SensoLyte® Rh110 Factor Xa Assay Kit
Fluorimetric

Revision Number: 1.1	*Last updated: October 2014*
Catalog #	**AS-72207**
Kit Size	100 Assays (96-well plate)

- ***Optimized Performance:*** This kit is optimized to detect Factor Xa enzyme activity
- ***Enhanced Value:*** It provides ample reagents to perform 100 assays in a 96-well plate 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	Rh110 Factor Xa substrate, Ex/Em=490 nm/520 nm upon cleavage	0.4 mM, 50 µL
Component B	Rh110, fluorescence reference standard, Ex/Em=490 nm/520 nm	0.4 mM, 10 µL
Component C	Purified Bovine Factor Xa	5 ng/µL, 20 µL
Component D	2X Assay Buffer	25 mL
Component E	Factor Xa Inhibitor	1 mM, 20 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 can be stored at room temperature for convenience.

Introduction

Factor Xa (FXa) is a serine endopeptidase composed of two disulfide-linked subunits. FXa leads to blood clot formation by converting prothrombin to thrombin through the prothrombinase complex. FXa is generated from zymogen Factor X via the intrinsic and extrinsic pathways and is the rate-limiting step in the propagation of thrombin generation. In the presence of Ca^{2+} ions, FXa forms prothrombinase with factor Va on the phospholipid membrane of the activated platelets.[1-3] FXa has emerged as an attractive target for drug discovery for thromboembolic diseases.[4,5]

The SensoLyte® Rh110 Factor Xa Assay Kit provides a convenient assay for screening of Factor Xa or continuous assay of enzyme activity using a fluorogenic substrate. Upon FXa protease cleavage, this substrate generates the Rh110 (rhodamine 110) fluorophore which has a bright green fluorescence that can be detected at excitation/emission=490 nm/520 nm. The longer-wavelength spectra and higher extinction coefficient of the Rh110 provide greater sensitivity and less interference from other reaction components. The detection limit can reach as low as 0.31 ng/mL.

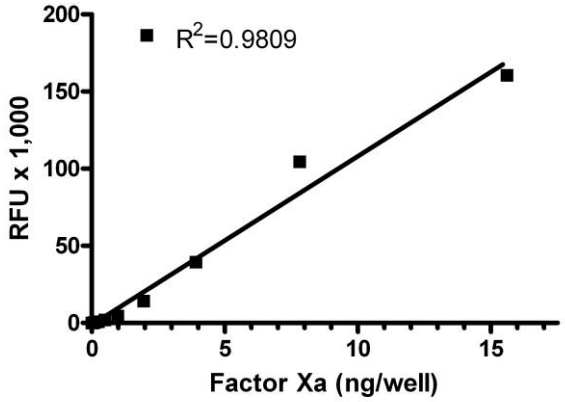

Figure 1. Detection of FXa with the SensoLyte® Rh110 Factor Xa Activity Assay Kit.

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 Factor Xa substrate solution: Dilute Factor Xa substrate (Component A) 1:100 in 1X assay buffer. Refer to Table 1. For each experiment, prepare fresh substrate solution. If not using the entire plate, adjust the amount of substrate to be diluted accordingly.

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

Components	Volume
Factor Xa substrate (Component A)	50 µL
1X assay buffer	4.95 mL
Total volume	5 mL

1.3 Factor Xa diluents: Dilute the Factor Xa enzyme (Component C), 1:200 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 Factor Xa inhibitor (Gabexate mesylate): Dilute the 1 mM inhibitor solution (Component E) 1:10 in 1X assay buffer to get a concentration of 0.1 mM. Add 10 µl of the diluted inhibitor 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 set up the following control wells, as deemed necessary:

- Positive control contains the diluted Factor Xa without test compound.
- Inhibitor control contains the diluted Factor Xa 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 the Factor Xa substrate solutions 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. 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.

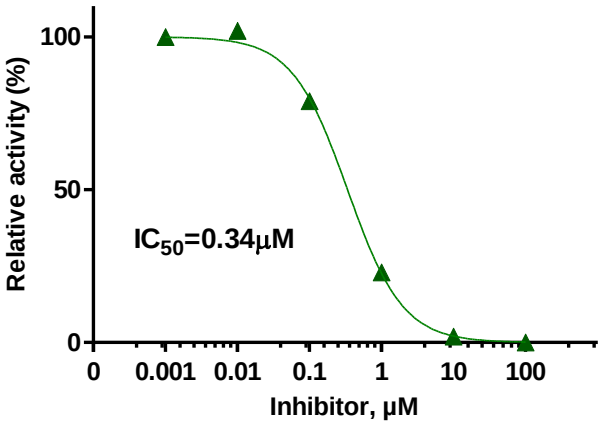

Figure 1. Inhibition of Factor Xa activity by gabexate mesylate as measured with SensoLyte® Rh110 Factor Xa Assay Kit.

Protocol B. Measuring Factor Xa activity in biological samples.

1. Prepare working solutions.

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

1.1 The Factor Xa substrate solution: Dilute Factor Xa substrate (Component A) in 2X assay buffer (Component D). Refer to Table 1. If not using the entire plate, adjust the amount of substrate to be diluted accordingly.

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

Components	Volume
Factor Xa 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 Factor Xa containing sample.

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

- Positive control contains purified active Factor Xa
- 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 the Factor Xa substrate solutions 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. 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.

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

- Fluorescence reference standard: Dilute the 0.4 mM fluorescence standard solution (Component B) to 4 μM in 1X assay buffer. Do 2-fold serial dilutions to get concentrations of 2, 1, 0.5, 0.25, 0.125, and 0.063 μM, include an assay buffer blank. Add 50 μL/well of these serially diluted reference solutions.
- Add 50 μL/well of the diluted Factor Xa substrate solution (refer to Protocol A, Step 1.2 for preparation).

  Note: The Factor Xa 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 reference standard curve as RFU (relative fluorescent units) versus concentration.

- The final concentrations of fluorescence reference standard are 2, 1, 0.5, 0.25, 0.125, 0.063, 0.032, 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 enzymatic reaction final product.

References :

1. Furie, B. and Furie, BC. *Cell.* 53:505–518 (1988).
2. Davie, EW. et al. *Biochemistry*. 30:10363–10370 (1991).
3. Mann, KG. *Thromb Haemost.* 82:165-174 (1999).
4. Camm, AJ. and Bounameaux, H. *Drugs.* 71:1503-1526 (2011).
5. Weitz, JI. *Thromb Res.* 127 Suppl 2:S5-S12 (2011).