SensoLyte® 520 Cathepsin K Assay Kit
Fluorimetric

Revision Number: 1.1	*Last updated: October 2014*
Catalog #	**AS-72171**
Kit Size	100 Assays (96-well plate)

- ***Optimized Performance:*** This kit is optimized to detect Cathepsin K 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 K substrate, Ex/Em=490 nm/520 nm upon cleavage	2 mM, 50 µL
Component B	HiLyte Fluor™ 488, fluorescence reference standard, Ex/Em=490 nm/520 nm	1 mM, 10 µL
Component C	Procathepsin K, human recombinant	0.15 mg/mL, 10 µL
Component D	Assay Buffer	20 mL
Component E	Cathepsin K activation buffer	100 µL
Component F	Cathepsin K Inhibitor	100 µM, 10 µL
Component G	DTT	1 M, 200 µL

Other Materials Required (but not provided)

- 96-well microplate: Black, flat-bottom, non-binding 96-well plate.
- 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 K is a cysteine protease that plays a role in the degradation of protein components of bone matrix during bone resorption.[1] Produced by bone resorbing macrophages and synovial fibroblasts, cathepsin K cleaves proteins such as collagen type I, collagen type II and osteonectin.[2] It has potential as a drug target in autoimmune diseases and osteoporosis.[3, 4]

The SensoLyte® 520 Cathepsin K Activity Assay Kit is a homogeneous assay that can be used in detecting Cathepsin K activity in biological samples or in purified enzyme preparations. A unique QXL™ 520/HiLyte Fluor™ 488 FRET peptide substrate is used in this kit. When active Cathepsin K cleaves this FRET substrate, it results in an increase of HiLyte Fluor™ 488 fluorescence, which can be monitored at Ex/Em = 490/520 nm. The fluorescent signal from HiLyte Fluor™ 488 is stable at low pH that is optimal for cathepsin activity.

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 K 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 subsequent 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 G)	50 µL
Total volume	10 mL

1.2 Cathepsin K substrate solution: Dilute Cathepsin K substrate (Component A) 100-fold in assay buffer. For each experiment, prepare fresh substrate solution. Refer to Table 2.

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

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

1.3 Prepare Cathepsin K activation buffer: Prepare fresh activation buffer for each experiment. Refer to Table 3.

Table 3. Activation buffer

Components	Volume
Activation buffer (Component E)	99 µL
1 M DTT (Component G)	1 µL
Total volume	100 µL

1.4 Cathepsin K diluent:

Activation of enzyme: Add 10 µL of activation buffer to the vial (10 µL) of Procathepsin K (Component C) and incubate 40 min. at room temperature. Dilute the activated enzyme 1:300 in assay buffer. This amount of enzyme is enough for a full 96-well plate. If not using an entire plate, adjust the amount of enzyme to be diluted accordingly.

Note: To activate enzyme, add an equal volume of activation buffer to the enzyme. This buffer adjusts the pH to 4.0, thereby activating enzyme. Prepare enzyme diluents immediately before use. Do not vortex the enzyme solution. Prolonged storage or vigorous agitation of the diluted enzyme will cause denaturation. Store on ice.

1.5 Cathepsin K inhibitor: Dilute 100 µM inhibitor solution (Component F) 1:1000 in assay buffer to get a concentration of 100 nM. Add 10 µl of the diluted compound 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 establish the following control wells, as deemed necessary:

- Positive control contains the enzyme without test compound.
- Inhibitor control contains Cathepsin K enzyme and inhibitor.
- Vehicle control contains Cathepsin K 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/well.

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 K 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 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 at Ex/Em=490 nm/520 nm.

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

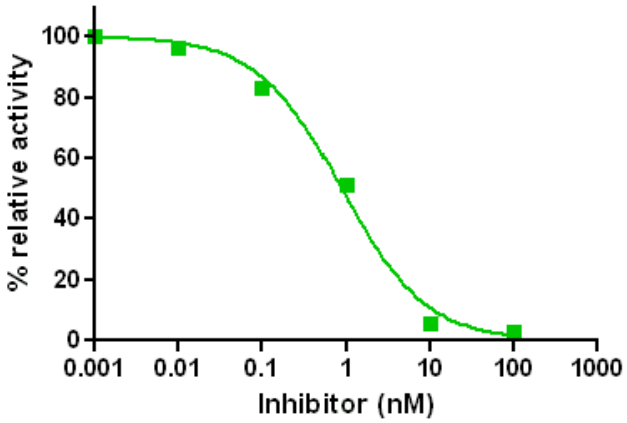

Figure 1. Inhibition of Cathepsin K activity measured with SensoLyte® 520 Cathepsin K Assay Kit.

Protocol B. Measuring Cathepsin K activity in biological samples.

1. Prepare Cathepsin K containing biological samples.

1.1 Prepare cell extracts:

Note: According to our data, Cathepsin L can also cleave FRET substrate provided in the kit, although at a slower rate. Since cells or tissues contain different cathepsins, you may need to inhibit Cathepsin L activity in your biological sample using a specific Cathepsin L inhibitor (Cat# 62935)

- Use freeze-thaw cycles for cell lysis.
- Suspension cells are collected by centrifugation at 500 X g for 5 min and washed by PBS.
- For adherent cells, aspirate the growth medium, trypsinize, followed by inactivation of trypsin using medium with 10% FBS. Wash cells with PBS before proceeding to the lysis step.
- Add an appropriate amount of assay buffer (see Step 2.1) to cells or cell pellet.
- Perform 3 freeze-thaw cycles to lyse cells.
- Centrifuge cell suspension for 10 min at 13,000X g, 4°C. Collect the supernatant and store at -70°C until use.

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. **Use this DTT-containing assay buffer in all the subsequent 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 G)	50 µL
Total volume	10 mL

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

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

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

2.3 Prepare Cathepsin K activation buffer: Prepare fresh activation buffer for each experiment. Refer to Table 3.

Table 3. Activation buffer.

Components	Volume
Activation buffer (Component E)	99 µL
1 M DTT (Component G)	1 µL
Total volume	100 µL

2.4 Cathepsin K diluent:

Activation of enzyme: If using purified Procathepsin K enzyme as a positive control, add an equal volume of activation buffer to the enzyme and incubate 40 min. at room temperature. Dilute the activated enzyme 1:300 in assay buffer. Adjust the amount of enzyme to be diluted accordingly.

Note: The activation buffer adjusts the pH to 4.0, thereby activating enzyme. Prepare enzyme diluents immediately before use. Do not vortex enzyme solutions. Prolonged storage or vigorous agitation of the diluted enzyme will cause denaturation. Store the enzyme solution on ice.

3. Set up enzymatic reaction.

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

3.2 Simultaneously establish the following control wells, as deemed necessary:

- Positive control contains active Cathepsin K diluents.
- Substrate control contains assay buffer.

3.3 Using assay buffer, bring the total volume of all samples to 50 µL/well.

3.3 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 K 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 at Ex/Em=490/520 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 at Ex/Em=490/520 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. 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 20 μM in assay buffer. Do 2-fold serial dilutions to get concentrations of 10, 5, 2.5, 1.25, 0.625 and 0.312 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 for preparation).

  Note: Cathepsin K 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 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 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 10, 5, 2.5, 1.25, 0.625, 0.312, 0.156 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.

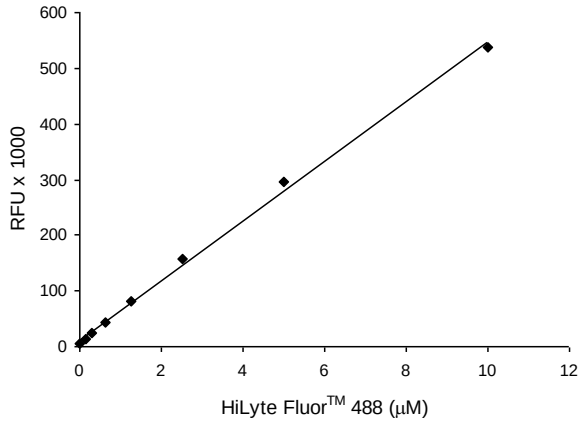

Figure 2. HiLyte Fluor™ 488 reference standard. HiLyte Fluor™ 488 was serially diluted in assay buffer, containing Cathepsin K substrate, and the fluorescence was recorded at Ex/Em=490 nm/ 520 nm. (Flexstation 384II, Molecular Devices)

References :

1. Motyckova, G. et al. *PNAS* **98** , 5798 (2001).
2. Hou, WS. et al. *Am. J. Pathol.* **159**, 2167 (2001).
3. Skoumal, M. et al. *Arthritis Res Ther.* **7**, 65(2005).
4. Asagiri, M. et al. *Science* **319**, 624 (2008).