SensoLyte® 390 Renin Assay Kit
Fluorimetric

Revision Number: 1.1	*Last updated: October 2014*
Catalog #	**AS-72039**
Kit Size	100 Assays (96-well)

- ***Optimized Performance:*** Optimal conditions for screening of renin inhibitors.
- ***Enhanced Value:*** Less expensive than the sum of individual components.
- ***High Speed:*** Minimal hands-on time.
- ***Assured Reliability:*** Detailed protocol and references are provided.

Kit Components, Storage and Handling

Component	Description	Quantity
Component A	Renin substrate Mc-Ala/Dnp FRET peptide Ex/Em=330 nm/390 nm upon cleavage	2 mM DMSO solution, 50 µL
Component B	Mc-Ala fluorescence reference standard Ex/Em=330 nm/390 nm	3 mM, 5 µL
Component C	Human recombinant renin	100 µL
Component D	Assay buffer	25 mL
Component E	Renin Inhibitor Ac-HPFV- (Sta)-LF-NH_2	1mM DMSO solution, 5 µL

Other Materials Required (but not provided)

- 96-well microplate: Black, flat-bottom 96-well plates with non-binding surface.
- Fluorescence microplate reader: Capable of detecting emission at 390 nm with excitation at 330 nm.

Storage and Handling

- Store all kit components, except Component C, at -20°C.
- Store Component C at -80°C. Aliquot as needed to avoid multiple freeze-thaw cycles.
- Keep Component A and B from light.
- If used frequently, Component D can be stored at room temperature for convenience.

Introduction

The renin–angiotensin system (RAS) plays a central role in the regulation of blood pressure and electrolyte homoeostasis.[1] At the first and rate-limiting step of the RAS cascade, renin (EC 3.4.23.15), a highly specific aspartyl protease, cleaves angiotensinogen, produced in the liver, to yield angiotensin I, which is further converted into angiotensin II by ACE (Angiotensin Converting Enzyme). Angiotensin II constricts blood vessels leading to increased blood pressure. It also increases the secretion of ADH and aldosterone, and stimulates the hypothalamus to activate the thirst reflex. Since an overactive renin-angiotensin system leads to hypertension, renin is an attractive target for the treatment of this disease. [2-4]

The SensoLyte® 390 Renin Assay Kit provides a convenient assay for high throughput screening of renin inhibitors and for continuous assay of renin activity using a Mc-Ala/Dnp fluorescence resonance energy transfer (FRET) peptide. The sequence of this FRET peptide is derived from the cleavage site of renin.[5] In the FRET peptide the fluorescence of Mc-Ala is quenched by Dnp. Upon cleavage into two separate fragments by renin, the fluorescence of Mc-Ala is recovered, and can be monitored at excitation/emission = 330/390 nm. Compared to an EDANS/DABCYL FRET substrate, the Mc-Ala/Dnp FRET substrate has higher sensitivity and can detect 8 ng/ml of renin.

The assays are performed in a convenient 96-well microplate format

Protocol

Note 1: For fluorometer calibration, please refer to Appendix II - recommended for first-time users.
Note 2: Warm all kit components until thawed to room temperature before starting the experiments.

Screening renin inhibitors using recombinant enzyme

1. Prepare working solutions.

1.1 Renin substrate solution: Dilute renin substrate (Component A) 100-fold in assay buffer (Component D). For each experiment prepare fresh substrate solution.

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

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

1.2 Renin diluents: Dilute renin (Component C) 100-fold in assay buffer (Component D). This amount of enzyme is sufficient 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 solutions. Prolonged storage or vigorous agitation of the diluted enzyme will cause denaturation. Store the enzyme solution on ice.

1.3 Renin inhibitor:[6] Dilute the 1 mM inhibitor solution (Component E) to 10 µM in assay buffer (Component D). Add 15 µL of the 10 µM inhibitor solution into each of the inhibitor control well (DMSO concentration should not exceed 1 %).

2. Set up enzymatic reaction.

2.1 Add test compounds and renin solution into the microplate wells. The suggested volume of renin solution for one well of a 96-well plate is 85 μL and test compound is 15 μL.

2.2 Set up the following controls at the same time as deemed necessary:

- Positive control contains diluted renin without test compound.
- Inhibitor control contains diluted renin and diluted renin inhibitor (provided as undiluted in the kit).
- Vehicle control contains diluted renin and diluted vehicle used to deliver test compound (e.g. DMSO).
- 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 100 μL.

3. Pre-incubation.

3.1 Incubate the plate at 37°C for 30 min. At the same time, also incubate the renin substrate solution at 37°C.

4. Initiate the enzymatic reaction.

4.1 Add 50 μL of renin substrate solution into each well. Mix the reagents completely by shaking the plate gently for no more than 30 seconds.

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 3 min for 15 min (37°C recommended).
- For end-point reading: Incubate the reaction at 37°C for 15 min. Keep plate from direct light. Then measure fluorescence intensity at Ex/Em=330 nm/390 nm.

4.3 Data analysis: Refer to Appendix I.

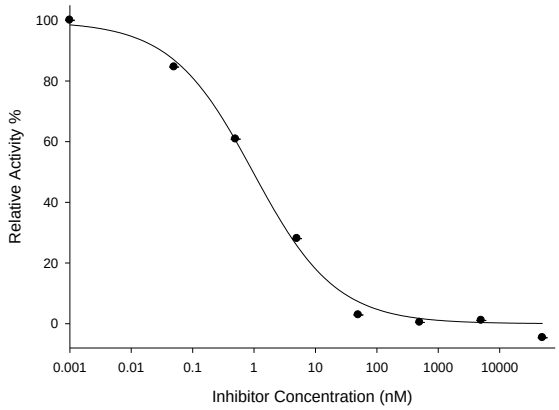

Figure 1. The inhibition of renin activity measured with SensoLyte® 390 Renin Assay Kit. The calculated IC_{50} for Ac-HPFV- (Sta)-LF-NH_2 is 0.97 nM.

Appendix I: Data Analysis

- The fluorescence reading from the substrate control well is the background fluorescence. This background reading should be subtracted from the readings of the other wells. The fluorescence readings are expressed in relative fluorescence units (RFU).
- For kinetic reading:
  - Plot data as RFU versus time for each sample. If you want to convert the RFU to the concentration of the product of enzymatic reaction, please refer to Appendix II for setting up a fluorescence reference standard.
  - Determine the range of initial time points during which the reaction is linear. Typically, 10-15% conversion will be the optimal range.
  - Obtain the initial reaction velocity (V_o) in RFU/min. Determine 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 reading:
  - 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

- Mc-Ala fluorescence reference standard: Dilute 3mM Mc-Ala (Component B) to 37.5 μM in assay buffer (Component D). Do 2-fold serial dilutions to get concentrations of 18.7, 9.4, 4.7, 2.3, 1.15, 0.58 μM, include an assay buffer blank. Add 100 μL/well of these serially diluted Mc-Ala reference solutions.
- Add 50 μL/well of the diluted renin substrate solution (refer to step 1.1 for preparation).

  Note: The renin substrate solution is added to the Mc-Ala 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 wells at Ex/Em=330 nm/390 nm. Use the same setting of sensitivity as used in the enzyme reaction.
- Plot the Mc-Ala fluorescent reference standard curve as RFU (relative fluorescent units) versus concentration as shown in **Figure 2**.

  Note: The final concentrations of Mc-Ala reference standard are 25, 12.5, 6.25, 3.125, 1.5, 0.78, 0.39, 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 renin enzymatic reaction.

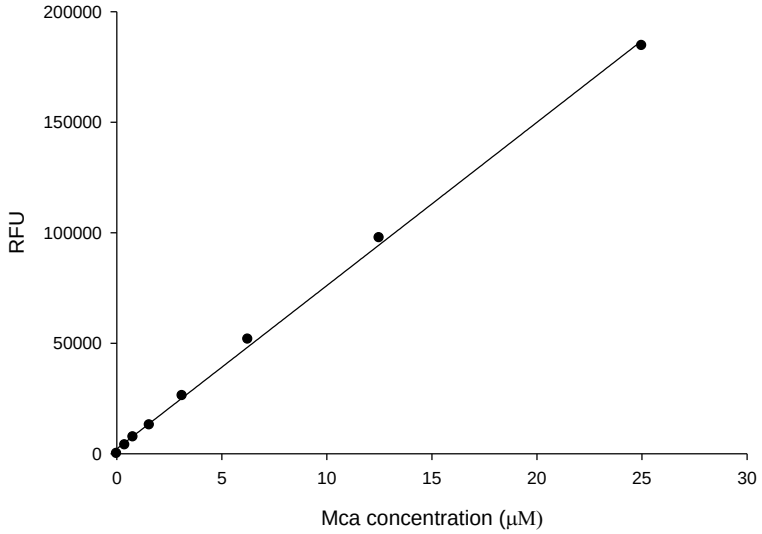

Figure 2. Mc-Ala reference standard. Mc-Ala was serially diluted in assay buffer containing substrate, and the fluorescence recorded at Ex/Em=330±20 nm/ 390±20 nm. (Flexstation 384II, Molecular Devices)

References:

1. He, F. et al. *J Renin Angiotensin Aldosterone Syst* **4**, 11 (2003).
2. Wood, JM. et al. *Hypertension*, **7**, 797 (1985).
3. Shibasaki, M. et al. *Am J Hypertens* **4**, 932 (1991).
4. Wood, JM.et al. *Biochem Biophys Res Comm* **308**, 698 (2003).
5. Paschalidou, K. et al. *Biochem J* **382**, 1031 (2004).
6. Hui, KY.et al. *J Med Chem* **31**, 1679 (1988).