Green Cathepsin B Kit

For Research Use Only

BIO-RAD

ICT9151 - 25 Tests

Component	Storage Conditions	Quantity
1 vial Rhodamine 110-$(RR)_2$	-20°C	25 tests
1 vial Hoechst 33342 (200 µg)	2-8°C	1 ml
1 bottle 10x Cellular Assay Buffer	2-8°C	15 ml

ICT9152 - 100 Tests

Component	Storage Conditions	Quantity
4 vials Rhodamine 110-$(RR)_2$	-20°C	25 tests
1 vial Hoechst 33342 (200 µg)	2-8°C	1 ml
1 bottle 10x Cellular Assay Buffer	2-8°C	60 ml

Both kits are provided in two boxes which should be stored at different temperatures:

- Box one contains the rhodamine substrate which should be stored at -20°C. Once reconstituted with DMSO, use immediately or store for up to 6 months at -20°C. Aliquot to avoid repeat freezing and thawing
- Box two contains all of the other kit components which should be stored at 2-8°C. Do not freeze

Background

Bio-Rad's Green Cathepsin B Kit enables researchers to quantitate and monitor intracellular cathepsin activity over time in vitro. The Rhodamine 110 Cathepsin B Substrate is a non-cytotoxic and membrane permeant substrate that fluoresces green upon cleavage by active cathepsin enzymes.

Cathepsins are a group of protease enzymes that were originally identified in lysosomes. They are classified based on the key catalytic group present in their active site, and categorized as aspartic, serine, or cysteine proteases. Cathepsins D and E are aspartic proteases, cathepsins A and G serine proteases, and cathepsins B, C, F, H, K, L, O, S, V, W, and X are cysteine proteases (Buhling F et al. 2000, Siklos M et al. 2015). 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 (Buhling F et al. 2000, Turk B et al. 2000, Turk V et al 2012). These enzymes exist in their processed form as disulfide-linked heavy and light chain subunits with molecular weights ranging from 20-35 kDa (Van Noorden CJ 1997). Cathepsin C is the noted exception, existing as an oligomeric enzyme with a MW ~200 kDa (Turk B et al. 2000).

Cathepsin B is thought to play important roles in many cellular functions, including intracellular protein degradation, antigen processing and presentation, and proenzyme processing, to name a few (Turk B et al. 2000). Elevated cathepsin enzyme activity in serum or the extracellular matrix often signifies a number of gross pathological conditions.

Cathepsin-mediated diseases include: neurodegenerative diseases such as Alzheimer's; autoimmune related diseases like arthritis; and the accelerated breakdown of bone structure seen with osteoporosis (Buhling F et al. 2000, Gerber A 2000, Lowry JR et al. 2018). Up-regulated cathepsin B activity has also been linked to several types of cancer. These include cancer of the colon, pancreas, ovaries, breast, lung, and skin (melanoma) (Bank U et al. 2000, Frosch B et al. 1999, Guinec N et al. 1993, Kos J et al. 2000).

Rhodamine 110 Cathepsin B Substrate utilizes the photostable green fluorophore, Rhodamine 110. It is comprised of Rhodamine 110 coupled to two copies of the amino acid sequence, arginine-arginine (RR), which is the preferential target sequence for cathepsin B. When bi-substituted via amide linkage to two cathepsin B target peptide sequences, Rhodamine110 is nonfluorescent. Following enzymatic cleavage at one or both arginine (R) amide linkage sites, the mono and non-substituted Rhodamine 110 fluorophores generate green fluorescence when excited at 500 nm.

To use the Green Cathepsin B Assay, simply add the Rhodamine 110 Cathepsin B Substrate [R110-$(RR)_2$] directly to the cell culture media (or 1x Cellular Assay Buffer), incubate, and analyze. Because R110-$(RR)_2$ is cell-permeant, it easily penetrates the cell membrane and the membranes of the internal cellular organelles - no lysis or permeabilization steps are required. R110-$(RR)_2$ will enter the cell in a nonfluorescent state. 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 R110-$(RR)_2$ 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-cathepsin forms of the enzymes. If the treatment or experimental conditions stimulate cathepsin activity, cells containing elevated levels of cathepsin activity will appear brighter green than cells with lower levels of cathepsin activity.

R110-$(RR)_2$ 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 R110-$(RR)_2$. 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.

Experimental Set Up

Staining with R110-$(RR)_2$ may require cell culture for a period of time to obtain the correct concentration of cells for cell lines and allow experimental treatment to take effect. The recommended staining is 10 µl per 500 µl of cells at a cell concentration of 0.3-1 x 10^6 cells/ml, however this may vary depending upon the cells and treatment used.

Include appropriate controls for your experiment, such as:

- Unlabeled treated and untreated cells
- Labeled treated, untreated, and vehicle (for example DMSO) treated cells
- A positive control of cells activated to elevate cathepsin activity. Note that the vehicle population may exhibit detectable levels of cathepsin activity, as cathepsin B is involved in normal cellular processes such as protein degradation within the lysosome
- A negative control of cells treated with a cathepsin B inhibitor
- A single stained sample of an appropriate viability dye is useful to determine underlying levels of necrosis within your sample
- A single stained sample of R110-$(RR)_2$ and any additional fluorophores may be required to determine compensation levels. Adherent cells may be stained before or after trypsinization but ensure sufficient washes are performed to remove excess trypsin

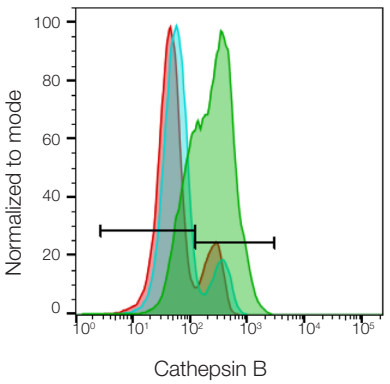
Reagent Preparation

Add 50 µl of DMSO to each vial of R110-$(RR)_2$ to form a 250x stock solution and gently vortex or swirl to ensure it is fully dissolved before using. The stock solution should be clear to slightly yellow in color. Once reconstituted, it can be aliquoted and stored at -20°C for up to 6 months.

Dilute Cellular Assay Buffer 1:10 with DI water. For example add 540 ml of DI water to 60 ml of buffer for a total of 600 ml. If a precipitate is visible, gently warm. Do not boil. 1x Assay Buffer may be stored at 2-8°C for 1 week or frozen and used within 6 months.

Flow Cytometry Protocol

1. Prepare samples (3 x 10^5 – 1 x 10^6 cells/ml) and appropriate controls resuspended in Cellular Assay Buffer.
2. Transfer 490 µl cell suspension per sample into 5 ml FACS tubes. Different sample volumes may be used; however, this will change the amount of R110-$(RR)_2$ needed for optimal staining and alter the number of tests per vial.
3. Reconstitute R110-$(RR)_2$ with 50 µl DMSO (250x stock).
4. Immediately before use, dilute the 250x stock 1:5 by adding 200 µL DI water (50x stock).
5. Add 10 µl staining solution to 490 µl cells (1:50).
6. Incubate 15-60 min at 37°C avoiding direct light. Gently resuspend cells approximately every 20 min throughout the staining process. If required a live dead marker can be added at this point.
7. Run unstained, compensation controls, experimental controls, and experimental samples and analyze.
8. Rhodamine 110 excites at 488 nm and can be detected using a 525/35 filter (similar to FITC).



Treatment	% Negative	% Positive
Untreated	37.6	62.6
CA-074Me	86.2	13.8
Leupeptin	81.6	18.4

Fig. 1. Cathepsin B staining in Jurkat T cells after treatment with inhibitors. Jurkat cells were treated with 10 mM CA-074 Me for 3 hr (blue histogram), 10 mM leupeptin for 1 hr (red histogram) or were untreated (green histogram). They were then stained for 1 hr with R110-$(RR)_2$. After incubation with the R110-(RR)2 substrate, the samples were analyzed by flow cytometry using a ZE5 Cell Analyzer (488 nm excitation and a 525/35 emission filter). Treatment with CA-074 Me or leupeptin decreased the fluorescence signal detected compared to the untreated control (see table), which displayed a baseline level of cathepsin activity.

Microscopy Protocol for Suspension Cells

1. Prepare samples (3 x 10^5-1 x 10^6 cells/ml) and appropriate controls resuspended in Cellular Assay Buffer.
2. When ready to label with the staining solution, cell concentrations may need to be increased to better optimize the number of cells in the frame of view when imaging. If necessary, concentrate cells by gentle centrifugation at 200 g for 5-10 min at RT. Remove the supernatant and resuspend with 1x Cellular Assay Buffer.
3. Transfer 500 µl of cell suspension into tubes. If desired, larger cell volumes can be used, but additional R110-$(RR)_2$ staining solution may be required.
4. Reconstitute R110-$(RR)_2$ with 50 µl DMSO (250x stock).
5. Immediately before use dilute the 250x stock 1:5 by adding 200 µl DI water (50x stock).
6. Add R110-$(RR)_2$ staining solution at a ratio of 1:25 to 1:50 (for instance 1:50 is 10 µl staining solution to 490 µl cells).
7. Incubate 15-60 min at 37°C avoiding direct light. Gently resuspend cells approximately every 20 min throughout the staining process. After the incubation, cells can be stained with Hoechst 33342 at approximately 0.5% v/v. For example, if the cell suspension is 500 µl, add 2.5 µl Hoechst 33342. Incubate 10-20 min at 37°C.
8. Place 15-20 µl of cell suspension onto a microscope slide and cover with a coverslip.
9. 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.

Microscopy Protocol for Adherent Cells

1. Seed 10^4-10^5 cells onto a sterile coverslip in a 35 mm petri dish, onto chamber slides, or grow in a plate.
2. Expose cells to the experimental conditions and create positive and negative controls.
3. Reconstitute R110-$(RR)_2$ with 50 µl DMSO (250x stock).
4. Immediately before use, dilute the 250x stock 1:5 by adding 200 µl DI water (50x stock).
5. Add R110-$(RR)_2$ staining solution at a ratio of 1:25 to 1:50 (for example, 1:50 is 10 µl staining solution to 490 µl Cellular Assay Buffer).
6. Incubate cells for 15-60 min at 37°C avoiding direct light.
7. At this point, cells can be mounted for analysis or stained with Hoechst 33342.
8. 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 min at 37°C.
9. Mount the coverslip with cells facing down onto a drop of PBS.
10. 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.

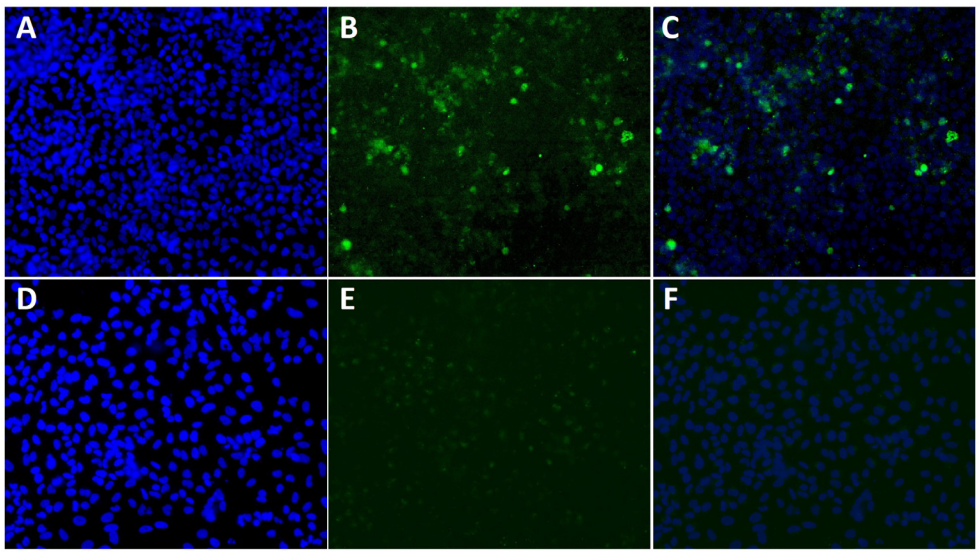


Fig. 2. Cathepsin B staining in HeLa cells after inhibitor treatment. HeLa cells were treated with 10 µM CA-074 Me or untreated and stained with Green Cathepsin B Kit (ICT9152) using Hoechst as a counterstain. **A**, Hoechst 33342; **B**, Green Cathepsin B; **C**, overlay on untreated cells; **D**, Hoechst 33342; **E**, Green Cathepsin B; **F**, overlay on CA-074 Me treated cells.

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