RayBio® Cathepsin H Activity Assay Kit

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RayBio® Cathepsin H 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-H Activity Assay kit is a fluorescence-based assay that utilizes the preferred cathepsin-H substrate Arginine labeled with AFC (amino-4-trifluoromethyl coumarin). Cell lysates or other samples that contain cathepsin-H will cleave the synthetic substrate R-AFC to release free AFC. The released AFC can easily be quantified using a fluorometer or fluorescence plate reader. The cathepsin-H 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
CH Cell Lysis Buffer	25 ml	WM	Item A
CH Reaction Buffer	5 ml	NM	Item B
CH Substrate R-AFC (10mM)	0.2 ml	Amber	Item C
CH Inhibitor (1mM)	20 µl	Red	Item D

III. REAGENT STORAGE & PREPARATION

- Store kit at –20°C (Store CH Cell Lysis Buffer and CH Reaction Buffer at 4°C after opening). Protect CH Substrate R-AFC from light. All reagents are stable for 6 months under proper storage conditions.

IV. Assay Procedure:

1. Collect cells (106) by centrifugation. If the sample is tissue, use 10 mg tissue. Lyse cells or tissue in 50 µl of chilled CH Cell Lysis Buffer. Incubate cells on ice for 10 minutes.Vortex for 5 minutes.
2. Centrifuge at top speed in a microcentrifuge for 5 min, transfer the lysate supernatant to a new tube. Measure protein concentration if desired.
3. Add 5-50 µl of the clear lysate into 96 wells depend on cathepsin H activity in the sample. Duplicate if desire. Add CH Cell Lysis Buffer to total 50 µl each well. Include a negative control for determining background using 50 µl CH Cell Lysis Buffer only without any lysate.
   Note: For negative control, add 2 µl of CH Inhibitor into samples (Optional).
4. Prepare Reaction Master Mix as follows for **each** reaction:
   - 50 µl of CH Reaction Buffer
   - 2 µl of CH Substrate R-AFC

   Note: Mix Well

5. Add 52 µl of Reaction Master Mix to each sample well.
6. Mix well and incubate at 37°C for 1-2 hours or longer if desired. Signals increase as incubation time increases.
7. 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 if desired

Cathepsin H activity can be expressed by Relative Fluorescence Units (RFU)/mg protein/min or RFU/million cells/min.

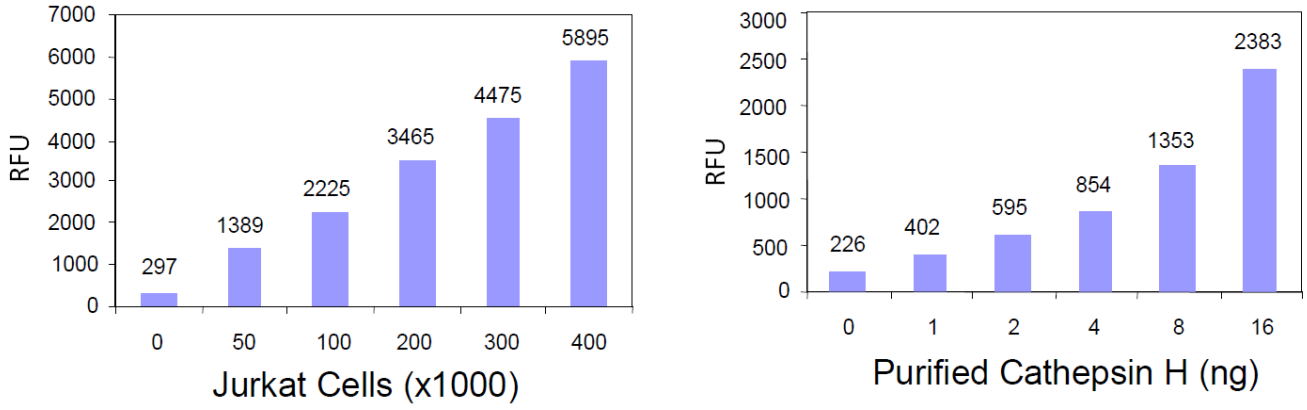

Figure 1. Cathepsin H Activity Assay. Cathepsin H assays were performed using indicated numbers of Jurkat cells (A) or indicated amounts of purified human liver cathepsin H (B). Results were analyzed using a fluorescence plate reader (Ex/Em = 400/505 nm) as described in the kit instructions.

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 lysis 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	• Cells did not initiate apoptosis • Very few cells used for analysis • Use of samples stored for a long time • Incorrect setting of the equipment used to read samples • Allowing the reagents to sit for extended times on ice	• Determine the time-point for initiation of apoptosis after induction (time-course experiment) • Refer to datasheet for appropriate cell number • Use fresh samples or aliquot and store and use within one month for the assay • Refer to datasheet and use the recommended filter setting • Always thaw and prepare fresh reaction mix before use
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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