SensoLyte® 520 Deubiquitination Assay Kit
Fluorimetric

Revision Number: 1.1	*Last updated: October 2014*
Catalog #	**AS-72203**
Kit Size	100 Assays (96-well plate)

- ***Optimized Performance:*** This kit is optimized to detect deubiquitin protease activity
- ***Enhanced Value:*** It provides ample reagents to perform 100 assays in a 96-well plate 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	520 DUB fluorogenic substrate, Ex/Em=490 nm/520 nm upon cleavage	2 mM, 50 µL
Component B	Fluorescence reference standard, Ex/Em=490 nm/520 nm	2 mM, 10 µL
Component C	Deubiquitin protease, Recombinant Human UCH-L3	0.5 mg/mL, 20 µL
Component D	2X Assay Buffer	25 mL
Component E	DTT	1 M, 100 µL
Component F	UCH-L3 Inhibitor	4 mM, 10 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

Modification of proteins with ubiquitin is a key step in protein degradation controlling many intracellular processes such as transcription, cell cycle progression, receptor internalization, and DNA repair.[1] Recent studies have demonstrated that regulation also occurs at the level of deubiquitination.[2,3] Deubiquitinating enzymes (DUBs) are proteases that reverse ubiquitin modifications.

Over 100 DUBs have been identified in humans. They are grouped into five distinct families based on their sequence similarities and mechanisms of action. Four of the families are cysteine proteases, while the fifth is a novel type of zinc-dependent metalloprotease.[4,5] The majority of human DUBs belong to two cysteine proteases families, the ubiquitin specific proteases (USPs) and the ubiquitin carboxyterminal hydrolases (UCHs). Both families hydrolyze the peptide bond downstream of the C terminus of ubiquitin, which is either a classic peptide bond in the proforms of ubiquitin or an isopeptide bond to an ε-amino group of a lysine residue within an ubiquitin modified protein. DUBs have been implicated in the pathogenesis of many human diseases, such as neurodegenerative disorders and cancer.[6,7] Consequently, they have become actively studied molecular targets for drug discovery.[8,9]

The SensoLyte® 520 Deubiquitination Assay Kit provides a convenient assay for inhibitors/activators screening in deubiquitinylation or for continuous assay of enzyme activity using a fluorogenic peptide. Upon protease cleavage, this substrate generates the bright green fluorophore that can be detected at excitation/emission=490/520 nm. The long wavelength spectra of the green fluorophore released after cleavage of the substrate provides less interference from other reaction components.

Protocol

Note 1: For instrument calibration, refer to Appendix II (optional).
Note 2: Please use Protocol A or B based on your needs.

Protocol A. Screening compounds using purified enzyme.

1. Prepare working solutions.

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

1.1 Prepare 1X assay buffer: Prepare fresh DTT-containing 1X assay buffer for each experiment. Refer to Table 1. **Use this DTT-containing assay buffer in all the following steps.** If not using the entire plate, adjust the amount of assay buffer to be diluted accordingly.

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

Components	Volume
2X assay buffer (Component D)	5 mL
1 M DTT (Component E)	10 μL
Deionized water	4.99 mL
Total volume	10 mL

1.2 The 520 DUB substrate solution: Dilute deubiquitin substrate (Component A) 1:100 in 1X assay buffer. Refer to Table 2. For each experiment, prepare fresh substrate solution. If not using the entire plate, adjust the amount of substrate to be diluted accordingly.

Table 2. The 520 DUB substrate solution for one 96-well plate (100 assays)

Components	Volume
520 DUB substrate (Component A)	50 µL
1X assay buffer	4.95 mL
Total volume	5 mL

1.3 Protease diluents: Dilute the enzyme, UCH-L3 (Component C), 1:200 in 1X 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 solution. Prolonged storage or vigorous agitation of the diluted enzyme will cause denaturation. Store the enzyme solution on ice.

1.4 UCH-L3 inhibitor: Dilute the 4 mM inhibitor solution (Component F) 1:10 in 1X assay buffer to get a concentration of 0.4 mM. Add 10 µl of the diluted inhibitor 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 one well of a 96-well plate is 40 µL and test compound is 10 µL.

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

- Positive control contains the diluted UCH-L3 without test compound.
- Inhibitor control contains the diluted UCH-L3 and inhibitor.
- Vehicle control contains enzyme and vehicle used in delivering test compound (e.g. DMSO, concentration not to exceed 1%).
- Test compound control contains 1X assay buffer and test compound. Some test compounds have strong autofluorescence and may give false results.
- Substrate control contains 1X 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 the 520 DUB substrate solutions 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. Mix the reagents and measure fluorescence intensity at Ex/Em=490 nm/520 nm.

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

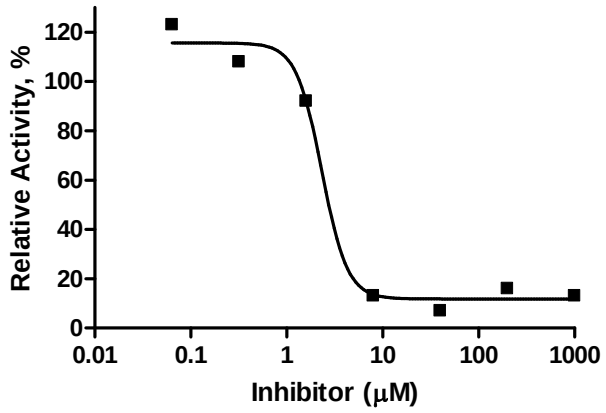

Figure 1. Inhibition of UCH-L3 activity as measured with SensoLyte® 520 Deubiquitination Assay Kit.

Protocol B. Measuring deubiquitination activity in biological samples.

1. Prepare working solutions.

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

1.1 The 520 DUB substrate solution: Dilute deubiquitin substrate (Component A) in 2X assay buffer (Component D). Refer to Table 1. If not using the entire plate, adjust the amount of substrate to be diluted accordingly.

Table 1. The 520 DUB substrate solution for one 96-well plate (100 assays)

Components	Volume
520 DUB substrate (Component A)	50 μL
1 M DTT (Component E)	10 μL
2X assay buffer (Component D)	4.94 mL
Total volume	5 mL

2. Set up the enzymatic reaction.

2.1 Add 50 μL of DUBs containing sample.

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

- Positive control contains purified active UCH-L3.
- Substrate control contains deionized water.

2.3 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 the 520 DUB substrate solutions 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. Mix the reagents and measure fluorescence intensity at Ex/Em=490 nm/520 nm.

3.3 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

- Fluorescence reference standard: Dilute the 2 mM fluorescence standard (Component B) to 20 μM in 1X DTT-containing assay buffer. Do 2-fold serial dilutions to get concentrations of 10, 5, 2.5, 1.25, 0.63, and 0.31 μM, include an assay buffer blank. Add 50 μL/well of these serially diluted reference solutions.
- Add 50 μL/well of the diluted 520 DUB substrate solution (refer to Protocol A, Step 1.2 for preparation).

  Note: The 520 DUB substrate solution is added to the reference standard to normalize for the intrinsic substrate fluorescence. 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 reference standard curve as RFU (relative fluorescent units) versus concentration.

- The final concentrations of fluorescence reference standard are 10, 5, 2.5,1.25, 0.63, 0.31, 0.16 and 0 μM. This reference standard is used to calibrate the variation of different instruments and different experiments.

References :

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