SensoLyte® 520 Cathepsin D Assay Kit
Fluorimetric

Revision Number: 1.1	*Last updated: October 2014*
Catalog #	**AS-72170**
Kit Size	100 Assays (96-well plate)

- ***Optimized Performance:*** This kit is optimized to detect Cathepsin D activity.
- ***Enhanced Value:*** Ample reagents to perform 100 assays in a 96-well format.
- ***High Speed:*** The entire process can be completed in one hour
- ***Assured Reliability:*** Detailed protocol and references are provided.

Kit Components, Storage and Handling

Component	Description	Quantity
Component A	QXL™ 520/HiLyte Fluor™ 488, Cathepsin D substrate, Ex/Em=490 nm/520 nm upon cleavage	1 mM, 50 µL
Component B	HiLyte Fluor™ 488, fluorescence reference standard, Ex/Em=490 nm/520 nm	1 mM, 10 µL
Component C	Cathepsin D, bovine spleen	0.1 mg/mL, 10 µL
Component D	Assay Buffer	20 mL
Component E	Pepstatin A	100 µM, 20 µL
Component F	DTT	1 M, 100 µL

Other Materials Required (but not provided)

- 96-well microplate: Black, flat bottom 96-well plate with non-binding surface.
- Fluorescence microplate reader: Capable of detecting emission at 520 nm with excitation at 490 nm.

Storage and Handling

- Store all kit components at -20°C, except for Component C
- Store Component C at -80°C
- Protect Components A and B from light and moisture.
- Component D can be stored at room temperature for convenience.

Introduction

Cathepsin D is the lysosomal aspartic proteinase implicated in intracellular protein degradation. It is involved in several pathological processes, such as inflammatory states, atherosclerosis, thrombosis, apoptosis, neoplastic proliferation and Alzheimer disease.[1-5]

The SensoLyte® 520 Cathepsin D Activity Assay Kit is a homogeneous assay that can be used to detect the activity of enzyme in biological samples or in purified enzyme preparations. The unique long wavelength FRET substrate was designed based on a sequence surrounding the cleavage site of Cathepsin D.[6, 7] A QXL™ 520/HiLyte Fluor™ 488 pair is used for optimal quenching of the intact substrate. When active Cathepsin D cleaves the FRET substrate, it results in an increase of HiLyte Fluor™ 488 fluorescence monitored at excitation/emission = 490 nm/520 nm. The fluorescent signal from HiLyte Fluor™ 488 is stable at low pH that is optimal for cathepsin activity. This assay can detect as low as 0.78 ng/mL active Cathepsin D.

Protocol

Note 1: For standard curve, please refer to Appendix II (optional).
Note 2: Please use protocol A or B based on your needs.

Protocol A. Screening Cathepsin D inhibitors using purified enzyme.

1. Prepare working solutions.

Note: Warm all kit components until thawed to room temperature before starting the experiments.

1.1 Prepare assay buffer: Prepare fresh assay buffer for each experiment. Refer to Table 1. **Use this DTT-containing assay buffer in all the following steps.**

Table 1. Assay buffer for one 96-well plate (100 assays).

Components	Volume
Assay buffer (Component D)	9.95 mL
1 M DTT (Component F)	50 µL
Total volume	10 mL

1.2 Cathepsin D substrate solution: Dilute Cathepsin D substrate (Component A) 100-fold in assay buffer. Prepare fresh assay buffer for each experiment. Refer to Table 2.

Table 2. Cathepsin D substrate solution for one 96-well plate (100 assays).

Components	Volume
Cathepsin D substrate (100X, Component A)	50 µL
Assay buffer	4.95 mL
Total volume	5 mL

1.3 Cathepsin D diluent: Dilute the enzyme (Component C) 1:400 in assay buffer. This amount of enzyme is enough for a full 96-well plate. If not using the entire plate, adjust the amount of enzyme to be diluted accordingly.

Note: Prepare enzyme diluents immediately before use. Do not vortex the enzyme solutions. Prolonged storage or vigorous agitation of the diluted enzyme will cause denaturation. Store the enzyme solution on ice.

1.4 Pepstatin A (Cathepsin D inhibitor): Dilute the 100 µM inhibitor solution (Component E) 1:1000 in assay buffer. The diluted pepstatin solution has a concentration of 100 nM. Add 10 µl of the diluted pepstatin into each of the inhibitor control well.

2. Set up the enzymatic reaction.

2.1 Add test compounds and diluted enzyme solution to the microplate wells. The suggested volume of enzyme solution for a 96-well plate is 40 µL/well and test compound is 10 µL/well.

2.2 Simultaneously set up the following control wells, as deemed necessary:

- Positive control contains the enzyme without test compound.
- Inhibitor control contains Cathepsin D enzyme and pepstatin.
- Vehicle control contains Cathepsin D enzyme and vehicle used in delivering test compound (e.g. DMSO, concentration not to exceed 1%).
- Test compound control contains assay buffer and test compound. Some test compounds have strong autofluorescence and may give false results.
- Substrate control contains assay buffer.

2.3 Using the assay buffer, bring the total volume of all controls to 50 µL.

2.4 Optional: Pre-incubate the plate for 10 min. at assay temperature. Any temperature (the *assay temperature*) from room temperature to 37°C may be used, as long as the subsequent incubations are performed at the same temperature.

3. Run the enzymatic reaction.

3.1 Add 50 µL of Cathepsin D substrate solution into each well. For best accuracy, it is advisable to have the substrate solution equilibrated to the assay temperature. Mix the reagents completely by shaking the plate gently for 30 sec.

3.2 Measure fluorescence signal:

- For kinetic reading: Immediately start measuring fluorescence intensity at Ex/Em=490 nm/520 nm continuously and record data every 5 min. for 30 to 60 min.
- For end-point reading: Incubate the reaction for 30 to 60 min. Keep plate from direct light. Then measure fluorescence intensity at Ex/Em=490 nm/520 nm.

For methods of data analysis: Refer to Appendix I.

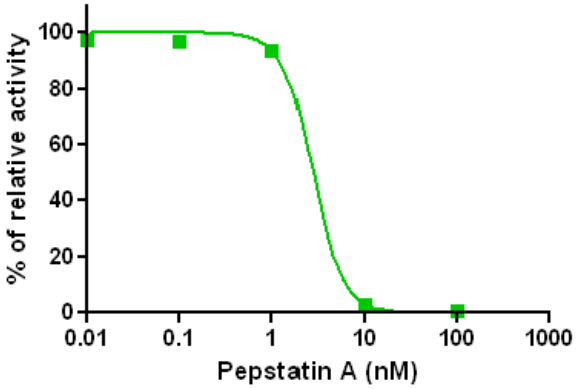

Figure 1. Inhibition of Cathepsin D activity by Pepstatin A as measured with SensoLyte® 520 Cathepsin D activity Assay Kit.

Protocol B. Measuring Cathepsin D activity in biological samples.

1. Prepare cathepsin containing biological samples.

1.1 Prepare cell extracts:

- Suspension cells are collected by centrifugation at 500 X g for 5 min. For adherent cells, simply aspirate the growth medium.
- Add an appropriate amount of assay buffer (see step 2.1) to cells or cell pellet. Collect the cell suspension in a microcentrifuge tube.
- Incubate the cell suspension at 4°C for 10 min.
- Centrifuge the cell suspension for 10 min at 2,500X g, 4°C. Collect the supernatant and store at -70°C until use.

Note: If the activity of cell extracts is low, prepare more concentrated samples by centrifugation at 20,000 x g. Use the precipitated pellets.

2. Prepare working solutions.

Note: Warm all kit components until thawed to room temperature before starting the experiments.

2.1 Prepare assay buffer: Prepare fresh assay buffer for each experiment. Refer to Table 1. U**se this DTT-containing assay buffer in all the following steps.**

Table 1. Assay buffer for one 96-well plate (100 assays).

Components	Volume
Assay buffer (Component D)	9.95 mL
1 M DTT (Component F)	50 µL
Total volume	10 mL

2.2 Cathepsin D substrate solution: Dilute Cathepsin D substrate (Component A) 1:100 in assay buffer. For each experiment, prepare fresh substrate solution. Refer to Table 2.

Table 2. Cathepsin D substrate solution for one 96-well plate (100 assays).

Components	Volume
Cathepsin D substrate (100X, Component A)	50 µL
Assay buffer	4.95 mL
Total volume	5 mL

2.3 Cathepsin D diluent: If using purified Cathepsin D as a positive control, then dilute the enzyme 1:500 in assay buffer. Add 50 µl of the diluted enzyme into each of the positive control well.

3. Set up enzymatic reaction.

3.1 Add 5-50 µL of Cathepsin D containing biological sample.

3.2 Set up the following control wells at the same time, as deemed necessary:

- Positive control contains purified active Cathepsin D.
- Substrate control contains assay buffer.

3.3 Using the assay buffer, bring the total volume of all controls to 50 µL.

3.4 Optional: Pre-incubate the plate for 10 min. at assay temperature. Any temperature (the *assay temperature*) from room temperature to 37°C may be used, as long as the subsequent incubations are performed at the same temperature.

4. Run the enzymatic reaction.

4.1 Add 50 µL of Cathepsin D substrate solution into each well. For best accuracy, it is advisable to have the substrate solution equilibrated to the assay temperature. Mix the reagents completely by shaking the plate gently for 30 sec.

4.2 Measure fluorescence signal:

- For kinetic reading: Immediately start measuring fluorescence intensity at Ex/Em=490 nm/520 nm continuously and record data every 5 min. for 30 to 60 min.
- For end-point reading: Incubate the reaction for 30 to 60 min. Keep plate from direct light. Then measure fluorescence intensity at Ex/Em=490 nm/520 nm.

4.3 For methods of data analysis: Refer to Appendix I.

Appendix I. Data Analysis

- The fluorescence reading from the substrate control well is used as the background fluorescence. This background reading should be subtracted from the readings of the other wells containing substrate. All fluorescence readings are expressed in relative fluorescence units (RFU).

- For kinetics analysis:
  - Plot data as RFU versus time for each sample. If you want to convert RFUs to the concentration of the product of the enzymatic reaction, please refer to Appendix II for establishing a fluorescence reference standard.
  - Determine the range of initial time points during which the reaction is linear. Typically, the first 10-15% of the reaction will be the optimal range.
  - Obtain the initial reaction velocity (V_o) in RFU/min by determining the slope of the linear portion of the data plot.
  - A variety of data analyses can be done, e.g., determining inhibition %, $EC_{50,}$ IC_{50}, K_m, K_i, etc.
- For endpoint analysis:
  - Plot data as RFU versus concentration of test compounds.
  - A variety of data analyses can be done, e.g., determining inhibition %, EC_{50}, IC_{50}, etc.

Appendix II. Instrument Calibration

- HiLyte Fluor™ 488 fluorescence reference standard: Dilute 1 mM HiLyte Fluor™ 488 (Component B) to 10 µM in assay buffer. Do 2-fold serial dilutions to get concentrations of 5,

2.5, 1.25, 0.625, 0.312, 0.156 and 0 μM, include an assay buffer blank. Add 50 μL/well of these serially diluted HiLyte Fluor™ 488 reference solutions.

- Add 50 μL/well of the diluted cathepsin substrate solution (refer to Protocol A, step 1.1 for preparation).

Note: The cathepsin substrate solution is added to the HiLyte Fluor™ 488 reference standard to correct for the absorptive quenching by the FRET peptide. If multiple concentrations of substrate are used, this step must be performed for each concentration.

- Measure the fluorescence intensity of the reference standard and substrate control wells at Ex/Em=490 nm/520 nm. Use the same setting of sensitivity as used in the enzyme reaction.
- Plot the HiLyte Fluor™ 488-fluorescence reference standard curve as RFU (relative fluorescent units) versus concentration as shown in Figure 2.
- The final concentrations of HiLyte Fluor™ 488 reference standard are 5, 2.5,1.25, 0.625, 0.312, 0.156 , 0.078 and 0 μM. This reference standard is used to calibrate the variation of different instruments and different experiments. It is also an indicator of the amount of final product of the enzymatic reaction.

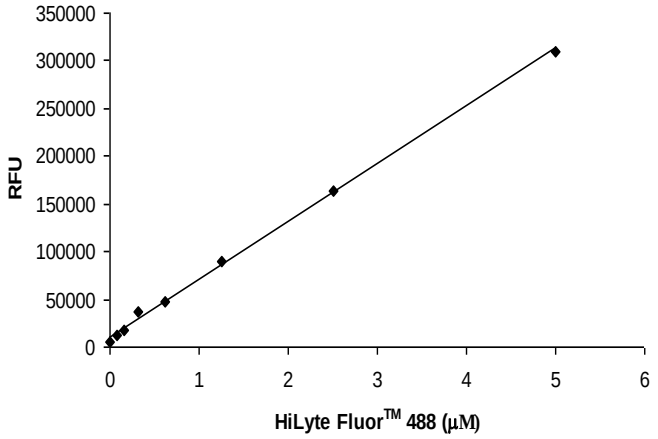

Figure 2. HiLyte Fluor™ 488 reference standard. HiLyte Fluor™ 488 was serially diluted in assay buffer, containing cathepsin D substrate, and the fluorescence recorded at Ex/Em=490 nm/520 nm. (Flexstation 384II, Molecular Devices)

References

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