SensoLyte® 520 ADAM10 Activity Assay Kit
Fluorimetric

Revision number: 1.1	*Last updated: 10 /14/14*
Catalog #	**AS-72226**
Kit Size	100 Assays (96-well plate)

- ***Optimized Performance:*** This kit is optimized to detect ADAM10 activity.
- ***Enhanced Value:*** 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	5-FAM /QXL™ 520 ADAM10 substrate Ex/Em=490 nm/520 nm upon cleavage	1 mM, 50µL
Component B	5-FAM fluorescence reference standard, Ex/Em=490 nm/520 nm	1 mM, 12 µL
Component C	Human recombinant ADAM10	0.1 mg/mL, 10 µL
Component D	Assay Buffer	25 mL
Component E	Inhibitor	1 mM, 10µL

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

ADAM10, a proteinase of the "A disintegrin and metalloprotease" (ADAM) family has been shown to be an effective α-secretase *in vitro*[1] and *in vivo*[2]. This enzyme acts as a sheddase to cleave cell surface proteins involved in neuropathology, inflammatory response and tumor progression.[3-6] ADAM10 is also a physiologically relevant, constitutive α-secretase for amyloid precursor protein (APP).[7] It plays an important role in the molecular pathogenesis of Alzheimer's disease.[8]

The SensoLyte® 520 ADAM10 Activity Assay Kit is an optimized assay that can be used to detect enzyme activity in biological samples or in purified enzyme preparations. The unique FRET substrate was designed to reduce the cross reactivity with ADAM17 (also called tumor necrosis factor-α-converting enzyme, TACE). When active ADAM10 cleaves the FRET substrate, it results in an increase of 5-FAM fluorescence, monitored at excitation/emission = 490 nm/520 nm. The long wavelength fluorescence of 5-FAM is also less interfered by the autofluorescence of components in biological samples and test compounds. This assay can detect as low as 0.25 ng/mL active ADAM10.

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 ADAM10 inhibitors using a purified enzyme.

1. Prepare working solutions.

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

1.1 ADAM10 substrate solution: Dilute ADAM10 substrate (Component A) 100-fold in assay buffer. Prepare fresh substrate solution for each experiment. Refer to Table 1.

Table 1. ADAM10 substrate solution for one 96-well plate (100 assays).

Components	Volume
ADAM10 substrate (100X, Component A)	50 µL
Assay buffer	4.95 mL
Total volume	5 mL

1.2 ADAM10 diluent:

Dilute ADAM10 enzyme 400-fold in assay buffer. This amount of enzyme is enough for a full 96-well plate. If not using an 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 enzyme denaturation. Store on ice.

1.3 Inhibitor (GM-6001): Dilute the 1 mM inhibitor solution (Component E) 1:100 in assay buffer. The diluted GM-6001 solution has a concentration of 10 µM. Add 10 µl of the diluted GM-6001 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 a 96-well plate is 40 µL/well and test compound is 10 µL/well.

2.2 Simultaneously set up the following control wells, as deemed necessary:

- Positive control contains the enzyme without test compound.

- Inhibitor control contains ADAM10 enzyme and GM-6001.
- Vehicle control contains ADAM10 enzyme and vehicle used in delivering test compound (e.g. DMSO, concentration not to exceed 1%).
- Test compound control contains assay buffer and test compound. Some test compounds have strong autofluorescence and may give false results.
- Substrate control contains assay buffer.

2.3 Use the assay buffer to bring the total volume of all controls to 50 µL.

3. Run the enzymatic reaction.

3.1 Add 50 µL of ADAM10 substrate solution into each well. For best accuracy, it is advisable to have the substrate solution equilibrated to the assay temperature (37ºC usually gives higher signal-to-background ratio). 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 at 37ºC for 30 to 60 min. Keep plate from direct light. Then measure fluorescence intensity at Ex/Em=490 nm/520 nm.

For methods of data analysis: Refer to Appendix I.

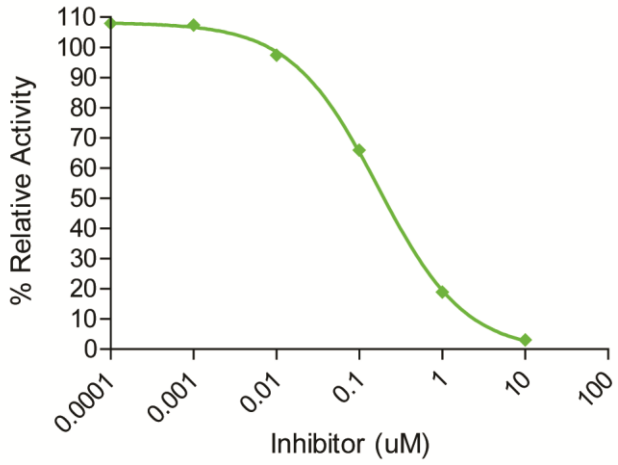

Figure 1. Inhibition of ADAM10 activity by GM-6001 as measured with SensoLyte® 520 ADAM10 Activity Assay Kit.

Protocol B. Measuring ADAM10 activity in biological samples.

1. Prepare ADAM10 containing biological samples.

1.1 Prepare sample from cell culture medium:

- Collect medium from culture.
- Spin the medium sample for 10-15 min. at 1,000X g, 4°C.
- Collect the supernatant and store at -70°C until use.

1.2 Prepare cell lysates:

- Cells are collected by centrifugation at 500 X g for 10 min.
- Add an appropriate amount of cold assay buffer to cell pellet. Collect the cell suspension to a microcentrifuge tube.
- Incubate the cell suspension on ice for at least 10 min.
- Pipette the cell suspension up and down for 5 times.
- Centrifuge the cell suspension for 5 min. at 10,000 X g, 4°C. Collect the supernatant and store at -70°C until use.

1.3 Prepare cellular membrane fractions:

- Wash cells with PBS and resuspend them in ice cold PBS with protease inhibitors.
- Samples are homogenized in cold assay buffer.
- Centrifuge homogenized cells 15 minutes at 20,000X g, 4°C.
- Wash pelleted membranes with PBS and resuspend after centrifugation in cold assay buffer. Store at -70°C until use.

1.4 Prepare tissue homogenate and lysate:

- Homogenize tissue samples in cold assay buffer as homogenate.
- Incubate homogenate on ice for an additional 15 min.
- Centrifuge for 15 min. at 10,000xg, 4°C. Collect the supernatant as tissue lysate. Store homogenate and/or lysate at -70°C until use.

Note 1: PBS is not provided.
Note 2: ADAM10 activity can be detected in live cells. In this case, the assay may need optimization. For example, the cells can be incubated with ADAM10 substrate (Component A) in an imaging solution (140 mM $NaCl_2$, 5 mM KCl, 8 mM $CaCl_2$, 1 mM $MgCl_2$, 20 mM HEPES, pH 7.4) or in other solutions.

2. Prepare working solutions.

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

2.1 Dilute ADAM10 substrate (Component A) 100-fold in assay buffer. Prepare fresh assay buffer for each experiment. Refer to Table 1.

Table 1. ADAM10 substrate solution for one 96-well plate (100 assays).

Components	Volume
ADAM10 substrate (100X, Component A)	50 µL

Assay buffer	4.95 mL
Total volume	5 mL

2.2 ADAM10 diluent: If using purified ADAM10 enzyme as a positive control, do 500-fold dilution to the enzyme with assay buffer. Add 50 µl of the diluted enzyme into each of the positive control well.

Note: Do not vortex enzyme solutions. Prolonged storage or vigorous agitation of the diluted enzyme will cause the enzyme denaturation. Store the enzyme solution on ice.

3. Set up enzymatic reaction.

3.1 Add 5-50 µL of ADAM10 containing biological sample.

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

- Positive control contains purified ADAM10 enzyme.
- Substrate control contains assay buffer.

3.3 Using the assay buffer, bring the total volume of all controls to 50 µL.

4. Run the enzymatic reaction.

4.1 Add 50 µL of ADAM10 substrate solution into each well. For best accuracy, it is advisable to have the substrate solution equilibrated to the assay temperature (37ºC usually gives higher signal-to-background ratio). 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 at 37 ºC for 30 to 60 min. Keep plate from direct light. Then 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 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

- 5-FAM fluorescence reference standard: Dilute 1 mM 5-FAM reference standard (Component B) to 10 µM in assay buffer. Do 2-fold serial dilutions to get concentrations of 5, 2.5, 1.25, 0.625, 0.312, 0.156 µM, and include 0 µM as an assay buffer blank. Add 50 µL/well of these serially diluted 5-FAMreference solutions.
- Add 50 µL/well of the diluted ADAM10 substrate solution (refer to Protocol A, step 1.1 for preparation).

  Note: The ADAM10 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.
- Measure the fluorescence intensity 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 5-FAM fluorescence reference standard curve as RFU (relative fluorescent units) versus concentration as shown in Figure 2.
- The final concentrations of 5-FAM reference standard are 5, 2.5, 1.25, 0.625, 0.312, 0.156, 0.078 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.

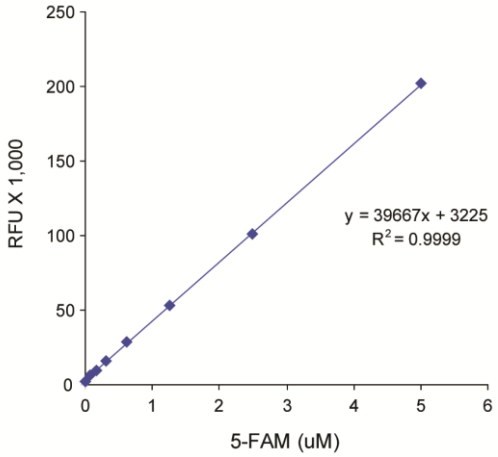

Figure 2. 5-FAM reference standard. 5-FAM was serially diluted in assay buffer, containing ADAM10 substrate, and the fluorescence recorded at Ex/Em=490 nm/520 nm (Flexstation 384 II, Molecular Devices).

References

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