SensoLyte® 520 ECEs Activity Assay Kit
Fluorimetric

Revision number: 1.0 *Last updated: 08/24/15*

Catalog #	AS-72243
Kit Size	100 Assays (96-well plate)

- ***Optimized Performance:*** This kit is optimized to detect ECEs (ECE-1 and ECE-2) 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 ECEs substrate Ex/Em=490 nm/520 nm upon cleavage	0.5 mM, 50µL
Component B	5-FAM fluorescence reference standard, Ex/Em=490 nm/520 nm	0.5 mM, 12 µL
Component C	Human recombinant ECE-1	0.1 mg/mL, 10 µL
Component D	2X Assay Buffer	20 mL
Component E	Inhibitor	200 µM, 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

Endothelin converting enzymes (ECEs), similar to neprilysin (NEP), belong to the M13 family of zinc metallopeptidases.[1-3] ECEs (ECE-1/ECE-2) are known as the rate-limiting enzymes in the formation of the vasoconstrictor peptide endothelin-1(ET-1) from its precursor proendothelin-1.[4] ECE-1 can also degrade amyloid β fragments both *in vitro* and *in vivo*.[5] Highly homologous and structurally comparable to ECE-1, ECE-2 was shown in homozygous knockout mice to have decreased Aβ degradation and significant elevated Aβ 1-40 and Aβ 1-42 in the brain.[6] Some studies indicate that carriers of ECE1 and ECE-2 gene variants may have an increased risk of developing late-onset Alzheimer's disease (AD).[3,7-9] However, the roles of ECE-1 and ECE-2 in AD progression have yet to be fully elucidated.

The SensoLyte® 520 ECEs Activity Assay Kit can be used to detect enzyme activity in biological samples or in purified enzyme preparations. When active ECEs cleave the FRET substrate, it results in an increase of 5-FAM fluorescence, and is monitored at excitation/emission = 490 nm/520 nm. The long wavelength fluorescence of 5-FAM shows less interference from autofluorescence of components in biological samples and test compounds. This assay can detect as low as 15.6 ng/mL active ECE-1 and 62.5 ng/mL active ECE-2.

Protocol

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

Protocol A. Screening ECEs 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 1X Assay buffer: Add 5 ml of 2X assay buffer (Component C) to 5 mL deionized water

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

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

Components	Volume
ECEs substrate (100X, Component A)	50 µL
1X Assay buffer	4.95 mL
Total volume	5 mL

1.3 ECEs diluent:

Dilute ECE-1 enzyme (Component C) 400-fold in 1X 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.

1.4 Inhibitor (Phosphoramidon): Dilute the 200 µM inhibitor solution (Component E) 1:100 in 1X assay buffer. The diluted Phosphoramidon solution has a concentration of 2 µM. Add 10 µl of the diluted Phosphoramidon 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 ECEs enzyme and Phosphoramidon.
- Vehicle control contains ECEs 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 Use the 1X assay buffer to bring the total volume of all controls to 50 µL.

3. Run the enzymatic reaction.

3.1 Add 50 µL of ECEs 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 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.

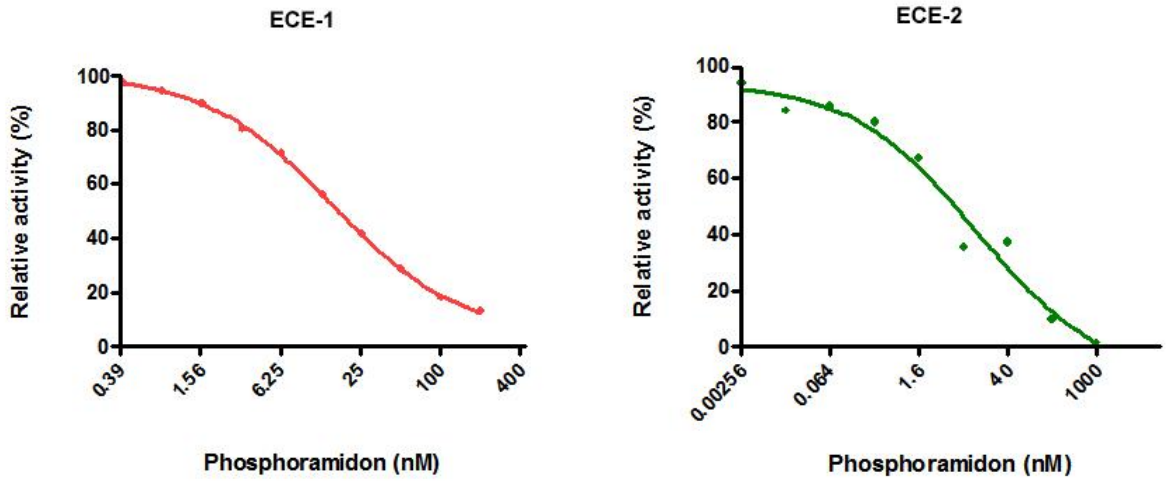

Figure 1. Inhibition of ECEs activity by Phosphoramidon as measured with SensoLyte® 520 ECEs Activity Assay Kit.

Protocol B. Measuring ECEs activity in biological samples.

1. Prepare ECEs containing biological samples.

1.1 Prepare sample from cell culture medium:

- Medium is collected from culture.
- Spin the medium sample for 10-15 min. at 1,000X g, 4°C.
- Collect the supernatant and store at -70°C until use.

1.2 Prepare cell lysates:

- Wash cell once with cold phosphate buffered saline (PBS) and collect cells by centrifugation at 1,000 X g for 5 min.
- Add an appropriate amount of "ice cold" 1X assay buffer to cell pellet.
- Incubate the cell suspension on ice for 30 min.
- Pipette the cell suspension up and down for 5 times.
- Centrifuge the cell suspension for 15 min. at 15,000 X g, 4°C. Collect the supernatant and store at -70°C until use.

1.3 Prepare cellular membrane fractions:

- Wash cells with PBS and resuspend them in ice cold PBS with protease inhibitors.
- Samples are homogenized in cold 1X assay buffer.
- Centrifuge homogenized cells 15 minutes at 20,000X g, 4°C.
- Wash pelleted membranes with PBS and resuspend them after centrifugation in cold 1X assay buffer. Store at -70°C until use.

Note 1: PBS is not provided.
Note 2: Users are advised to optimize the protocol according to their experimental requirements.
Note 3: Please perform the extraction procedure at 4°C.

2. Prepare working solutions.

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

2.1 Dilute ECEs substrate (Component A) 100-fold in 2X assay buffer. Prepare fresh substrate solution for each experiment.

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

Components	Volume
ECEs substrate (100X, Component A)	50 µL
2X Assay buffer	4.95 mL
Total volume	5 mL

2.2 ECEs diluent: If using purified ECEs enzyme as a positive control, dilute enzyme to the concentration of 0.2 ng/ µl with 1X 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 ECEs containing biological sample.

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

- Positive control contains purified ECEs enzyme.
- Substrate control contains deionized water.

3.3 Using the 1X assay buffer, bring the total volume of all controls to 50 μL.

4. Run the enzymatic reaction.

4.1 Add 50 μL of ECEs 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 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 0.5 mM 5-FAM reference standard (Component B) to 5 μM in 1X assay buffer. Do 2-fold serial dilutions to get concentrations of 2.5, 1.25, 0.625, 0.312, 0.156, 0.078 μM, and include 0 μM as an assay buffer blank. Add 50 μL/well of these serially diluted 5-FAMreference solutions.
- Add 50 μL/well of the diluted ECEs substrate solution (refer to Protocol A, step 1.1 for preparation).

  Note: The ECEs 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 2.5, 1.25, 0.625, 0.312, 0.156, 0.078, 0.039 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.

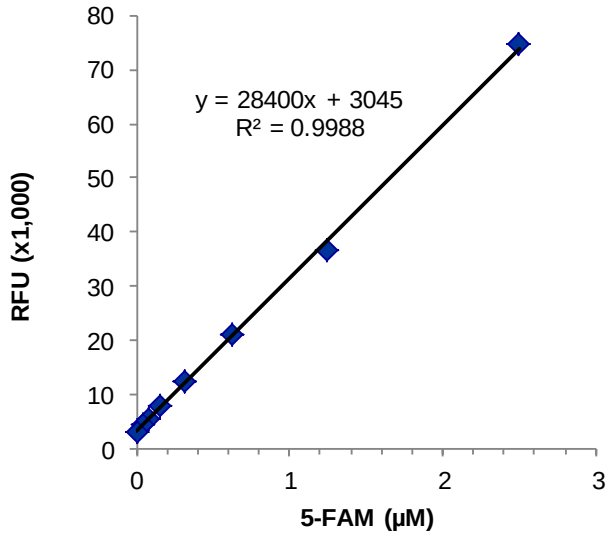

Figure 2. 5-FAM reference standard. 5-FAM was serially diluted in assay buffer, containing ECEs substrate, and the fluorescence recorded at Ex/Em=490 nm/520 nm (Flexstation 384 II, Molecular Devices).

References

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