Cathepsin D Inhibitor Screening Kit (Fluorometric)

(Catalog #BN00428; 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 D Activity Assay kit is a fluorescence-based assay that utilizes the preferred cathepsin D substrate sequence GKPILFFRLK(DNP)-D-R-NH2) labeled with MCA. Cathepsin D will cleave the synthetic substrate to release the quenched fluorescent group MCA, which can then easily be measured using a fluorometer or fluorescence plate reader at Ex/Em = 320/420 nm. The relative efficacy of test inhibitors are compared to the positive control inhibitor, Pepstatin A (IC_{50} < 0.1 nM). The Cathepsin D assay is simple, straightforward, and can be adapted to 96-well plate assays and is suitable for high throughput screening (HTS).

II. Kit Contents:

Components	100 Assays	Cap Color	Part Number
CD Reaction Buffer	10 ml	WM	BN00428-1
CD Substrate (1 mM)	0.2 ml	Brown	BN00428-2
Cathepsin D, human (lyophilized)	1 vial	Yellow	BN00428-3
Pepstatin A (1 mM in DMSO)	20 µl	Blue	BN00428-4

III. Storage and Stability:

Store kit at –20°C (Store CD Reaction Buffer at 4°C after opening). Protect CD Substrate from light. Store Cathepsin D vial at -80°C. All reagents are stable for 6 months under proper storage conditions.

IV. Reagent Preparation: (Please read entire protocol before proceeding)

1. Cathepsin D: Reconstitute with 1 ml of dH_2O, aliquot and store at -80°C. Avoid repeated Freeze/Thaw cycles.
2. Pepstatin A: Store at -20°C. To make working solution, take 2 µl of Pepstatin A stock solution and dilute with 798 µl CD Reaction Buffer. Working solution is stable for a day at RT & then should be discarded.

V. Cathepsin D Assay Protocol:

1. Prepare Positive Control by mixing 10 µl reconstituted Cathepsin D with 40 µl CD Reaction Buffer.
2. Prepare a Background Control with 50 µl of CD Reaction Buffer alone.
3. Prepare an Inhibitor Reference Control by mixing 10 µl reconstituted Cathepsin D with 10 µl of Pepstatin A working solution and 30 µl CD Reaction Buffer.
4. Prepare Test Inhibitor Samples by mixing 10 µl reconstituted Cathepsin D with 10 µl of the Test Inhibitor and 30 µl CD Reaction Buffer.
5. Pre-incubate Controls and Test Samples at 37 °C for 10 min.
6. Prepare Substrate Mix:

   CD Substrate 2 µl
   Reaction Buffer 48 µl

   Add 50 µl to each well containing Controls and Test Samples. Tap plate gently to mix then incubate at 37°C for 1-2 hour.
7. Read samples in a fluorometer equipped with a 320-nm excitation filter and 420-nm emission filter.
8. **Calculation:**

$$\% \text{ Inhibition} = \frac{(RFU\ Test\ Inhibitor - RF\quad Background\ Control))}{(RFU\ Positive\ Control - RFU\ Background\ Control)} \times 100$$

Or

% Inhibition Relative to Pepstatin A

$$= \frac{(RFU\ Test\ Sample - RFU\ Background\ Control))}{(RFU\ Negative\ Control - RFU\ Background\ Control)} \times 100$$

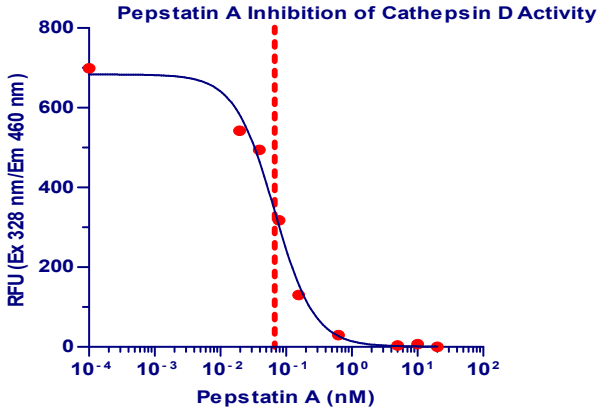

Figure 1. Typical Pepstatin A Inhibition Profile of Cathepsin D Activity. Red line denotes an IC_{50} value of 0.067 nM. Results were analyzed by fluorescence plate reader according to the kit instructions.

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GENERAL TROUBLESHOOTING GUIDE

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.		