SensoLyte® 520 IDE Activity Assay Kit
Fluorimetric

Revision number: 1.1	*Last updated: 10 /14/14*
Catalog #	**AS-72231**
Kit Size	100 Assays (96-well plate)

- ***Optimized Performance:*** This kit is optimized to detect IDE 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	5-FAM /QXL® 520 IDE substrate Ex/Em=490 nm/520 nm upon cleavage	1 mM, 50µL
Component B	5-FAM fluorescence reference standard, Ex/Em=490 nm/520 nm	1 mM, 12 µL
Component C	Human recombinant IDE	0.1 mg/mL, 10 µL
Component D	Assay Buffer	25 mL
Component E	Inhibitor	1 M, 10µL

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

Insulin degrading enzyme (IDE), an extremely conserved Zn^{2+}metalloendopeptidase, belongs to the M16A family of metallopeptidases.[1-3] IDE is expressed in almost all tissues and distributed in the cytosol. With great capability to degrade various peptides such as IGF-II, TGF-α, glucagon, amylin, calcitonin, β amyloid and amyloid precursor protein (APP), IDE is considered a physiological and pathological relevant enzyme.[4-6] Growing evidences have indicated that IDE may be involved in the pathogenesis of Alzheimer's disease and diabetes mellitus type 2. [2, 7, 8]

The SensoLyte® 520 IDE Activity Assay Kit can be used to detect enzyme activity in biological samples or in purified enzyme preparations. The unique FRET substrate was derived from an APP sequence designed to reduce cross reactivity with Neprilysin, ADAM10, TACE, BACE-1, and BACE-2. When active IDE cleaves the FRET substrate, it results in an increase of 5-FAM fluorescence, which is monitored at excitation/emission = 490 nm/520 nm. The long wavelength fluorescence of 5-FAM is also less interfered by the autofluorescence of components in biological samples and test compounds. This assay can detect as low as 0.8 ng/mL active IDE.

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 IDE inhibitors using a purified enzyme.

1. Prepare working solutions.

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

1.1 IDE substrate solution: Dilute IDE substrate (Component A) 100-fold in assay buffer. Prepare fresh assay buffer for each experiment. Refer to Table 1.

Table 1. IDE substrate solution for one 96-well plate (100 assays).

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

1.2 IDE diluent:

Dilute IDE enzyme (Component C) 400-fold 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: Prepare enzyme diluents immediately before use. Do not vortex the enzyme solution. Prolonged storage or vigorous agitation of the diluted enzyme will cause enzyme denaturation. Store on ice.

1.3 Inhibitor (1, 10-Phenanthroline): Dilute the 1 M inhibitor solution (Component E) 1:100 in assay buffer. The diluted 1, 10-Phenanthroline solution has a concentration of 10 mM. Add 10 µl of the diluted 1, 10-Phenanthroline 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 IDE enzyme and 1, 10-Phenanthroline.
- Vehicle control contains IDE 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 Use the assay buffer to bring the total volume of all controls to 50 µL.

3. Run the enzymatic reaction.

3.1 Add 50 µL of IDE substrate solution into each well. For best accuracy, it is advisable to have the substrate solution equilibrated to the assay temperature (37ºC usually gives higher signal-to-background ratio). 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 at 37ºC 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.

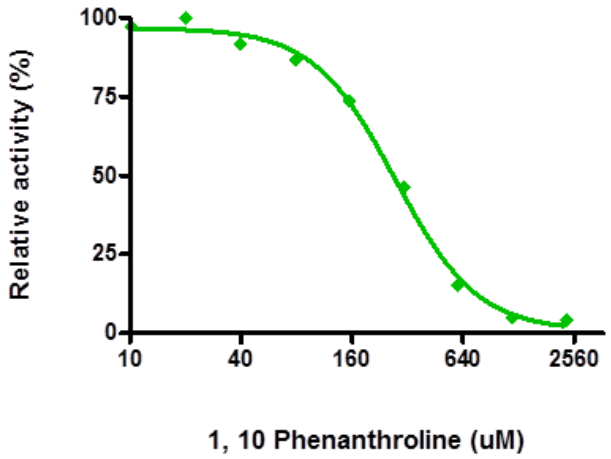

Figure 1. Inhibition of IDE activity by 1, 10-Phenanthroline as measured with SensoLyte® 520 IDE Activity Assay Kit.

Protocol B. Measuring IDE activity in biological samples.

1. Prepare IDE containing biological samples.

1.1 Prepare cell lysates:

- Wash cell once with phosphate buffered saline (PBS) and collect cells by centrifugation at 1500 X g for 5 min.
- Add an appropriate amount of "ice-cold" assay buffer to cell pellet.
- Incubate the cell suspension on ice for at least 15 min.
- Pipette up and down the cell suspension for 5 times.
- Centrifuge the cell suspension for 10 min. at 10,000 X g, 4°C. Collect the supernatant and store at -70°C until use.

1.2 Prepare tissue homogenate and lysate:

- Homogenize tissue samples in ice-cold assay buffer as homogenate.
- Incubate homogenate on ice for an additional 15 min.
- Centrifuge for 15 min. at 10,000xg, 4°C. Collect the supernatant as tissue lysate. Store homogenate and/or lysate at -70°C until use.

Note: PBS is not provided. Cell or tissue lysate are used as the enzyme source for enzyme activity measurement.

2. Prepare working solutions.

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

2.1 Dilute IDE substrate (Component A) 100-fold in assay buffer. Prepare fresh assay buffer for each experiment. Refer to Table 1.

Table 1. IDE substrate solution for one 96-well plate (100 assays).

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

2.2 IDE diluent: If using purified IDE enzyme as a positive control, dilute enzyme 500-fold with assay buffer. Add 50 µl of the diluted enzyme into each of the positive control well.

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

3. Set up enzymatic reaction.

3.1 Add 5-50 µL of IDE containing biological sample.

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

- Positive control contains purified IDE enzyme.
- Substrate control contains assay buffer.

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

4. Run the enzymatic reaction.

4.1 Add 50 µL of IDE substrate solution into each well. For best accuracy, it is advisable to have the substrate solution equilibrated to the assay temperature (37ºC usually gives higher

signal-to-background ratio). 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 at 37 ºC 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. 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

- 5-FAM fluorescence reference standard: Dilute 1 mM 5-FAM reference standard (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 μM, and include 0 μM as an assay buffer blank. Add 50 μL/well of these serially diluted 5-FAM reference solutions.
- Add 50 μL/well of the diluted IDE substrate solution (refer to Protocol A, Step 1.1 for IDE Substrate Solution preparation).

Note: The IDE substrate solution is added to the 5-FAM 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 5-FAM fluorescence reference standard curve as RFU (relative fluorescent units) versus concentration as shown in Figure 2.
- The final concentrations of 5-FAM 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.

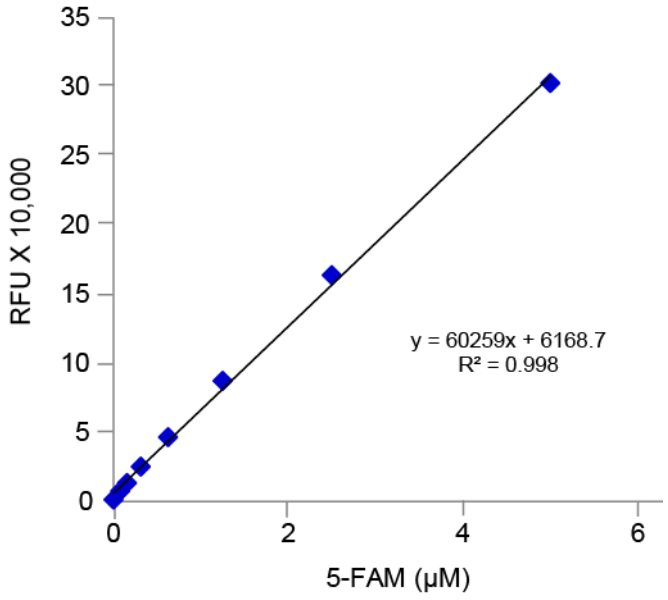

Figure 2. 5-FAM reference standard. 5-FAM was serially diluted in assay buffer, containing IDE substrate, and the fluorescence recorded at Ex/Em=490 nm/520 nm (Flexstation 384 II, Molecular Devices).

References

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