



SensoLyte[®] 520 Thrombin Activity Assay Kit *Fluorimetric*

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| Revision Number: 1.1 | Last updated: October 2014 |
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| Catalog # | AS-72129 |
| 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 | 5-FAM/QXL™ 520 Thrombin substrate, Ex/Em=490 nm/520 nm upon cleavage | 2 mM, 50 µL |
| Component B | 5-FAM, Fluorescence reference standard, Ex/Em=490 nm/520 nm | 2 mM, 10 µL |
| Component C | 2X Assay buffer | 20 mL |
| Component D | Purified human thrombin enzyme | 0.1 mg/mL, 40 µL |
| Component E | Thrombin inhibitor | 60 µM, 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 detection at Ex/Em= 490 nm/520 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 angiogenesis⁵ and tumor invasion.⁶

The SensoLyte[®] 520 Thrombin Assay Kit is optimized for detecting activity of thrombin. This kit contains a novel internally quenched 5-FAM/QXL FRET substrate for thrombin. Thrombin cleaves the FRET substrate into two separate fragments resulting in release of 5-FAM fluorescence which can be monitored at excitation /emission= 490 nm/520 nm. The long wavelength fluorescence of 5-FAM is less interfered by the autofluorescence of components in biological samples and test compounds. This assay can detect as low as 6.25 ng/mL active thrombin.

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 μ L |
| 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 μ L |
| 1X Assay buffer | 3.96 mL |
| Total volume | 4 mL |

1.4 Thrombin inhibitor (Argatroban): Dilute the 60 μ M inhibitor solution (Component E) 100-fold to 600 nM in 1X assay buffer. Add 10 μ l of the 600 nM 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=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 μL of stop solution 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.

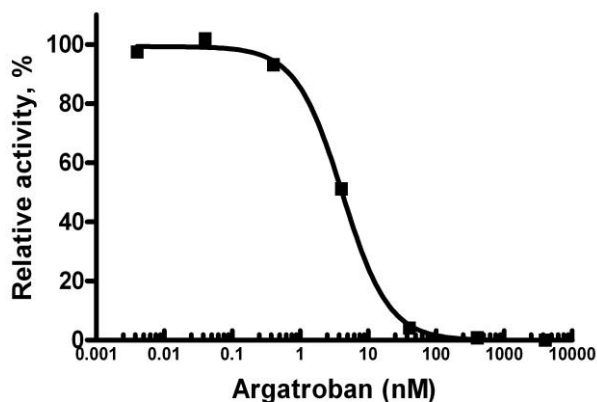


Figure 1. Inhibition of thrombin activity was measured using SensoLyte[®] 520 Thrombin Assay Kit.

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 μ L 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 2X 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 μ L |
| 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 μ L 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=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 μL of stop solution (Component F) to each well. Mix the reagents and measure fluorescence intensity at Ex/Em=490 nm/520 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

- 5-FAM fluorescence reference standard: Dilute 2 mM 5-FAM (Component B) 100-fold to 20 μM with 1X assay buffer. Do 2-fold serial dilutions to get concentrations of 10, 5, 2.5, 1.25,

0.625 and 0.312 μM , include an assay buffer blank. Add 50 μL /well of these serially diluted 5-FAM 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 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.

- (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=490nm/520nm. 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.
- The final concentrations of 5-FAM reference standard are 10, 5, 2.5, 1.25, 0.625, 0.312, 0.156, 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.

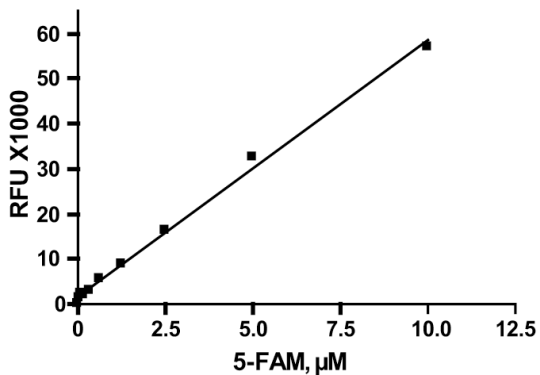


Figure 2. 5-FAM reference standard. 5-FAM was serially diluted in assay buffer containing substrate, and the fluorescence recorded at Ex/Em=490 nm/520 nm. (Flexstation 384II, Molecular Devices).

References

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