 0.5% v/v. For example, if the cell suspension is 500 µL, add 2.5 µL Hoechst 33342. Incubate 10-20 mins at 37°C. Go to Step 10.11.

10.10 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:

10.10.1 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 50 µL to 450 µL cell suspension.

10.10.2 Incubate for 30 mins at 37°C.

10.10.3 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 10.11.

10.10.4 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:

- Gently pellet cells at 200 *x g* for 5-10 mins at RT.
- Remove and discard supernatant.
- Resuspend cells in 500 µL or a similar volume of PBS in which the cells were originally suspended.

10.11 Place 15-20 µL of cell suspension onto a microscope slide and cover with a coverslip.

10.12 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 appear yellowish green instead of red.

11. Microscopy analysis of adherent cells

11.1 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.

11.2 Grow cells until 80-90% confluent. This usually takes about 24 hrs 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.

11.3 Expose cells to the experimental conditions and create positive and negative controls (Section 6).

11.4 Reconstitute Magic Red (Section 7) to form the concentrated stock solution at 250X.

11.5 When ready to stain cells, dilute the 250X stock concentrate 1/10 in diH_2O to form the staining solution at 25X:

11.5.1 Add 450 µL diH_2O to the 25 tests vial.

11.5.2 Add 1,800 µL diH_2O to the 100 tests vial.

11.6 Add Magic Red staining solution at approximately 1/25 and gently mix to ensure an even distribution of Magic Red. For example, add 20 µL staining solution to 480 µL cells forming a final volume of 500 µL.

Δ Note: Do not add Magic Red to cells that will be stained with AO: add a placebo instead, such as diH_2O.

11.7 Incubate 30-60 mins at 37°C protected from light.

11.8 Remove the media from the cell monolayer surface and rinse twice with PBS, 1 min per rinse. At this point, cells can be analyzed or stained with Hoechst 33342 (Section 8). Unstained cells can be labeled with AO (Section 9).

11.9 If cells are to be labeled with Hoechst 33342, add it at approximately 0.5% v/v. For example, add 2.5 µL Hoechst

33342 to 500 µL cells labeled with Magic Red and control samples. Incubate 10-20 mins at 37°C. Go to Step 11.11.

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 50 µL diluted AO to 450 µL cell media.

b. Incubate 30 mins at 37°C.

c. Remove the media from the cell monolayer surface. Rinse twice with PBS, 1 min per rinse.

11.11 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.

11.12 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.

11.13 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.

12. Fluorescence plate reader analysis

12.1 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.

12.2 Expose cells to the experimental conditions and create positive and negative controls (Section 6).

12.3 If using suspension cells, transfer 280 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 will be required per sample.

12.4 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.

12.5 Reconstitute Magic Red (Section 7) to form the concentrated stock solution at 150X.

12.6 When ready to stain cells, dilute the 150X stock concentrate 1/10 in diH_2O to form the staining solution at 15X:

12.6.1 Add 450 µL diH_2O to the 25 tests vial.

12.6.2 Add 1,800 µL diH_2O to the 100 tests vial.

12.7 Add 20 µL Magic Red staining solution directly to 280 µL cell sample and gently mix. If different cell volumes are used, add Magic Red staining solution at approximately 1/15. Due to sensitivity limitations, plate readers require a higher concentration of Magic Red for detection compared to microscopes.

12.8 Incubate cells for 30-60 mins at 37°C protected from light. As cells settle to the bottom, gently resuspend them approximately every 10-20 mins to ensure Magic Red is evenly dispersed among all cells.

12.9 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.

12.10 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.

13. Notes

Technical Support

For all technical or commercial enquiries please go to:
www.abcam.com/contactus

www.abcam.cn/contactus (China)

www.abcam.co.jp/contactus (Japan)