RayBio® Cathepsin L Activity Assay Kit

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RayBio® Cathepsin L Activity Assay Kit Protocol

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RayBiotech, Inc.

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I. INTRODUCTION

Apoptosis can be mediated by mechanisms other than the traditional caspase-mediated cleavage cascade. There is growing recognition that alternative proteolytic enzymes such as the lysosomal cathepsin proteases may initiate or propagate proapoptotic signals. Cathepsins are lysosomal enzymes that are also used as sensitive markers in various toxicological investigations. The Cathepsin-L Activity Assay kit is a fluorescence-based assay that utilizes the preferred cathepsin-L substrate sequence FR labeled with AFC (amino-4-trifluoromethyl coumarin). Cell lysates or other samples that contain cathepsin-L will cleave the synthetic substrate FR-AFC to release free AFC. The released AFC can easily be quantified using a fluorometer or fluorescence plate reader. The cathepsin-L assay is simple, straightforward, and can be adapted to 96-well plate assays. Assay conditions have been optimized to obtain the maximal activity.

II. REAGENTS

Components	ASP-S100	Cap Code	Part Number
CL Buffer	30 ml	WM	Item A
DTT	100µL	BLUE	Item B
Cathepsin L Positive Control	1 vial	Green	Item C
CL Substrate Ac-FR-AFC (10 mM)	0.2 ml	Brown	Item D
CL Inhibitor (1mM)	20 µl	Red	Item E

III. REAGENT STORAGE & PREPARATION

Store kit at –20°C (Store CL Buffer at 4°C after opening). Substrate Ac-FRAFC from light. All reagents are stable for 6 months under proper storage conditions.

Dissolve positive control in 25µL of CL Buffer

IV. Assay Procedure:

1. Collect cells (1-5 x 10^6) by centrifugation.
   Note: Use 50-200 µg cell lysates (in 50 µl of CL Buffer) if protein concentration has been measured.
2. Lyse cells in 50 µl of chilled CL Buffer. Incubate cells on ice for 10 minutes.
3. Centrifuge at top speed in a microcentrifuge for 5 min, transfer the supernatant to a new tube. Add 50µl of cell lysate to a 96-well plate.
4. If a positive control well is desired, add 45µL CL Buffer and 5µL of reconstituted positive control to a separate well.
5. Add 50 µl of CL Buffer to each sample and control wells.
6. Add 1 µL of DTT to each well
7. Add 2µL of the 10mM Ac-FR-AFC substrate (200 µM final concentration).
   Note: For negative control, add 2 µl of Cathepsin L Inhibitor (Optional).
8. Incubate at 37°C for 1-2 hour.

9. Read samples in a fluorometer equipped with a 400-nm excitation filter and 505-nm emission filter. For a plate-reading set-up, transfer the samples to a 96-well plate. You may also perform the entire assay directly in a 96-well plate.

Fold-increase in Cathepsin L activity can be determined by comparing the relative fluorescence units (RFU) with the level of the uninduced control or the negative control sample. If desired, the units of cathepsin L can be determined by generating a standard curve using free AFC under your assay conditions.

IV. General Troubleshooting Guide:

Problems	Cause	Solution
Assay not working	• Cells did not lyse completely • Experiment was not performed at optimal time after apoptosis induction • Plate read at incorrect wavelength	• Resuspend the cell pellet in the buffer and incubate as described in the datasheet • Perform a time-course induction experiment for apoptosis • Check the wavelength listed in the datasheet and the filter settings of the instrument
High Background	• Increased amount of cell lysate used • Increased amounts of components added due to incorrect pipetting • Incubation of cell samples for extended periods • Use of expired kit or improperly stored reagents • Contaminated cells	• Refer to datasheet and use the suggested cell number to prepare lysates • Use calibrated pipettes • Refer to datasheet and incubate for exact times • Always check the expiry date and store the individual components appropriately • Check for bacteria/ yeast/ mycoplasma contamination
Lower signal levels	• Improperly thawed components • Use of expired kit or improperly stored reagents • Allowing the reagents to sit for extended times on ice • Incorrect incubation times or temperatures • Incorrect volumes used	• Thaw all components completely and mix gently before use • Always check the expiry date and store the components appropriately • Always thaw and prepare fresh reaction mix before use • Refer datasheet & verify correct incubation times and temperatures • Use calibrated pipettes and aliquot correctly
Samples with erratic readings	• Uneven number of cells seeded in the wells • Samples prepared in a different buffer • Adherent cells dislodged and lost at the time of experiment • Cell/ tissue samples were not completely homogenized • Samples used after multiple freeze-thaw cycles • Presence of interfering substance in the sample • Use of old or inappropriately stored samples	• Seed only equal number of healthy cells (correct passage number) • Use the cell lysis buffer provided in the kit • Perform experiment gently and in duplicates/triplicates; apoptotic cells may become floaters • Use Dounce homogenizer (increase the number of strokes); observe efficiency of lysis under microscope • Aliquot and freeze samples, if needed to use multiple times • Troubleshoot as needed • Use fresh samples or store at correct temperatures until use
Unanticipated results	• Measured at incorrect wavelength • Cell samples contain interfering substances	• Check the equipment and the filter setting • Troubleshoot if it interferes with the kit (run proper controls)
General issues	• Improperly thawed components • Incorrect incubation times or temperatures • Incorrect volumes used • Air bubbles formed in the well/tube • Substituting reagents from older kits/ lots • Use of a different 96-well plate	• Thaw all components completely and mix gently before use • Refer to datasheet & verify the correct incubation times and temperatures • Use calibrated pipettes and aliquot correctly • Pipette gently against the wall of the well/tubes • Use fresh components from the same kit • Fluorescence: Black plates; Absorbance: Clear plates
Note: The most probable list of causes is under each problem section. Causes/ Solutions may overlap with other problems.		

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