



SensoLyte[®] Rh110 Plasmin Activity Assay Kit *Fluorimetric*

Revision Number: 1.1	Last updated: October 2014
Catalog #	AS-72125
Kit Size	100 Assays (96-well plate)

- **Optimized Performance:** This kit is optimized to detect plasmin activity.
- **Enhanced Value:** It provides 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	Plasmin substrate, Ex/Em=490 nm/520 nm upon cleavage	5 mM, 50 μ L
Component B	Rh110, Fluorescence reference standard, Ex/Em=490 nm/520 nm	5 mM, 10 μ L
Component C	Human plasmin	250 μ g/mL, 10 μ L
Component D	2X Assay Buffer	10 mL
Component E	Plasmin Inhibitor	1 mM, 10 μ L
Component F	Stop Solution	5 mL

Other Materials Required (but not provided)

- 96-well 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 and B from light and moisture.
- Components D and F can be stored at room temperature for convenience.

Introduction

Plasmin is a serine protease derived from the conversion of plasminogen in blood plasma by plasminogen activators.¹ Its primary function is to degrade fibrin in blood clots.¹ Plasmin activators and inhibitors are thought to be the key participants in the balance between proteolytic and antiproteolytic activities that regulate extracellular matrix degradation.² In addition to its established role in fibrinolysis, plasmin is also involved in several pathological and physiological processes such as inflammation, neoplasia, metastasis, wound healing, angiogenesis, embryogenesis, and ovulation.²

The SensoLyte[®] Rh110 Plasmin Assay Kit provides a convenient assay for screening of enzyme inhibitors and activators or for continuous assay of enzyme activity using a fluorogenic substrate. Upon plasmin protease cleavage, this substrate generates the Rh110 (rhodamine 110) fluorophore which has a bright green fluorescence and can be detected at excitation/emission=496 nm/520 nm. The longer-wavelength spectra and higher extinction coefficient of the Rh110 provide greater sensitivity and less interference from other reaction components.

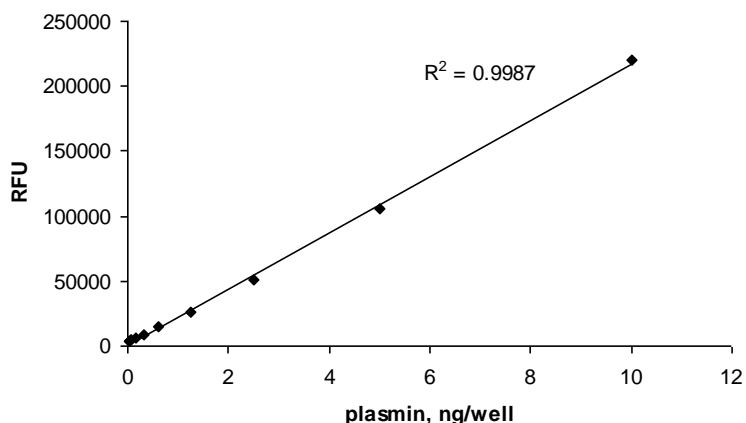


Figure 1. Detection of plasmin with the SensoLyte[®] Rh110 Plasmin Activity Assay Kit. The detection limit can reach as low as 0.02 ng.

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 compounds using purified enzyme.

1. Prepare working solutions.

Note: Warm 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 of deionized water.

1.2 Plasmin substrate solution: Dilute plasmin substrate (Component A) 1:100 in 1X assay buffer according to Table 1. For each experiment, prepare fresh substrate solution.

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

Components	Volume
Plasmin substrate (Component A)	50 μ L
1X assay buffer	4.95 mL
Total volume	5 mL

1.3 Plasmin diluents: Dilute the enzyme (Component C) 1:1000 in 1X assay buffer. 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 solution. Prolonged storage or vigorous agitation of the diluted enzyme will cause denaturation. Store the enzyme solution on ice.

1.4 Plasmin inhibitor: Dilute the 1 mM inhibitor solution (Component E) 1:100 in 1X assay buffer to get a concentration of 10 μ M. Add 10 μ l of the diluted compound 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 one well of a 96-well plate is 40 μ L and test compound is 10 μ L.

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

- Positive control contains the diluted plasmin without test compound.
- Inhibitor control contains the diluted plasmin and inhibitor.
- Vehicle control contains 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 Using the assay buffer, bring the total volume of all controls to 50 μ L.

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 μ L of plasmin 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 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 for 30 to 60 min. Keep plate from direct light. Optional: Add 50 μ L of stop solution (Component F) to each well. Mix the reagents and measure fluorescence intensity at Ex/Em=490 nm/520 nm.

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

Protocol B. Measuring plasmin activity in biological samples.

1. Prepare working solutions.

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

- 1.1** Plasmin substrate solution: Dilute plasmin substrate (Component A) in 2X assay buffer (Component D) according to Table 1.

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

Components	Volume
Plasmin substrate (Component A)	50 μ L
2X assay buffer (Component D)	4.95 mL
Total volume	5 mL

2. Set up the enzymatic reaction.

- 2.1** Add 50 μ L of plasmin containing sample.
- 2.2** Set up the following control wells at the same time, as deemed necessary:
- Positive control contains plasmin positive sample or purified active plasmin.
 - Substrate control contains deionized water.
- 2.3** Bring the total volume of all controls to 50 μ L.
- 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 μ L of plasmin 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.
- 3.4** Measure fluorescence signal:
- For kinetic reading: Immediately start measuring fluorescence 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 for 30 to 60 min. Keep plate from direct light. Optional: Add 50 μ L of stop solution (Component F) to each well. Mix the reagents and measure fluorescence intensity at Ex/Em=490 nm/520 nm.
- 3.5** 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

- Rh110 fluorescence reference standard: Dilute 5 mM Rh110 (Component B) to 50 μ M in 1X assay buffer. Do 2-fold serial dilutions to get concentrations of 25, 12.5, 6.25, 3.1, 1.5, and 0.75 μ M, include an assay buffer blank. Add 50 μ L/well of these serially diluted Rh110 reference solutions.
- Add 50 μ L/well of the diluted plasmin substrate solution (refer to Protocol A for preparation).

Note: The plasmin 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 $Ex/Em=490\text{ nm}/520\text{ nm}$. 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 25, 12.5, 6.25, 3.1, 1.5, 0.75, 0.375, and 0 μ M. 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 plasmin enzymatic reaction final product.

References

1. Collen, D. *Circulation*, 93, 857 (1996).
2. Vassalli, J.D. et al, *J. Clin. Invest.* 88, 1067 (1991).