SensoLyte® AFC Plasmin Activity Assay Kit *Fluorimetric*

Revision Number: 1.1	*Last updated: October 2014*
Catalog #	**AS-72124**
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=380 nm/500 nm upon cleavage	3 mM, 50 µL
Component B	AFC, fluorescence reference standard, Ex/Em=380 nm/500 nm	3 mM, 10 µL
Component C	Human plasmin	250 µg/mL, 10 µL
Component D	2X Assay Buffer	10 mL
Component E	Plasmin Inhibitor	1 mM, 10 µL
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 500 nm with excitation at 380 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® AFC 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. This substrate releases the AFC (7-amido-4-trifluoromethylcoumarin) fluorophore upon plasmin cleavage. AFC emits a yellow-green fluorescence, which can be quantified at excitation/emission=380 nm/500 nm.

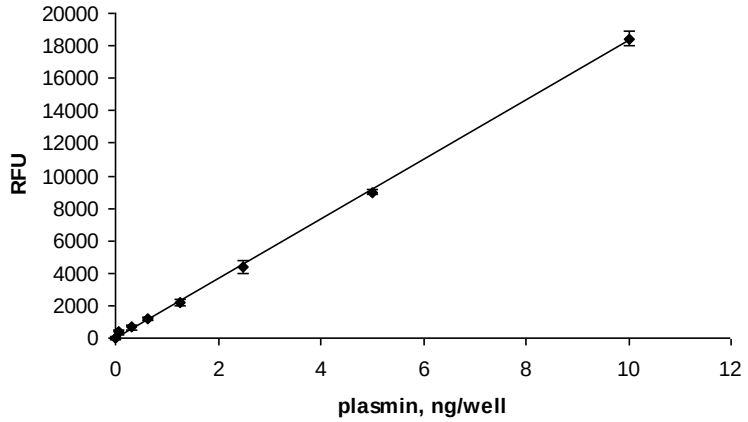

Figure 1. Detection of plasmin with the SensoLyte® AFC Plasmin Activity Assay Kit. The detection limit can reach as low as 0.08 ng.

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 µL
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 solutions. 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 μM. Add 10 μl 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 μL and test compound is 10 μL.

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 μL.

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 μL 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=380 nm/500 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 μL of stop solution (Component F) to each well. Mix the reagents and measure fluorescence intensity at Ex/Em=380 nm/500 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 µL
2X assay buffer (Component D)	4.95 mL
Total volume	5 mL

2. Set up the enzymatic reaction.

2.1 Add 50 µL 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 µL.

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 µL 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=380 nm/500 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 µL of stop solution (Component F) to each well. Mix the reagents and measure fluorescence intensity at Ex/Em=380 nm/500 nm.

3.3 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

- AFC fluorescence reference standard: Dilute 3 mM AFC (Component B) to 30 μM in 1X assay buffer. Do 2-fold serial dilutions to get concentrations of 15, 7.5, 3.75, 1.9, 0.95, and 0.47 μM, include an assay buffer blank. Add 50 μL/well of these serially diluted AFC reference solutions.
- Add 50 μL/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=380 nm/500 nm. Use the same setting of sensitivity as used in the enzyme reaction.
- Plot the AFC reference standard curve as RFU (relative fluorescent units) versus concentration as shown in Figure 2.
- The final concentrations of AFC reference standard are 15, 7.5, 3.75, 1.9, 0.95, 0.47, 0.23 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 enzymatic reaction.

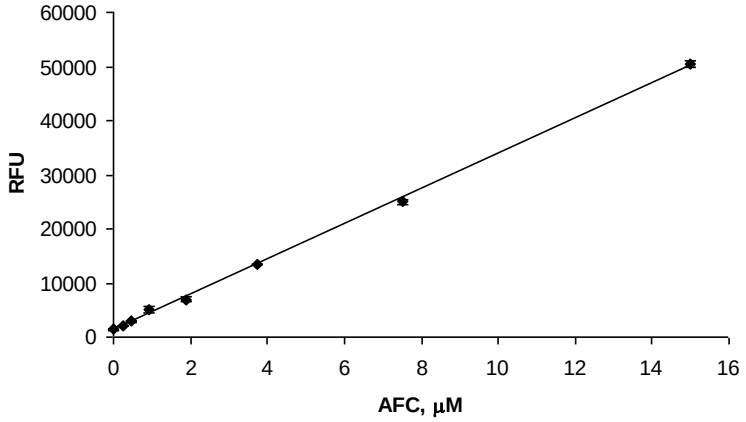

Figure 2. AFC reference standard. AFC was serially diluted in 1X assay buffer, containing plasmin substrate, and the fluorescence was recorded at Ex/Em=380 nm/500 nm. (Flexstation 384II, Molecular Devices)

References

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