Cathepsin H Activity Fluorometric Assay Kit

(Catalog #BN00425; 100 assays; Store at –20° C)

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. Kit Contents:

Components	100 Assays	Cap Code	Part No.
CH Cell Lysis Buffer	25 ml	WM	BN00425-1
CH Reaction Buffer	5 ml	NM	BN00425-2
CH Substrate R-AFC (10 mM)	0.2 ml	Amber	BN00425-3
CH Inhibitor (1 mM))	20 µl	Red	BN00425-4

III. Storage and Stability:

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

IV. Cathepsin H Assay Protocol:

1. Collect cells (10^6) 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 13000 rpm for 5 min in bench-top micro-centrifuge to remove insoluble materials. Transfer the clear lysate into 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 desired. Add CH Cell Lysis Buffer to total 50 µl each well. Do a negative control as background using 50 µl CH Cell Lysis Buffer only without lysate.

   Note: For negative control, add 2 µl of CH Inhibitor into samples (Optional).

   We recommend using a flat bottom, opaque, white or black 96-well plate for enhanced sensitivity.
4. Prepare Reaction master mix. For each reaction:

   50 µl of CH Reaction Buffer
   2 µl of CH Substrate R-AFC
   Mix well.
5. Add 52 µl of the master mix into each reaction.
6. Mix and Incubate at 37° C for 1-2 hour or longer. Signal increase as incubation time increase.
7. Read samples with a Fluorometer equipped with a 400-nm excitation and 505-nm emission filters.

   Cathepsin H activity can be expressed by Relative Fluorescence Units (RFU)/mg protein/min or RFU/million cells/min. If desire, cathepsin H activity can be determined by generating a standard curve using free AFC under your assay conditions. Free AFC is available from Assay Genie.

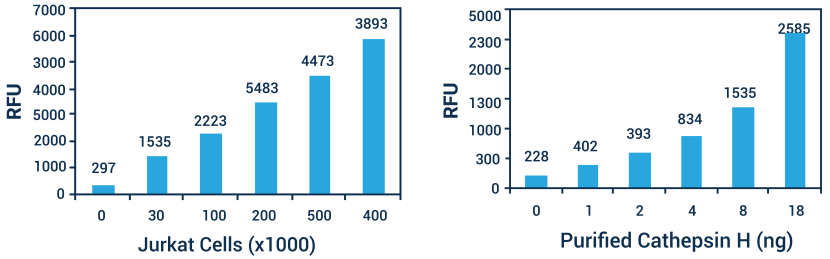

Figure 1. Cathepsin H Activity Assay. Cathepsin H assays were performed using various numbers of Jurkat cells (A) or various amounts of purified human liver cathepsin H (B), as indicated. Results were analyzed using a fluorescence plate reader (Ex/Em = 400/505 nm) as described in the kit instructions.

GENERAL TROUBLESHOOTING GUIDE FOR CATHEPSIN FLUOROMETRIC KITS:

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