Cathepsin L Activity Fluorometric Assay Kit

(Catalog # BN00395; 100 assays; Store kit 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-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. Kit Contents:

Components	BN00395	Color Code	Part Number
CL Buffer	30 ml	WM	BN00395-1
DTT	100 µl	Blue	BN00395-2
Cathepsin L Positive Control	1 vial	Green	BN00395-3
CL Substrate Ac-FR-AFC (10 mM)	0.2 ml	Brown	BN00395-4
CL Inhibitor (1 mM)	20 µl	Red	BN00395-5

III. Storage and Stability:

- Store kit at –20° C (Store CL Buffer at 4°C after opening). Protect CL Substrate Ac-FR-AFC from light. All reagents are stable for 6 months under proper storage conditions.
- Dissolve positive control in 25 µl CL Buffer.

IV. Cathepsin L Assay Protocol:

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 min.
3. Centrifuge at top speed in a microcentrifuge for 5 min, transfer the supernatant to a new tube. Add 50 µl of cell lysate to a 96-well plate.
   Note: We recommend using a flat bottom, opaque, white or black 96-well plate for enhanced sensitivity.
4. If a positive control well is desired, add 45 µl CL Buffer and 5 µl of reconstituted positive control to a separate well.
5. Add 50 µl of CL Buffer to all sample and control wells.
6. Add 1 µl of DTT to each well
7. Add 2 µl of the 10 mM Ac-FR-AFC substrate (200 µM final concentration).
   Note: For negative control, add 2 µl of Cathepsin L Inhibitor (Optional).
6. Incubate at 37°C for 1-2 hour.
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.

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. Free AFC is available from Assay Genie.

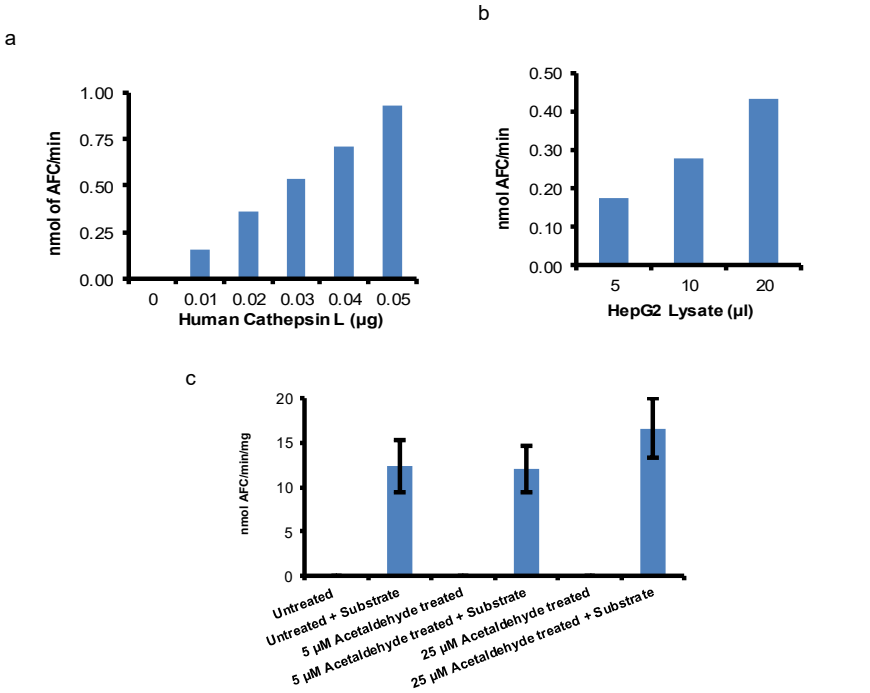

Figure: a. Measurement of recombinant human Cathepsin L activity. b. Measurement of Cathepsin L activity in HepG2 lysate. c. Measurement of Cathepsin L activity in untreated and treated (5 or 25 µM Acetaldehyde) HepG2 lysates. Lysate without the addition of substrate was used as background control. Free AFC was used to obtain AFC standard curve. The protein amount in lysate obtained after treatment with 25 µM of acetaldehyde was lower as compared to untreated or 5 µM treated cells. Assays were performed according to the kit protocol.

FOR RESEARCH USE ONLY! Not to be used on humans.

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.		