

# Cathepsin D Inhibitor Screening Kit (Fluorometric)

(Catalog #K148-100; 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 Inhibitor Screening Kit** utilizes the ability of an active Cathepsin D to cleave a synthetic 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 be easily measured using a fluorometer or fluorescence plate reader at Ex/Em = 320/420 nm. The relative efficacy of test inhibitors is compared with the positive control inhibitor, Pepstatin A (IC<sub>50</sub> < 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.

## II. Kit Contents:

Components	100 Assays	Cap Color	Part Number
CD Reaction Buffer	10 ml	WM	K148-100-1
CD Substrate (1 mM)	0.2 ml	Amber	K148-100-2
Cathepsin D, human (lyophilized)	1 vial	Green	K148-100-3
Pepstatin A (1 mM in DMSO)	20 µl	Blue	K148-100-4

## 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)

- Cathepsin D, human (lyophilized):** Reconstitute the vial in 500 µl of dH<sub>2</sub>O. Divide into aliquots and store at -80°C. Avoid repeated Freeze/Thaw cycles.
- Pepstatin A (1 mM in DMSO):** Store at -20 °C. To make a 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 room temperature (RT) & then should be discarded.

## V. Cathepsin D Assay Protocol:

- Prepare Positive Control by mixing 5 µl of reconstituted Cathepsin D with 45 µl CD Reaction Buffer into one of the wells.
- Prepare a Background Control well with 50 µl of CD Reaction Buffer alone.
- Prepare an Inhibitor Reference Control by mixing 5 µl reconstituted Cathepsin D with 10 µl of Pepstatin A working solution and 35 µl CD Reaction Buffer into one of the wells.
- Prepare Test Inhibitor Samples by mixing 5 µl reconstituted Cathepsin D with 10 µl of the Test Inhibitor and 35 µl CD Reaction Buffer into one of the wells.
- Pre-incubate the Controls and Test Samples at 37 °C for 10 min.
- Prepare Substrate Mix:

CD Substrate	2 µl
Reaction Buffer	48 µl

Add 50 µl to each wells containing Controls and Test Samples. Tap plate gently to mix then incubate at 37 °C for 1-2 hr.

- Read Samples in a fluorometer equipped with a 320 nm excitation filter and 420 nm emission filter.

## 8. Calculation:

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

Or

% Inhibition Relative to Pepstatin A

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

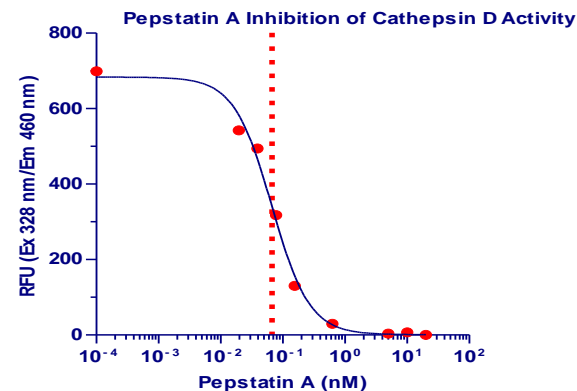


Figure 1. Typical Pepstatin A Inhibition Profile of Cathepsin D Activity. Red line denotes an IC<sub>50</sub> value of 0.067 nM. Results were analyzed using fluorescence plate reader according to the kit instructions.

## VI. Related Products:

- Apoptosis Detection Kits & Reagents
  - Annexin V Kits & Bulk Reagents
  - Caspase Assay Kits & Reagents
  - Mitochondrial Apoptosis Kits & Reagents
  - Nuclear Apoptosis Kits & Reagents
  - Apoptosis Inducers and Set
  - Apoptosis siRNA Vectors
- Cathepsin Activity Assay Kits & Reagents
- Cell Damage & Repair
  - HDAC and HAT Fluorometric & Colorimetric Assays & Drug Discovery Kits
  - DNA Damage Quantification Kit
- Proteins & Enzymes
- Proteases and Protease Inhibitors

## GENERAL TROUBLESHOOTING GUIDE

Problems	Cause	Solution
Assay not working	<ul style="list-style-type: none"> <li>• Cells did not lyse completely</li> <li>• Experiment was not performed at optimal time after apoptosis induction</li> <li>• Plate read at incorrect wavelength</li> </ul>	<ul style="list-style-type: none"> <li>• Resuspend the cell pellet in the lysis buffer and incubate as described in the datasheet</li> <li>• Perform a time-course induction experiment for apoptosis</li> <li>• Check the wavelength listed in the datasheet and the filter settings of the instrument</li> </ul>
High Background	<ul style="list-style-type: none"> <li>• Increased amount of cell lysate used</li> <li>• Increased amounts of components added due to incorrect pipetting</li> <li>• Incubation of cell samples for extended periods</li> <li>• Use of expired kit or improperly stored reagents</li> <li>• Contaminated cells</li> </ul>	<ul style="list-style-type: none"> <li>• Refer to datasheet and use the suggested cell number to prepare lysates</li> <li>• Use calibrated pipettes</li> <li>• Refer to datasheet and incubate for exact times</li> <li>• Always check the expiry date and store the individual components appropriately</li> <li>• Check for bacterial/ yeast/ mycoplasma contamination</li> </ul>
Lower signal levels	<ul style="list-style-type: none"> <li>• Cells did not initiate apoptosis</li> <li>• Very few cells used for analysis</li> <li>• Use of samples stored for a long time</li> <li>• Incorrect setting of the equipment used to read samples</li> <li>• Allowing the reagents to sit for extended times on ice</li> </ul>	<ul style="list-style-type: none"> <li>• Determine the time-point for initiation of apoptosis after induction (time-course experiment)</li> <li>• Refer to datasheet for appropriate cell number</li> <li>• Use fresh samples or aliquot and store and use within one month for the assay</li> <li>• Refer to datasheet and use the recommended filter setting</li> <li>• Always thaw and prepare fresh reaction mix before use</li> </ul>
Samples with erratic readings	<ul style="list-style-type: none"> <li>• Uneven number of cells seeded in the wells</li> <li>• Samples prepared in a different buffer</li> <li>• Adherent cells dislodged and lost at the time of experiment</li> <li>• Cell/ tissue samples were not completely homogenized</li> <li>• Samples used after multiple freeze-thaw cycles</li> <li>• Presence of interfering substance in the sample</li> <li>• Use of old or inappropriately stored samples</li> </ul>	<ul style="list-style-type: none"> <li>• Seed only equal number of healthy cells (correct passage number)</li> <li>• Use the cell lysis buffer provided in the kit</li> <li>• Perform experiment gently and in duplicates/triplicates; apoptotic cells may become floaters</li> <li>• Use Dounce homogenizer (increase the number of strokes); observe efficiency of lysis under microscope</li> <li>• Aliquot and freeze samples, if needed to use multiple times</li> <li>• Troubleshoot as needed</li> <li>• Use fresh samples or store at correct temperatures until use</li> </ul>
Unanticipated results	<ul style="list-style-type: none"> <li>• Measured at incorrect wavelength</li> <li>• Cell samples contain interfering substances</li> </ul>	<ul style="list-style-type: none"> <li>• Check the equipment and the filter setting</li> <li>• Troubleshoot if it interferes with the kit (run proper controls)</li> </ul>
General issues	<ul style="list-style-type: none"> <li>• Improperly thawed components</li> <li>• Incorrect incubation times or temperatures</li> <li>• Incorrect volumes used</li> <li>• Air bubbles formed in the well/tube</li> <li>• Substituting reagents from older kits/ lots</li> <li>• Use of a different 96-well plate</li> </ul>	<ul style="list-style-type: none"> <li>• Thaw all components completely and mix gently before use</li> <li>• Refer to datasheet &amp; verify the correct incubation times and temperatures</li> <li>• Use calibrated pipettes and aliquot correctly</li> <li>• Pipette gently against the wall of the well/tubes</li> <li>• Use fresh components from the same kit</li> <li>• Fluorescence: Black plates; Absorbance: Clear plates</li> </ul>

**Note#** The most probable cause is listed under each section. Causes may overlap with other sections.