SensoLyte® AFC Thrombin Activity Assay Kit **Fluorimetric**

Revision Number: 1.1	*Last updated: October 2014*

Catalog #	**AS-72130**
Kit Size	100 Assays (96-well plate)

- ***Optimized Performance:*** This kit is optimized to detect thrombin activity
- ***Enhanced Value:*** It provides enough 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	AFC Thrombin substrate, Ex/Em=380 nm/500 nm	5 mM, 50 µL
Component B	AFC, Fluorescence reference standard, Ex/Em=380 nm/500 nm	5 mM, 10 µL
Component C	2X Assay buffer	20 mL
Component D	Purified human thrombin enzyme	0.01 mg/mL, 40 µL
Component E	Thrombin inhibitor	10 mM, 10 µL
Component F	Stop solution	5 mL

Other Materials Required (but not provided)

- 96-well microplate: Black microplates provide better signal to noise ratio.
- Fluorescence microplate reader: Capable of detection at Ex/Em= 380 nm/500 nm.

Storage and Handling

- Store all kit components, except Component D, at -20°C.
- Store Component D at -80°C. Aliquot as needed to avoid multiple freeze-thaw cycles.
- Protect Components A and B from light and moisture.
- Components C and F can be stored at room temperature for convenience.

Introduction

Thrombin is a serine protease functioning as a main executioner of the coagulation cascade. It is the last enzyme in the clotting cascade, converting soluble fibrinogen into an insoluble fibrin clot. Thrombin also catalyzes other coagulation-related reactions, and promotes platelet aggregation.[1] In addition to these procoagulant effects, thrombin also influences a number of normal and pathological processes, including inflammation,[2] tissue repair,[3] embryogenesis,[4] angiogenesis,[5] and tumor invasion.[6]

The SensoLyte® AFC Thrombin Assay Kit is optimized for screening of enzyme inhibitors. This kit contains a fluorogenic substrate with a high reactivity and low background. Thrombin cleaves the substrate resulting in release of AFC (7-amido-4-trifluoromethylcoumarin) fluorophore. Fluorescence can be monitored at excitation /emission= 380 nm/500 nm.

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 5 ml of 2X assay buffer (Component C) to 5 mL deionized water.

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

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

Components	Volume
Thrombin substrate (Component A)	50 µL
1X Assay buffer	4.95 mL
Total volume	5 mL

1.3 Thrombin diluent: Dilute thrombin enzyme (Component D) 100-fold 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.

Table 2. Thrombin solution for one 96-well plate (100 assays)

Components	Volume
Thrombin (Component D)	40 µL
1X Assay buffer	3.96 mL
Total volume	4 mL

1.4 Thrombin inhibitor (Argatroban): Dilute the 10 mM inhibitor solution (Component E) 100-fold to100 µM in 1X assay buffer. Add 10 µl of the 100 µM inhibitor solution into each of the inhibitor control well of a 96-well plate.

2. Set up the enzymatic reaction.

2.1 Add test compounds and diluted enzyme solution to the microplate wells. For one well of a 96-well plate, the suggested volume of enzyme solution is 40 µL and 10 µL of test compound.

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

- Positive control contains the thrombin without test compound.
- Inhibitor control contains thrombin and inhibitor.
- Vehicle control contains thrombin 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 1X 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 thrombin substrate solution from Step 1.2 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=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 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.

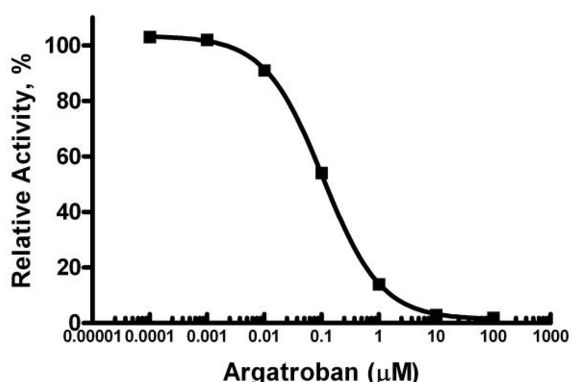

Figure 1. Inhibition of thrombin activity was measured using SensoLyte® AFC Thrombin Assay Kit.

Protocol B. Measuring thrombin activity in biological samples.

1. Prepare thrombin containing biological samples.

1.1 Prepare blood plasma samples:

- Collect whole blood sample with anticoagulant (such as mixing 140 µL citrate-phosphate-dextrose solution with each milliliter of whole blood).
- Centrifuge samples for 25 min at 2,500x g, 4°C.
- Collect the plasma supernatant and store at -70°C until use.

1.2 Prepare cell extract samples:

- Collect cells (such as from synovial fluid or cultured synovial fibroblast-like cells) and wash cell pellets with phosphate buffered saline (PBS).
- Lyse cells, and centrifuge at 3,000x g for 5 min, 4°C.
- Collect the supernatant and store at -70°C until use.

1.3 Prepare tissue extract samples:

- Collect tissues (such as synovial tissues obtained from rheumatoid arthritis patients at joint surgery)
- Homogenize tissue samples, and centrifuge at 3,000x g for 5 min at 4°C.
- Collect the supernatant and store at -70°C until use.

Note 1: PBS is not provided. The plasma, cell and tissue extract should be diluted and used as the enzyme source to measure thrombin activity.

Note 2: It is optional to use our assay buffer (1X) for preparation of biological samples. In this case, the thrombin substrate (Component A) should be diluted in 1X assay buffer instead of 2X assay buffer.

2. Prepare working solutions.

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

2.1 Thrombin substrate solution: Dilute thrombin substrate (Component A) 100-fold in assay buffer (Component C) according to Table 2. For each experiment, prepare fresh substrate solution.

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

Components	Volume
Thrombin substrate (Component A)	50 µL
2X Assay buffer (Component C)	4.95 mL
Total volume	5 mL

2.2 Thrombin diluent: If purified thrombin enzyme is used as a positive control, then dilute the enzyme 100-fold in 1X assay buffer. Adjust the appropriate amount of enzyme to be diluted accordingly.

3. Set up enzymatic reaction.

3.1 Add 50 µL of thrombin containing biological sample.

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

- Positive control contains purified active thrombin enzyme.
- Substrate control contains deionized water.

3.3 Bring the total volume of all controls to 50 µL.

3.4 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.

4. Run the enzymatic reaction.

4.1 Add 50 µL of thrombin substrate solution into each well. For best accuracy, it is advisable to have the thrombin 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=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.

4.3 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 kinetic 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 5 mM AFC (Component B) 100-fold to 50 µM with 1X assay buffer. Do 2-fold serial dilutions to get concentrations of 25, 12.5, 6.25, 3.13 1.57 and 0.78, include an assay buffer blank. Add 50 µL/well of these serially diluted AFC reference solutions.

- Add 50 µL/well of the diluted thrombin substrate solution (refer to Protocol A, step 1.2 for preparation).

  Note: Thrombin substrate solution is added to the AFC reference standard to normalize for the intrinsic substrate fluorescence. If multiple concentrations of substrate are used, this step must be performed for each concentration.

- (Optional) If the stop solution (component F) was added into the enzymatic reaction before taking the end-point reading, the same volume of stop solution should be added to the reference standard wells to obtain a better comparison.
- Measure the fluorescence of the reference stand and substrate control wells at Ex/Em=380nm/500nm. Use the same setting of sensitivity as used in the enzyme reaction.
- Plot the AFC fluorescent reference standard curve as RFU (relative fluorescent units) versus concentration as shown in Figure 2.
- The final concentrations of AFC reference standard are 25, 12.5, 6.25, 3.13, 1.57, 0.78, 0.39, 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.

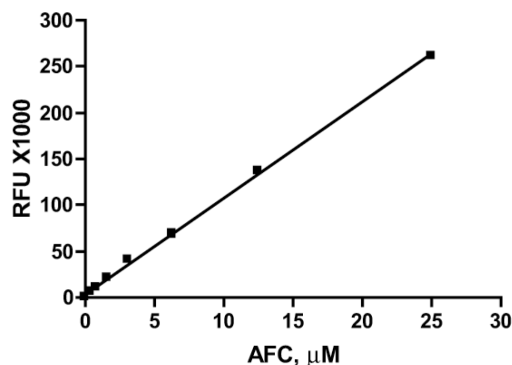

Figure 2. AFC reference standard. AFC was serially diluted in 1X assay buffer containing substrate, and the fluorescence was recorded at Ex/Em=380 nm/500 nm. (Flexstation 384II, Molecular Devices).

References :

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