RayBio® Cathepsin D Activity Assay Kit

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RayBio® Cathepsin D 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-D Activity Assay kit is a fluorescence-based assay that utilizes the preferred cathepsin-D substrate sequence GKPILFFRLK (Dnp)-DR-NH2 labeled with MCA. Cell lysates or other samples that contain cathepsin-D will cleave the synthetic substrate to release fluorescence, which can then easily be quantified using a fluorometer or fluorescence plate reader at Ex/Em = 328/460 nm. The cathepsin-D 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	100 Assays	Cap Code	Part Number
CD Cell Lysis Buffer	25 ml	WM	Item A
CD Reaction Buffer	5 ml	NM	Item B
CD Substrate (1mM)	0.2 ml	Brown	Item C

III. REAGENT STORAGE & PREPARATION

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

IV. Assay Procedure:

1. Collect cells (1-2 x 10^6) by centrifugation.
2. Lyse cells in 200 µl of chilled CD Cell Lysis Buffer. Incubate cells on ice for 10 min.
3. Centrifuge at top speed in a microcentrifuge for 5 min, transfer the supernatant to a new tube. Add 5-50 µl of cell lysate to a 96-well plate for each assay.
4. Bring up the volume to 50 µl of CD Reaction Buffer for each sample.
5. Prepare a master assay mix for each assay. Each assay needs: 50 µl of Reaction Buffer + 2µl CD substrate. Mix well.
6. Add 52 µl of master mixed into each assay well. Mix well
7. Incubate at 37°C for 1-2 hours.
8. Read samples in a fluorometer equipped with a 328-nm excitation filter and 460-nm emission filter.

Fold-increase in Cathepsin D activity can be determined by comparing the relative fluorescence units (RFU) per million cells, or RFU per microgram of protein in your sample, or RFU fold increase of treated versus untreated control or negative control samples.

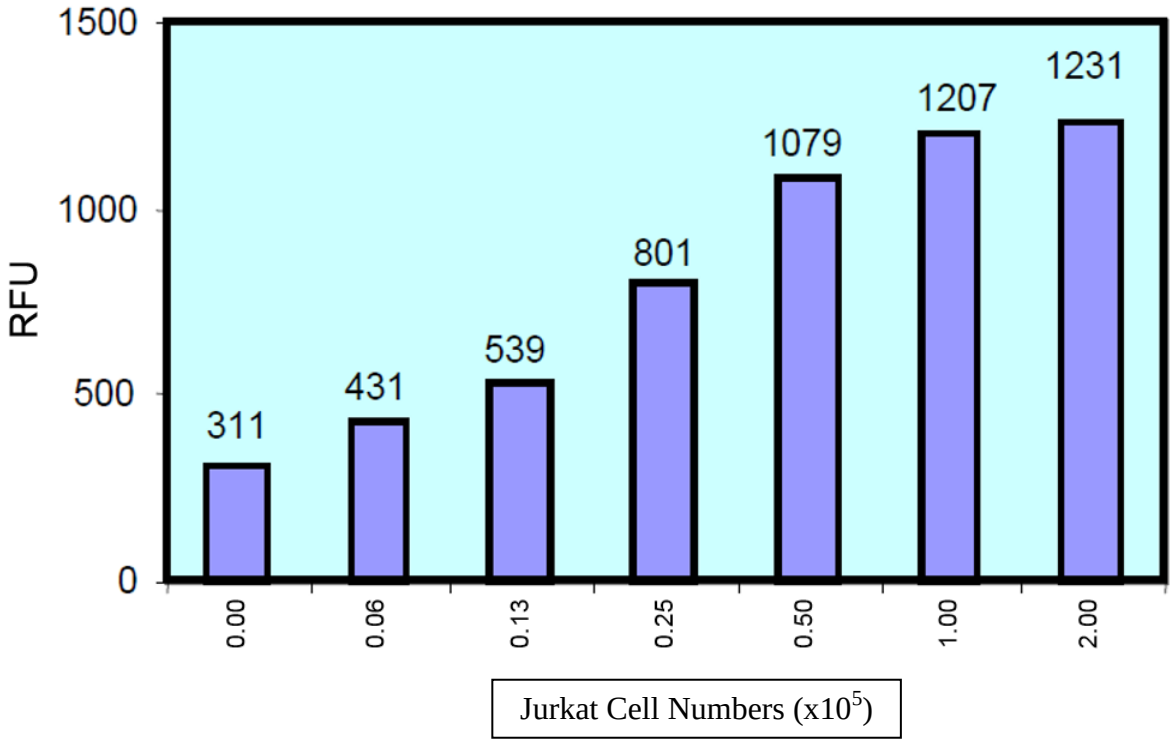

Figure 1. Cathepsin D assays were performed using various numbers of Jurkat Cells as indicated. Assay was performed and results were analyzed by fluorescence plate reader analysis according to kit instructions.

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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