SensoLyte® 520 Mouse Renin Assay Kit
Fluorimetric

Revision Number: 1.1	*Last updated: October 2014*
Catalog #	**AS-72161**
Kit Size	100 Assays (96-well)

- ***Optimized Performance:*** Optimal conditions for screening of renin inhibitors.
- ***Enhanced Value:*** Ample reagents to perform 100 assays in a 96-well format.
- ***Assured Reliability:*** Detailed protocol and references are provided.

Kit Components, Storage and Handling

Component	Description	Quantity
Component A	Mouse renin substrate 5-FAM/QXL™520 FRET peptide	50 µL
Component B	5-FAM, fluorescence reference standard	100 µM, 10 µL
Component C	Mouse Prorenin	0.5 mg/mL, 20 µL
Component D	Assay buffer	25 mL
Component E	Trypsin Activation buffer	300 µL
Component F	Trypsin Inhibitor	10 mM, 20 µL
Component G	Renin Inhibitor Ac-HPFV- (Sta)-LF-NH_2	1 mM, 10 µL

Other Materials Required (but not provided)

- Microplate: Black, flat-bottom, non-binding 96-well plate.
- 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, B, F, and G 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 Mouse Renin Assay Kit provides a convenient assay for high throughput screening of renin inhibitors and for continuous assay of mouse 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 mouse 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 easily adapted to a 384-well microplate format.

Protocol

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

1. Activate Prorenin

1.1 Dilute 0.5 mg/mL of mouse Prorenin (Component C) 5-fold to 0.1 mg/mL in Trypsin Activation buffer (Component E). Incubate at 37°C for 1 hr.

1.2 Dilute Trypsin inhibitor (Component F) 5-fold in assay buffer (Component D) to get a concentration of 2 mM. Add diluted trypsin inhibitor to renin enzyme in 1:1 volume ratio. Incubate an additional 30 min. at room temperature.

Note: Activate mouse prorenin immediately before the experiment.

2. Prepare working solutions

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

2.1 Mouse substrate solution: Dilute mouse renin substrate (Component A) 100-fold in assay buffer (Component D). Dilute only the amount needed for each experiment. Substrate solution must be prepared fresh.

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

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

2.2 Mouse renin diluent: Dilute activated renin (from step 1.2) 30-fold 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.

2.3 Renin inhibitor:[5] Dilute the 1 mM inhibitor solution (Component G) 100-fold to 10 µM in assay buffer (Component D). Add 10 µl of the inhibitor solution into each of the inhibitor control well (DMSO concentration should not exceed 1%).

3. Set up enzymatic reaction

3.1 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 40 µL and test compound is 10 µL.

3.2 Simultaneously set up the following controls as deemed necessary:

- Positive control contains the enzyme without test compound.
- Inhibitor control contains mouse renin enzyme and renin inhibitor.
- Vehicle control contains enzyme and vehicle used in delivering test compound.
- 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.

3.3 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 mouse 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=490 nm/520 nm continuously and record data every 5 min for 30 to 60 min (37°C recommended).
- For end-point reading: Incubate the reaction at 37°C for 30 to 60 min. Keep plate from direct light. Then measure fluorescence intensity at Ex/Em=490 nm/520 nm.

4.3 Data analysis: Refer to Appendix I.

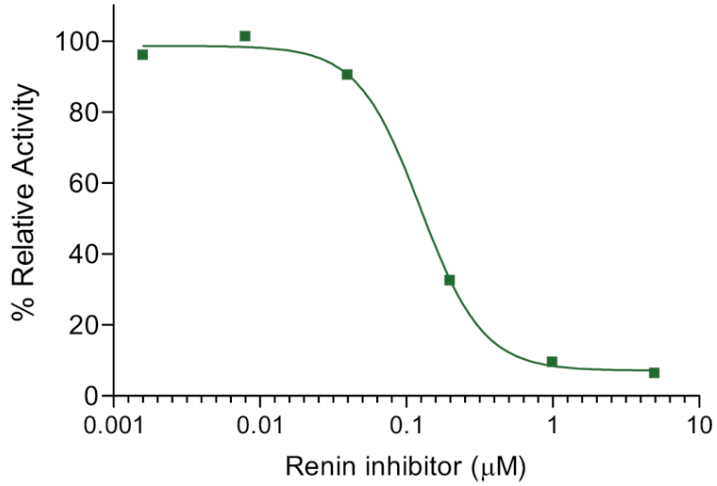

Figure 1. Inhibitory curve of renin inhibitor, Ac-HPFV- (Sta)-LF-NH_2

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. Fluorescence readings are expressed in relative fluorescence units (RFU).
- For kinetic reading:
  - Plot data as RFU versus time for each sample. To convert RFU to the concentration of the product of enzymatic reaction, refer to Appendix II for instructions on 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 100 μM 5-FAM (Component B) 100-fold to 1 μM in assay buffer (Component D). Prepare 2-fold serial dilutions to get concentrations of 500, 250, 125, 62.5, 31.25, and 15.6 nM, include an assay buffer blank. Add 50 μL/well of these serially diluted 5-FAM reference solutions.

- Add 50 µL/well of the diluted mouse renin substrate solution (refer to step 2.1).

  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.

- Measure the fluorescence intensity of the reference standard wells at Ex/Em=490 nm/520 nm. Use the same setting of sensitivity as used in the enzyme reaction.
- 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 500, 250, 125, 62.5, 31.25, 15.6, 7.8 and 0 n M. 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.

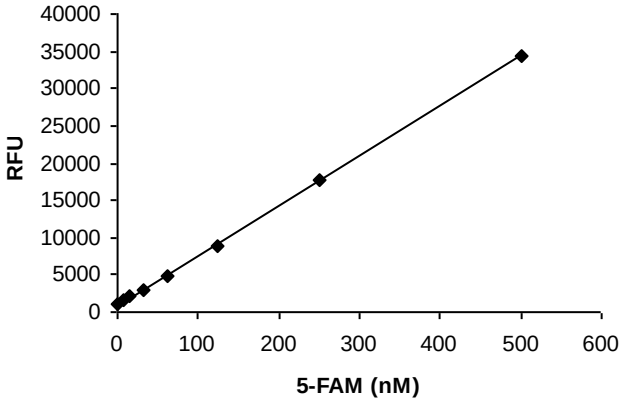

Figure 2. 5-FAM reference standard. 5-FAM was serially diluted in assay buffer containing substrate, and fluorescence was recorded at Ex/Em=490/520 nm. (Flexstation 384II, Molecular Devices).

References:

1. He, FJ. and GA. MacGregor, *J. Renin Angiotensin Aldosterone Syst.* **4**, 11 (2003).
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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).