SensoLyte® 390 ACE2 Activity Assay Kit
Fluorimetric

Revision Number: 1.1	*Last updated: October 2014*
Catalog #	**AS-72086**
Kit Size	100 Assays (96-well plate)

- ***Optimized Performance:*** This kit is optimized to detect ACE2 activity.
- ***Enhanced Value:*** It provides enough 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	Mca/Dnp, ACE2 substrate, Ex/Em=330 nm/390 nm upon cleavage	5 mM, 50 µL
Component B	Mca fluorescence reference standard, Ex/Em=330 nm /390 nm	1 mM, 10 µL
Component C	Assay Buffer	20 mL
Component D	Inhibitor of ACE2	100 µM, 10 µL
Component E	Stop Solution	10 mL

Other Materials Required (but not provided)

- ACE2 source: The active enzyme (Calbiochem, Cat# 176872), cell lysates, tissue extracts.
- 96-well microplate: Black, flat-bottom, non-binding 96-well plate.
- Fluorescence microplate reader: Capable of detecting emission at 390 nm with excitation at 330 nm.

Storage and Handling

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

Introduction

Angiotensin I converting enzyme 2 (ACE2), the newest member of the renin angiotensin system (RAS), is a zinc metallopeptidase that plays a central role in the control of angiotensin peptides.[1,2] ACE2 has direct effects on cardiac function,[3] and is expressed predominantly in vascular endothelial cells of the heart and the kidneys.[2] It has been reported that ACE2 might protect kidneys in early stages of diabetes.[4] In addition to its role in the regulation of hypertension, ACE2 is a functional receptor for coronavirus that causes severe acute respiratory syndrome (SARS).[5] ACE2 is considered an important therapeutic target for controlling cardiovascular diseases, kidney disease and severe acute respiratory syndrome (SARS) outbreaks.

The SensoLyte® 390 ACE2 Activity Assay Kit provides a convenient assay for high throughput screening of ACE2 inhibitors and inducers and for continuous assay of ACE2 activity using Mca/Dnp fluorescence resonance energy transfer (FRET) peptide. In the FRET peptide the fluorescence of Mca is quenched by Dnp. Upon cleavage into two separate fragments by the enzyme, the fluorescence of Mca is recovered, and can be monitored at excitation/emission = 330 nm /390 nm. The assay can detect the activity of subnanogram level of ACE2. Assays are performed in a convenient 96-well microplate format.

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 ACE2 inhibitors using purified enzyme.

1. Prepare working solutions.

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

1.1 ACE2 substrate solution: Dilute ACE2 substrate (Component A) 1:100 in assay buffer (Component C). For each experiment prepare fresh substrate solution.

Table 1. ACE2 substrate solution for one 96-well plate (100 assays)

Components	Volume
ACE2 substrate (100X, Component A)	10 µL
Assay buffer (Component C)	0.99 mL
Total volume	1 mL

1.2 ACE2 diluent: Dilute enzyme to an appropriate concentration in assay buffer (Component C).

1.3 ACE2 inhibitor (DX600): Dilute 100 µM inhibitor solution (Component D) to 1 µM in assay buffer (Component C). Add 10 µl of the 1 µM inhibitor solution into each of the inhibitor control well of a 96-well plate.

2. Set up the enzymatic reaction.

2.1 Add test compounds and diluted enzyme solution to the microplate wells. For one well of 96-well plate, the suggested volume of enzyme solution is 40 µL and 10 µL of test compound.

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

- Positive control contains ACE2 enzyme without test compound.
- Inhibitor control contains ACE2 enzyme and a known ACE2 inhibitor.

- Vehicle control contains ACE2 enzyme and vehicle used in delivering test compound (e.g. DMSO, concentration not to exceed 1%).
- Test compound control contains assay buffer (Component C) and test compound.
- Substrate control contains assay buffer (Component C).

2.3 Using the assay buffer (Component C), 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 ACE2 substrate solution into each well. For best accuracy, it is advisable to have the ACE2 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=330 nm/390 nm continuously and record data every 5 min. for 30 min.
- For end-point reading: Incubate the reaction for 30 min. Keep plate from direct light. Optional: Add 50 µL of stop solution (Component E) to each well. Mix the reagents and measure fluorescence intensity at Ex/Em=330 nm/390 nm.

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

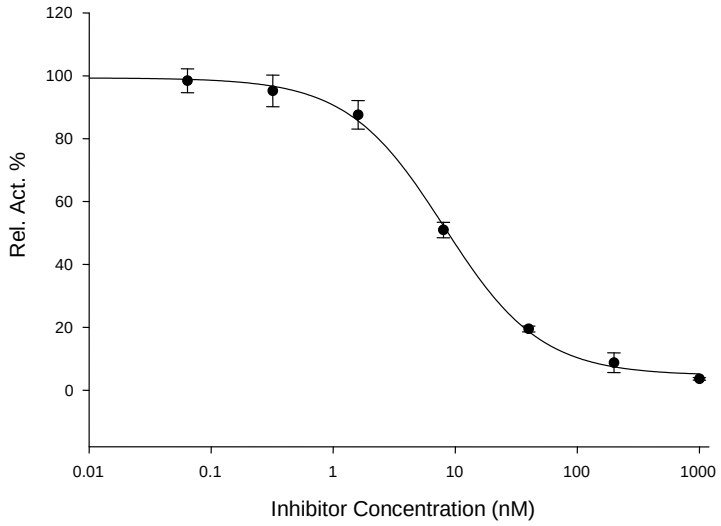

Figure 1. DX600 inhibition of ACE2 activity measured with SensoLyte® 390 ACE2 Activity Assay Kit.

Protocol B. Measuring ACE2 activity in biological samples.

1. Prepare ACE2 containing biological samples.

1.1 Prepare cell lysates:

- Wash cells with PBS.
- Add an appropriate amount of assay buffer (Component C) containing 0.1% (v/v) Triton-X 100 to cells or cell pellet. Collect the cell suspension in a microcentrifuge tube.
- Incubate the cell suspension at 4°C for 10 minutes.
- Centrifuge the cell suspension for 10 minutes at 20,000X g, 4°C. Collect the supernatant and store at -70°C until use.

1.2 Prepare tissue samples:

- Homogenize tissue samples in assay buffer (Component C).
- Incubate for 15 min. at 4°C.
- Centrifuge for 10 min. at 20,000xg at 4°C and collect the supernatant. Store at -70°C until use.

Note 1: Triton-X 100 and PBS are not provided.

2. Prepare working solutions.

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

2.1 ACE2 substrate solution: Dilute ACE2 substrate (Component A) 1:100 in assay buffer (Component C). For each experiment prepare fresh substrate solution.

Table 1. ACE2 substrate solution for one 96-well plate (100 assays)

Components	Volume
ACE2 substrate (100X, Component A)	50 µL
Assay buffer (Component C)	4.95 mL
Total volume	5 mL

2.2 ACE2 diluent: If purified ACE2 is used as a positive control, then dilute the enzyme to an appropriate concentration in assay buffer (Component C).

3. Set up enzymatic reaction.

3.1 Add 50 µL of ACE2 containing biological sample.

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

- Positive control contains purified active ACE2.
- Substrate control contains assay buffer.

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

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 ACE2 substrate solution into each well. For best accuracy, it is advisable to have the ACE2 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=330 nm/390 nm continuously and record data every 5 min. for 30 min.
- For end-point reading: Incubate the reaction for 30 min. Keep plate from direct light. Optional: Add 50 µL of stop solution (Component E) to each well. Mix the reagents and measure fluorescence intensity at Ex/Em=330 nm/390 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

- Mca fluorescence reference standard: Dilute 1 mM Mca (Component B) to 10 μM in assay buffer (Component C). Do 2-fold serial dilutions to get concentrations of 5, 2.5, 1.2, 0.6, 0.3, 0.15 μM, include an assay buffer blank. Add 50 μL/well of these serially diluted Mca reference solutions.
- Add 50 μL/well of the diluted ACE2 substrate solution (refer to Protocol A, step 1.1 for preparation).

  Note: The ACE2 substrate solution is added to the Mca reference standard to correct the absorptive quenching by the FRET peptide. If multiple concentrations of substrate are used, this step must be performed for each concentration.
- Plot the Mca fluorescent reference standard curve as RFU (relative fluorescent units) versus concentration as shown in Figure 2.
- The final concentrations of Mca reference standard are 5, 2.5, 1.25, 0.6, 0.3, 0.15, 0.075, 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.

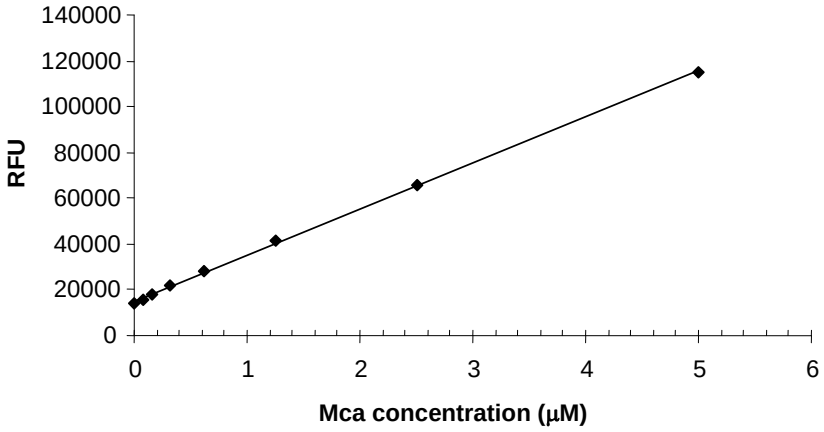

Figure 2. Mca reference standard, Mca was serially diluted in assay buffer containing substrate, and the fluorescence recorded at Ex/Em=330 nm/ 390 nm. (Flexstation 384II, Molecular Devices)

References

1. Katovich, MJ. et al. *Exp. Physiol.* **90**, 299 (2005).
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