SensoLyte® 520 ADAMTS13 Activity Assay Kit *Fluorimetric*

Revision#1.2	*Last Updated: August 2017*
Catalog #	**AS-72232**
Kit Size	96-well plate format

- ***Optimized Performance:*** This kit is optimized to detect ADAMTS13 activity.
- ***High Speed:*** The entire process can be completed in 1 hour.
- ***Assured Reliability:*** Detailed protocol and references are provided.

Kit Components, Storage and Handling

Component	Description	Quantity
Component A	5-FAM/QXL® 520 ADAMTS13 substrate, Ex/Em=490/520 nm upon cleavage	50 µL
Component B	5-FAM, fluorescence reference standard, Ex/Em=490/520 nm	0.1 mM, 15 µL
Component C	Recombinant human ADAMTS13	50µg/mL, 20 µL
Component D	2X Assay Buffer	30 mL
Component E	ADAMTS13 Inhibitor	1 M, 15 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 520nm with excitation at 490nm.
- Plate cover: To prevent liquid evaporation during incubation steps
- Amount of positive control ADAMTS13 enzyme provided is enough for 10 assays; additional enzyme can be purchased from R&D Systems, Cat# 4245-AD. All other components are supplied to run 100 assays.

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

ADAMTS13 (A Disintegrin-like and Metalloprotease with Thrombospondin type-1 motifs 13) is a metalloproteinase that is primarily synthesized in the liver cells and secreted into blood circulation.[1] ADAMTS13 specifically cleaves von Willebrand factor (vWF) at the Tyr1605-Met1606 peptide bond.[2] vWF is a multimeric glycoprotein that mediates adhesion and aggregation of platelets. ADAMTS13 is essential for preventing platelet aggregation in the circulation. Deficiency of plasma ADAMTS13 activity may lead to thrombotic thrombocytopenic purpur (TTP), a potentially lethal syndrome characterized by the formation of VWF-platelet-rich thrombi in the arterioles and capillaries.[3] Studies also have shown that ADAMTs13 is involved in angiogenesis, inflammation, atherosclerosis, and liver cirrhosis.[4-7] Measurement of ADAMTS13 activity in plasma has been used for supporting the acute diagnosis of TTP.[8]

The SensoLyte®520 ADAMTS13 Assay Kit employs an internally quenched vWF73 FRET peptide substrate for the detection of enzyme activity.[9] This substrate contains a novel 5-FAM/QXL® 520 FRET pair. ADAMTS13 cleaves this FRET substrate into two separate fragments resulting in the release of 5-FAM fluorescence, which can be monitored at Ex/Em= 490/520 nm. The long wavelength fluorescence of 5-FAM is less interfered by the autofluorescence of components in biological samples.

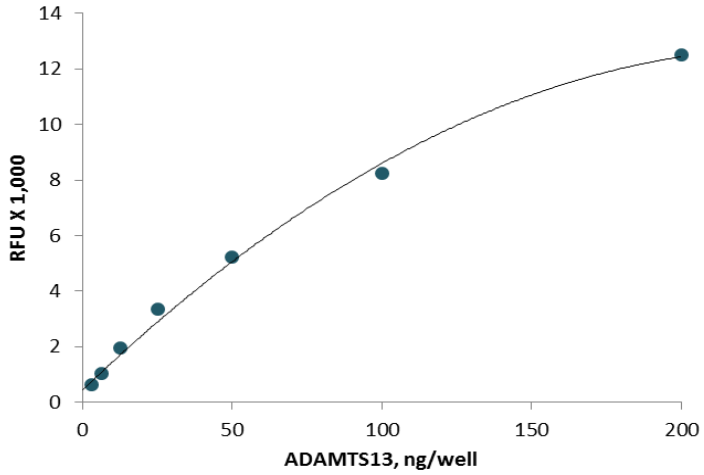

Figure 1. Sensitivity of the assay has been tested using serial dilutions of recombinant ADAMTS13. 5-FAM/QXL® 520 FRET substrate was incubated with the indicated amount of enzyme and fluorescence was measured after 60 min (FlexStation 384II, Molecular Devices). The assay can detect as low as 10 ng/mL of active ADAMTS13.

Protocol

Note 1: To prepare a standard curve, please refer to Appendix II (optional).
Note 2: Please use Protocol A or B based on your needs.

Protocol A. **Screening ADAMTS13 regulators using purified enzyme.**

Prepare working solutions.

Note: Bring all kit components to room temperature before starting the experiments.

1.1 1X assay buffer: Mix 10 mL of 2X assay buffer (Component D) with 10 mL of deionized water.

1.2 ADAMTS13 substrate solution: Dilute ADAMTS13 substrate (Component A) 100-fold in 1X assay buffer. Refer to the 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. ADAMTS13 substrate solution for one 96-well plate (100 assays)

Components	Volume
ADAMTS13 substrate (Component A)	50 µL
1X assay buffer	4.95 mL
Total volume	5 mL

1.3 ADAMTS13 diluents: Dilute ADAMTS13 enzyme (Component C) 20-fold in 1X assay buffer. Enzyme is provided for positive control only (enough for 10 assays).

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 ADAMTS13 inhibitor 1,10-Phenanthroline: Dilute 1 M inhibitor solution (Component E) 100-fold in 1X assay buffer to make the 10 mM diluted inhibitor solution. Add 10 µl of the diluted inhibitor solution 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 diluted ADAMTS13 without test compound.
- Inhibitor control contains diluted ADAMTS13 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 1X assay buffer bring the total volume of all controls to 50 µL.

2.4 Optional: Pre-incubate 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 ADAMTS13 substrate solutions into each well. For best accuracy, it is advisable to have substrate solution equilibrated to the assay temperature. Mix all 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.

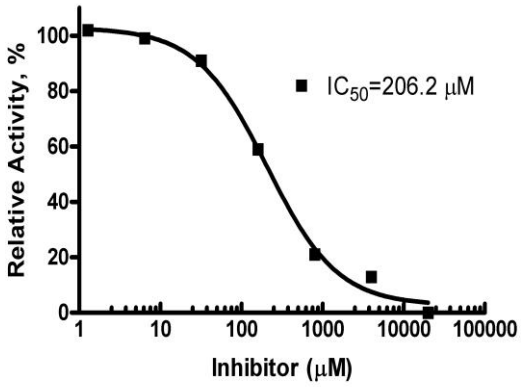

Figure 2. Inhibition of ADAMTS13 activity by 1,10-Phenanthroline as measured with SensoLyte® 520 ADAMTS13 Activity Assay Kit.

Protocol B. Measuring ADAMTS13 activity in biological samples.

1. Prepare ADAMTS13 containing biological samples.

1.1 Prepare blood plasma:

- Collect whole blood sample into 3.2 % trisodium citrate anticoagulant solution to give a ratio of whole blood: anticoagulant of 9:1. **Do Not Use EDTA**.
- Centrifuge samples for 15 min at 10,000x g.
- Collect the plasma supernatant and store at -70°C until use (up to 6 months).

1.2 Prepare cell culture medium

- Centrifuge collected medium at 10,000x g for 15 min, 4°C to remove insoluble content and concentrate samples using centrifugal filter units.
- Store samples at -80°C until use.

1.3 Prepare cell extract samples:

- Collect cells (such as from isolated rat hepatic stellate cells).
- Lyse cells, and centrifuge at 10,000x g for 15 min, 4°C.
- Collect the supernatant and store at -80°C until use.

Note: It is optional to use our assay buffer (1X) for preparation of biological samples. In this case, the ADAMTS13 substrate (Component A) should be diluted in 1X assay buffer instead of 2X assay buffer.

2. Prepare working solutions.

Note: Bring all kit components to room temperature before starting the experiments.

2.1 ADAMTS13 substrate solution: Dilute ADAMTS13 substrate (Component A) 100-fold in 2X assay buffer (Component D) according to the 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. ADAMTS13 substrate solution for one 96-well plate (100 assays)

Components	Volume
ADAMTS13 substrate (Component A)	50 μL
2X Assay buffer (Component D)	4.95 mL
Total volume	5 mL

2.2 ADAMTS13 diluent: Dilute ADAMTS13 enzyme (Component C) 25-fold in deionized water and add 50 µL per well for positive control.

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.

3. Set up enzymatic reaction.

3.1 Add 50 µL of ADAMTS13 containing biological sample.

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

- Positive control contains purified active ADAMTS13 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 ADAMTS13 substrate solution into each well. For best accuracy, it is advisable to have the ADAMTS13 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. 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.

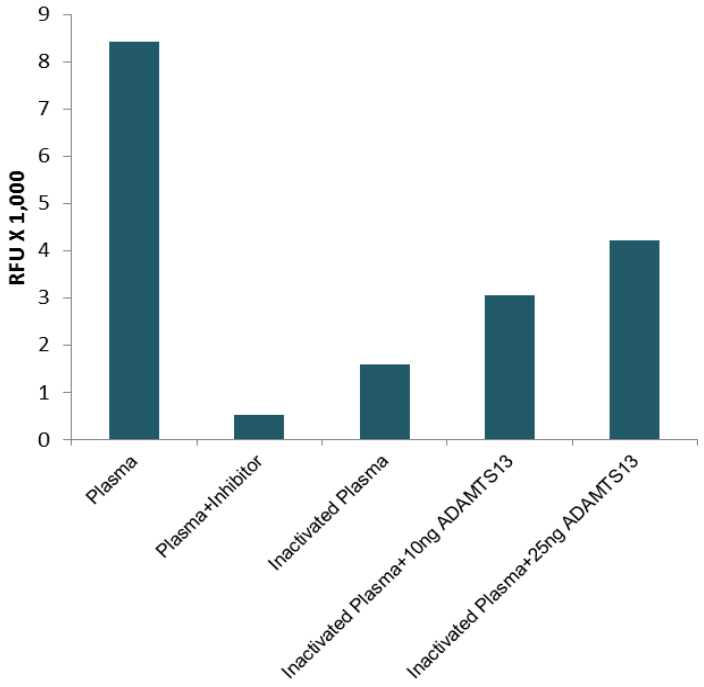

Figure 3. Human plasma samples assayed for ADAMTS13 activity. Human plasma was spiked with ADAMST13 inhibitor (1,10-Phenanthroline). Inactivated human plasma was spiked with recombinant ADAMTS13. Samples were incubated at 37°C for 1 hour and fluorescence was measured at Ex/Em=490/520nm (FlexStation 384II, Molecular Devices).

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 0.1 mM fluorescence standard solution (Component B) 100-fold to 1 μM in 1X assay buffer. Do 2-fold serial dilutions to get concentrations of 0.5, 0.25, 0.13, 0.06, 0.03, and 0.015 μM, include an assay buffer blank. Add 50 μL/well of these serially diluted reference solutions.
- Add 50 μL/well of the diluted ADAMTS13 substrate solution (Step 2.1 of Protocol A).

  Note: The ADAMTS13 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 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 0.5, 0.25, 0.13, 0.06, 0.03, 0.015, 0.008 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.

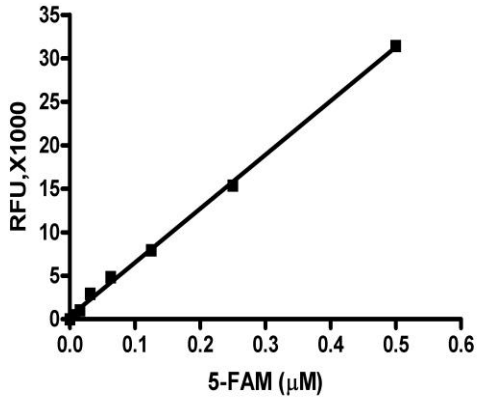

Figure 4. 5-FAM reference standard. 5-FAM standard solution was serially diluted with the assay buffer containing ADAMTS13 substrate, and the fluorescence was recorded at Ex/Em=490/520 nm. (Flexstation 384II, Molecular Devices).

References

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