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ab270787
Cathepsin B Assay Kit

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For the detection of Cathepsin B activity in cultured cells.

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

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1. Overview

The Cathepsin B Assay Kit (ab270787) enables researchers to quantitate and monitor intracellular cathepsin activity over time in vitro. The Rhodamine 110 Cathepsin B substrate reagent is a non-cytotoxic and membrane permeant substrate that fluoresces green upon cleavage by active cathepsin enzymes.

To use this kit, add the Rhodamine 110-(RR)2 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, they will cleave off the two arginine-arginine cathepsin B targeting sequences and allow the rhodamine 110 fluorophore to become fluorescent upon excitation.

By varying the duration and concentration of exposure to the Rhodamine 110-(RR)2 substrate, a picture can be obtained of the relative abundance and intracellular location of cathepsin enzymatic activity. Positive cells will fluoresce green, while negative cells will exhibit very low levels of background green fluorescence. 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 green than cells with lower levels of cathepsin activity.

The Rhodamine 110-(RR)2 substrate has an optimal excitation of 500 nm and emission of 525 nm. Hoechst 33342 is included with the kit to concurrently label nuclei after labeling with the Rhodamine substrate. Hoechst 33342 is revealed under a microscope using a UV-filter with excitation at 365 nm and emission at 480 nm. Cells can be easily analyzed by flow cytometry or fluorescence microscopy.

2. Materials Supplied and Storage

Store kit components at the indicated temperatures immediately on receipt.

Δ Note: Once reconstituted with DMSO, use Rhodamine Substrate immediately, or store at ≤-20°C for up to 6 months, protected from light and thawed no more than twice during that time.

Item	Quantity 25 tests	Quantity 100 Tests	Storage temperature
Hoechst 33342, 200 µg/mL	1 vial	1 vial	+4°C
Rhodamine 110-(RR)2 substrate	1 vial	4 vials	-20°C
10X Cellular Assay Buffer	1 bottle (15 mL)	1 Bottle (60mL)	+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 µL per vial to reconstitute Rhodamine Substrate
- DiH_2O, 200 µL per vial to dilute Rhodamine Substrate
- 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
- Slides and coverslips
- Ice or refrigerator
- Fluorescence microscope
- Flow cytometer

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 Rhodamine 110-(RR)2 substrate can be completed within a few hours. However, Rhodamine 110-(RR)2 substrate 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 Rhodamine 110-(RR)2 substrate 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 elevated in the cells.

5.3 The recommended volume of the Rhodamine 110-(RR)2 substrate staining solution is 10 µL per 500 µL of cells at between 3-5 $x10^5$ cells/mL; the ideal amount may vary based on the experimental conditions and method of analysis. Each investigator should adjust the amount of Rhodamine 110-(RR)2 substrate 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 Rhodamine 110-(RR)2 substrate 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 green fluorescence with Rhodamine 110-(RR)2 substrate 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 Rhodamine 110-(RR)2 substrate used to stain cells and the incubation time.

5.6 Hoechst 33342 can be used with Rhodamine 110-(RR)2 substrate to label nuclei. Because of the overlap in emissions, dual staining of cells with both Rhodamine 110-(RR)2 substrate and AO will yield confusing results and is not recommended; these dyes should be used separately. Do not use Rhodamine

110-(RR)2 substrate 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 Rhodamine Substrate make 4 control populations:

Control #	
1 and 2	Unlabeled, stimulated and non-stimulated populations.
3 and 4	Rhodamine 110-(RR)2 substrate-labeled, stimulated and non-stimulated populations.

Δ Note: If dual staining samples for flow cytometry analysis, additional single and dual-stained controls will need to be prepared to assist with compensation.

7. Preparation of Rhodamine 110-(RR)2 substrate

- Rhodamine 110-(RR)2 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 250X stock concentrate, and then diluted 1:5 with diH2O to form the final 50X staining solution.
- The staining solution is typically used to stain cells at approximately 1:50 for flow cytometry analysis (Section 10) or between 1:25 - 1:50 for microscopy analysis (Sections 11-12). Protect from light and use gloves when handling

7.1 Create the stock solution by Rhodamine 110-(RR)2 substrate.

7.1.1 Reconstitute each vial with 50 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. 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 1:50 with diH_2O by adding 200 µL to form the staining solution. This yields 250 µL of the 50X staining solution.

7.3.1 For other amounts, dilute the stock concentrate 1/50 in diH_2O. For example, add 10 µL stock to 490 µL diH_2O; this yields 500 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 Rhodamine 110-(RR)2 substrate to label nuclei.

To use, add to samples at 0.5% v/v, and incubate 10-20 minutes at 37°C . For example, add 2.5 µL of Hoechst to a 500 µL suspension cell sample.

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. 1X Cellular Assay Buffer

- The 1X Cellular Assay Buffer is an isotonic solution used to stabilize cells when staining with Rhodamine 110-(RR)2 substrate. It contains mammalian proteins to stabilize cells, and sodium azide to retard bacterial growth (1X Cellular Assay Buffer contains 0.01% w/v sodium azide).
- Alternative solutions including cell culture media containing FBS and other additives may be used to stabilize cells during staining instead of 1X Cellular Assay Buffer.
- 10X Cellular Assay Buffer may form precipitates during cold storage. If this happens, gently warm it until all crystals have dissolved. Do not boil.
- Dilute 10X Cellular Assay Buffer 1:10 in diH2O. For example, add 60 mL 10X Cellular Assay Buffer to 540 mL diH2O for a total of 600 mL.

ΔNote: 1X Cellular Assay Buffer may be stored at 4°C and used within 1 week or frozen and used within 6 months.

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 1X Cellular Assay Buffer.

10.4 Transfer 500 µL cell suspension into 12 x 75 mm glass or polypropylene tubes. If desired, larger cell volumes can be used, but additional Rhodamine 110-(RR)2 substrate staining solution may be required.

10.5 Reconstitute Rhodamine 110-(RR)2 substrate (see Section 7) to form the concentrated stock solution.

10.6 When ready to stain cells, dilute the stock concentrate 1:5 by adding 200 µL diH_2O to form the R110-(RR)2 staining solution.

10.7 Add the Rhodamine 110-(RR)2 substrate staining solution to each sample at a ratio between 1:25 - 1:50 and gently mix to ensure an even distribution of Rhodamine 110-(RR)2 substrate. For example, if staining with Rhodamine Substrate at a 1:50 ratio, add 10 µL staining solution to 490 µL cells, forming a final volume of 500 µL.

Δ Note: Microscopy analysis is generally not as sensitive as flow cytometry and may require greater amounts of staining solution.

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 Rhodamine 110-(RR)2 substrate. After the incubation, cells can be stained with Hoechst 33342 (Section 8).

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.

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

10.11 Observe cells under a fluorescence microscope using a bandpass filter (excitation 490 nm, emission >520 nm) to view green fluorescence. Hoechst 33342 can be seen using a UV-filter with excitation at 365 nm and emission at 480 nm.

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 Rhodamine 110-(RR)2 substrate (Section 7) to form the concentrated stock solution.

11.5 When ready to stain cells, dilute the stock 1:5 by adding 200 µL diH2O to form the R110-(RR)2 staining solution.

11.6 Add Rhodamine Substrate staining solution at approximately 1/25-1/50 and gently mix to ensure an even distribution of Rhodamine Substrate. For example, add 10 µL staining solution to 490 µL cells forming a final volume of 500 µL.
Δ **Note:** Microscopy analysis is generally not as sensitive as flow cytometry and may require greater amounts of staining solution.

11.7 Incubate 15-60 mins at 37°C protected from light.

11.8 At this point, cells can be mounted for analysis (Step 10) or stained with Hoechst 33342 (step 11.9).

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 Rhodamine Substrate and control samples. Incubate 10-20 mins at 37°C.

11.10 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.

11.11 Observe cells under a fluorescence microscope using a bandpass filter (excitation 490 nm, emission >520 nm) to view green fluorescence. Hoechst 33342 can be seen using a UV-filter with excitation at 365 nm and emission at 480 nm.

12. Flow cytometry analysis

12.1 Expose cells to the experimental conditions and prepare control cell populations; also prepare an unstained control for gating (Section 6).

12.2 Initial cell concentration should be 3 x 10^5 - 1 x 10^6 cells/mL. The optimal cell concentration for staining will vary based on the experimental conditions. In general, flow cytometric analysis has lower cell density requirements than analyzing with a microscope.

12.3 Transfer 490 µL cell suspension per sample into FACS tubes. Different sample volumes may be used; however, this will change the amount of Rhodamine Substrate needed for optimal staining and alter the number of tests per vial.

12.4 Reconstitute vial of Rhodamine 110-(RR)2 substrate by adding 50 µL DMSO to form the concentrated 250X stock solution (Section 9).

12.5 When ready to stain cells, dilute the 250X stock 1:5 by adding 200 µL diH2O to form the 50X Rhodamine 110-(RR)2 substrate staining solution (Section 7).

12.6 Add the 50X Rhodamine Substrate staining solution at approximately 1:50 and gently mix to ensure an even distribution of Rhodamine 110-(RR)2 substrate. For example, add 10 µL staining solution to 490 µL cells, forming a final volume of 500 µL.

12.7 Incubate 15-60 minutes at 37°C protected from light. Gently resuspend cells approximately every 20 minutes throughout the staining process.

12.8 Run the unstained controls. Generate a FSC vs SSC dot or density plot and gate on the population of interest. Adjust the voltages, if necessary, so that the cell population is easily distinguished.

12.9 For single-color analysis, a 488 nm blue argon laser or comparable can be used with the emission filter pairing that best approximates 530/30 (often FL-1/FITC channel).

12.10 Run single color controls. Generate a histogram with the log FL-1 on the x-axis versus the number of cells on the y-axis. Cathepsin negative cells will fall within the lower log fluorescence output decades of the FL-1 x-axis and the cathepsin-positive cells will appear as a shoulder or as a separate peak on the right side of the negative peak histogram. Adjust the voltage on FL-1, if necessary, to ensure

fluorescence is on scale and cathepsin positive and negative populations are distinguished.

12.11 For dual-color analyses, run each single color control. Adjust compensation to remove spectral overlap from interfering FL channels.

12.12 Run experimental samples and analyze.

13. Notes

Technical Support

Austria
wissenschaftlicherdienst@abcam.com | 019-288-259
France
supportscientifique@abcam.com | 01.46.94.62.96
Germany
wissenschaftlicherdienst@abcam.com | 030-896-779-154
Spain
soportecientifico@abcam.com | 91-114-65-60

Switzerland
technical@abcam.com
Deutsch: 043-501-64-24 | Français: 061-500-05-30
UK, EU and ROW
technical@abcam.com | +44(0)1223-696000

Canada
ca.technical@abcam.com | 877-749-8807
US and Latin America
us.technical@abcam.com | 888-772-2226

Asia Pacific
hk.technical@abcam.com | (852) 2603-6823
China
cn.technical@abcam.com | 400 921 0189 | +86 21 2070 0500

Japan
technical@abcam.co.jp | +81-(0)3-6231-0940
Singapore
sg.technical@abcam.com | 800 188-5244

Australia
au.technical@abcam.com | +61-(0)3-8652-1450
New Zealand
nz.technical@abc.com | +64-(0)9-909-7829