SensoLyte® 520 Rat Renin Assay Kit

Fluorimetric

Catalog #	AS-72140
Kit Size	100 Assays (96-well)

- ***Optimized Performance:*** Optimal conditions for screening of renin inhibitors.
- ***Enhanced Value:*** It provides enough reagents to perform 100 assays in a 96-well format.
- ***High Speed:*** The entire process can be completed within 30-60 min.
- ***Assured Reliability:*** Detailed protocol and references are provided.

Kit Components, Storage and Handling

Component	Description	Quantity
Component A	Rat renin substrate 5-FAM/QXL™520 FRET peptide	1 mM, 25 µL
Component B	5-FAM, fluorescence reference standard	1 mM, 10 µL
Component C	Recombinant rat renin	40 µl
Component D	Assay buffer	25 mL
Component E	Renin Inhibitor Ac-HPFV- (Sta)-LF-NH_2	1 mM, 10 µL

Other Materials Required (but not provided)

- 96-well microplate: Black microplate provides better signal to noise ratio.
- 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

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, 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® 520 Rat Renin Assay Kit provides a convenient assay for high throughput screening of renin inhibitors and for continuous assay of rat renin activity using a 5-FAM/QXL™ 520 fluorescence resonance energy transfer (FRET) peptide. In the FRET peptide, the fluorescence of 5-FAM is quenched by QXL™ 520. Upon cleavage into two separate fragments by rat renin, the fluorescence of 5-FAM is recovered, and can be monitored at excitation/emission = 490/520 nm. With a high fluorescence quantum yield and long emission wavelength, the signal of 5-FAM can be detected with less interference from the autofluorescence of cell components and test compounds. The assays are performed in a convenient 96-well microplate format and can be adjusted to 384-well microplate format. The assay can detect as low as 0.4 μg/mL active rat renin.

Protocol

Note: For standard curve, please refer to Appendix II (optional).

1. Prepare working solutions.

Note: Warm all kit components to room temperature before starting the experiment.

1.1 Renin substrate solution: Dilute rat renin substrate (Component A) 1:200 in assay buffer (Component D). For each experiment, prepare fresh substrate solution.

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

Components	Volume
Renin substrate (200X, Component A)	25 μL
Assay buffer (Component D)	4.975 mL
Total volume	5 mL

1.2 Rat renin diluent: Dilute renin (Component C) 1:100 in assay buffer (Component D). 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 solutions. Prolonged storage or vigorous agitation of the diluted enzyme will cause denaturation. Store the enzyme solution on ice.

1.3 Renin inhibitor: [5] Dilute the 1 mM inhibitor solution (Component E) to 10 μM (1:100) in assay buffer (Component D). Add 10 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 is 40 µL and test compound is 10 µL.

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

- Positive control contains the enzyme without test compound.
- Inhibitor control contains rat renin enzyme and renin inhibitor.
- Vehicle control contains enzyme and vehicle used to deliver test compound (e.g. DMSO, concentration not to exceed 1%).
- Test compound control contains assay buffer (Component D) and test compound. Some test compounds have strong auto-fluorescence and may give false results.
- Substrate control contains assay buffer (Component D).

Note: Use the assay buffer (Component D) to bring the total volume of all controls to 50 µL.

2.3 Incubate the plate at 37°C for 10 min. At the same time, also incubate the renin substrate solution at 37°C.

3. Initiate the enzymatic reaction.

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

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 min to 60 min (37°C recommended).
- For end-point reading: Incubate the reaction at 37°C for 30 min to 60 min. Keep plate from direct light. Then measure fluorescence intensity at Ex/Em=490 nm/520 nm.

3.3 Data analysis: Refer to Appendix I

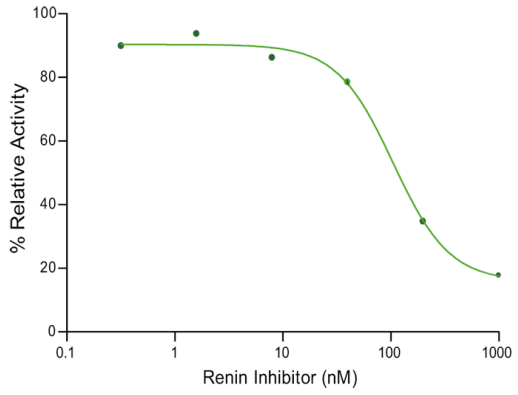

Figure 1. The inhibitory curve of renin inhibitor, Ac-HPFV- (Sta)-LF-NH_2

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 RFU to the concentration of the product of enzymatic reaction, 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

- 5-FAM fluorescence reference standard: Dilute 1 mM 5-FAM (Component B) to 5 µM (1:200) in assay buffer (Component D). Do 2-fold serial dilutions to get concentrations of 2.5, 1.25, 0.625, 0.3125, 0.156, and 0.078, include an assay buffer blank. Add 50 µL/well of these serially diluted 5-FAM reference solutions.
- Add 50 µL/well of the diluted rat renin substrate solution (refer to 1.1).

  Note: The renin 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.
- Plot the 5-FAM fluorescent reference standard curve as RFU (relative fluorescent units) versus concentration as shown in Figure 2.

  Note: The final concentrations of 5-FAM reference standard are 2.5, 1.25, 0.625, 0.3125, 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 renin enzymatic reaction.

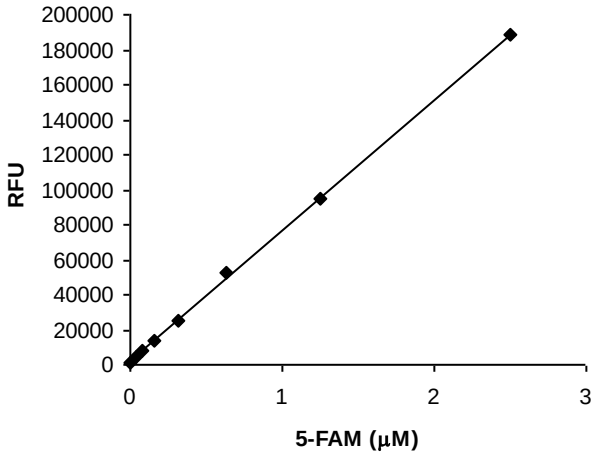

Figure 2. 5-FAM reference standard.
5-FAM was serially diluted in assay buffer containing substrate, and the fluorescence was recorded at Ex/Em=490/520 nm. (Flexstation 384II, Molecular Devices).

References

1. He, FJ. and GA. MacGregor, *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. Hui, KY.et al., *J. Med. Chem.* **31**, 1679 (1988).