SensoLyte® Rh110 Plasma Kallikrein Activity Assay Kit *Fluorimetric*

Revision number: 3.0 *Last updated: 05 /09/18*

Catalog #	AS-72255
Kit Size	100 Assays (96-well plate)

- ***Optimized Performance:*** This kit is optimized to detect plasma kallikrein 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	Plasma kallikrein substrate Ex/Em=490 nm/520 nm upon cleavage	100 µL
Component B	Rh110 fluorescence reference standard, Ex/Em=490 nm/520 nm	40 µL
Component C	Human Plasma Kallikrein	0.1 mg/mL, 10 µL
Component D	2X Assay Buffer	12 mL
Component E	Plasma Kallikrein Inhibitor	10 µL
Component F	Plasma Prekallikrein Activator	500 µL

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.

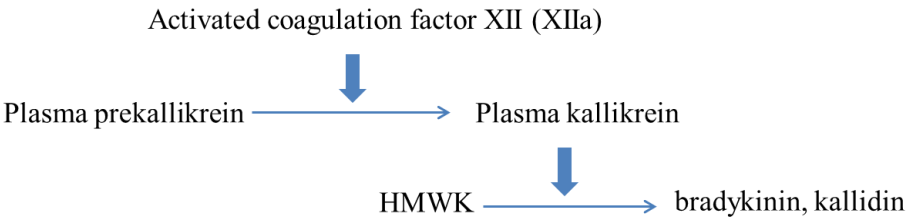
Storage and Handling

- 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 and F can be stored at room temperature for convenience.

Introduction

Human plasma kallikrein, a serine protease, is first synthesized as an inactive plasma prekallikrein in liver and circulates in the plasma. Once activated by activated coagulation factor XII (FXIIa), plasma kallikrein releases bradykinin (an inflammatory mediator) from high molecular weight kininogen (HMWK) (Figure 1). Plasma kallikrein is involved in several physiological and pathological processes such as blood coagulation, the classical complement cascade pathway and the activation of the alternative complement pathway.[1, 2-5] Plasma kallikrein also plays a role in the induction of elastase release from neutrophils and conversion of prourokinase to urokinase in fibrinolysis. [6-9]

The SensoLyte®Rh110 Plasma Kallikrein Activity Assay Kit employs a fluorescence peptide substrate for the detection of enzyme activity. This substrate contains rhodamine 110 fluorophore (Rh110). Plasma kallikrein cleaves this Rh110 substrate and results in the release of a bright green fluorescence and can be detected at excitation/emission=496 nm/520 nm. The longer wavelength spectra and higher extinction coefficient of Rh110 provide greater sensitivity and less interference from other reaction components. This assay can detect as low as 1 ng/mL active plasma kallikrein. This kit can be used to detect enzyme activity in purified enzyme preparations, biological samples and can also be applied for compound screening.



Scheme 1. Plasma kallikrein activation cascade

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 Plasma Kallikrein 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 10 ml of 2X Assay Buffer (Component D) to 10 mL deionized water. Prepare fresh 1X assay buffer for each experiment.

1.2 Plasma Kallikrein substrate solution: Dilute plasma kallikrein substrate (Component A) 50-fold in 1X assay buffer. Refer to Table 1.

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

Components	Volume
Plasma Kallikrein substrate (50X, Component A)	100 µL
1X assay buffer	4.90 mL
Total volume	5 mL

1.3 Plasma Kallikrein diluent:
Dilute Plasma Kallikrein enzyme (Component C) 400-fold in 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 (PKSI-527): Dilute the 5 mM inhibitor solution (Component E) 1:100 in 1X assay buffer. The diluted inhibitor solution has a concentration of 50 µM. Add 10 µl of the diluted PKSI-527 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 Plasma Kallikrein enzyme and PKSI-527.
- Vehicle control contains Plasma Kallikrein 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 Plasma Kallikrein 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 room temperature 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.

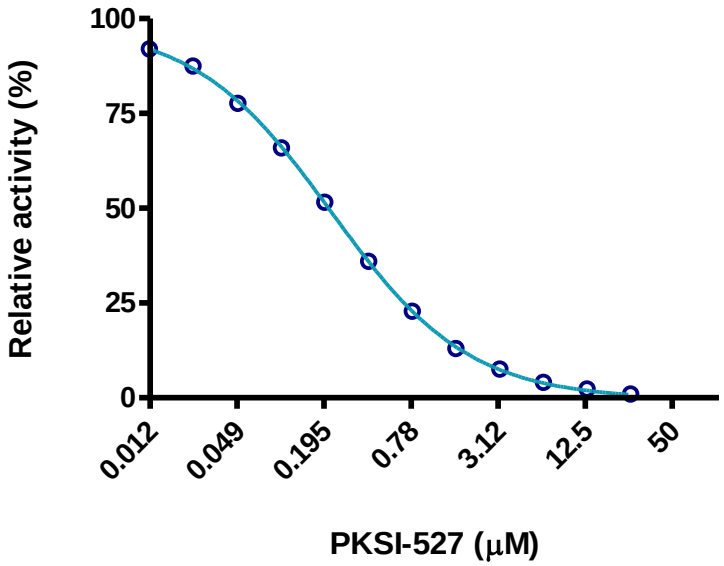


Figure 1. Inhibition of Kallikrein activity by PKSI-527 measured with SensoLyte® Rh110 Plasma Kallikrein Activity Assay Kit. Serial dilutions of PKSI-5127 were added to 10 ng purified plasma kallikrein. Fluorescence signal was detected at Ex/Em=490/520 nm (SpectraMax M5^e).

Protocol B. Measuring Plasma Kallikrein activity in biological samples.

1. Prepare Plasma Kallikrein containing biological samples.

1.1 Prepare plasma sample:

- Plasma sample is further diluted by 1X Assay Buffer for activity detection (See example in Figure 3). Recommended dilution range: 2-fold to 10-fold (optimization may be needed due to batch-to-batch variation of plasma sample).
- Prekallikrein is also present in the plasma sample. The user could proceed to the prekallikrein activation for evaluating both kallikrein and prekallikrein activities in the plasma sample. The procedure is listed below:

Prekallikrein activation

- Add equal amount of Plasma Prekallikein Activator (Component F) to the tube containing plasma
- Mix sample well and incubate the sample at 37°C for 15 minutes
- Activated plasma sample then could be further diluted for assay detection (See example in Figure 3). Recommended dilution range: 2-fold to 5-fold (optimization may be needed due to batch-to-batch variation of plasma sample)

Note 1: Chloroform treatment (optional): Add equal amount of cold chloroform to the tube containing plasma sample. Invert the tube for 1 minute and spin the sample for 5 min. at 16,000X g, 4°C. Carefully collect the treated plasma from the top layer.

Note 2: Plasma Prekallikrein Activator (Component F: dextran sulfate) is used for activating coagulation factor XII (XIIa) which further converts prekallikrein to kallikrein in the plasma. Therefore, only use the plasma prekallikrein activator for biological sample preparation.

Note 3: The substrate in this kit can also be cleaved by tissue kallikreins: KLK-4, 5, 6, 8, 12, 13, and -14. If several kallikreins are coexisting in your samples and you want to specifically measure plasma kallikrein activity, then plasma

kallikrein must first be isolated by immuno-affinity purification or other methods before measuring its specific activity using current assay kit.

2. Prepare working solutions.

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

2.1 Dilute Plasma Kallikrein substrate (Component A) 50-fold in 2X Assay Buffer. Refer to Table 2.

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

Components	Volume
Plasma Kallikrein substrate (50X, Component A)	100 µL
2X Assay Buffer	4.90 mL
Total volume	5 mL

2.2 Plasma Kallikrein diluent:

If using purified Plasma Kallikrein enzyme as a positive control, dilute enzyme 500-fold with 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 the enzyme denaturation. Store the enzyme solution on ice.

3. Set up enzymatic reaction.

3.1 Add 50 µL of Plasma Kallikrein containing biological sample.

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

- Positive control contains purified Plasma Kallikrein enzyme.
- Substrate control contains 1X assay buffer.

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 Plasma Kallikrein 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 room temperature 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.

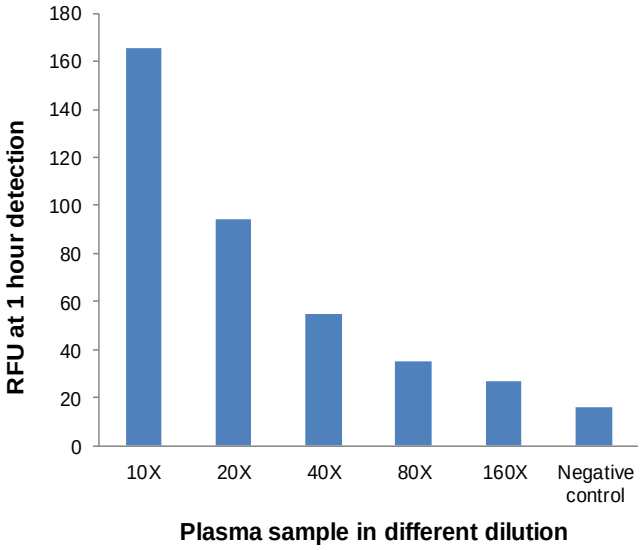


Figure 2. Kallikrein activity present in plasma samples was detected by SensoLyte® Rh110 Plasma Kallikrein Activity Assay Kit. Plasma samples were diluted by 1X Assay Buffer. The fluorescence signal was measured after 1 hour incubation of samples with substrate.

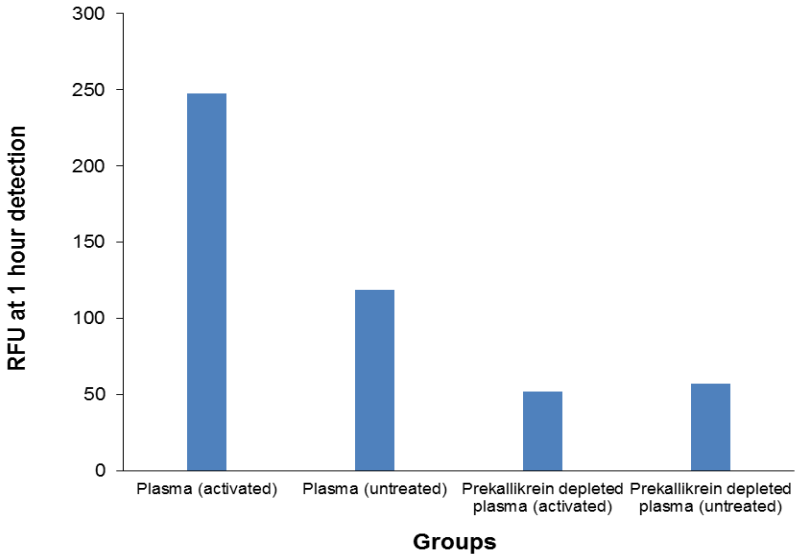


Figure 3. The specificity of SensoLyte® Rh110 Plasma Kallikrein Acitivty Assay Kit was tested with prekallikrein depleted human plasma. Plasma were treated with equal volume of plasma prekallikrein activator for 15 minutes at 37°C. Lack of prekallikrein in the depleted plasma resulted in no conversion of prekallikrein to kallikrein.

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

- Rh110 fluorescence reference standard: Dilute 2 mM Rh110 reference standard (Component B) to 40 μM in 1X assay buffer. Do 2-fold serial dilutions to get concentrations of 20, 10, 5, 2.5, 1.25, 0.62, 0.31 μM, and include 0 μM as an assay buffer blank. Add 50 μL/well of these serially diluted Rh110 reference solutions.
- Add 50 μL/well of the diluted Plasma Kallikrein substrate solution (refer to Protocol A, step 1.1 for preparation).

  Note: The Plasma Kallikrein substrate solution is added to the reference standard to normalize for the intrinsic substrate fluorescence. If multiple concentrations of substrate are used, this step must be performed for each concentration.
- Measure the fluorescence 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 Rh110 reference standard curve as RFU (relative fluorescent units) versus concentration.
- The final concentrations of Rh110 reference standard are 20, 10, 5, 2.5, 1.25, 0.62, 0.31, and 0 μM. This reference standard is used to calibrate the variation of different instruments and different experiments. Since the proteolytic cleavage of the Rh110 substrate consists of two steps, with both the intermediate and final products having fluorescence, the Rh110 reference standard cannot serve as an indicator of the amount of plasma kallikrein enzymatic reaction final product.

References

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