SensoLyte® 520 Aggrecanase-1 Assay Kit **Fluorimetric**

Revision number: 1.2	*Last updated: October 2014*
Catalog #	AS-72114
Kit Size	100 Assays (96-well plate)

- ***Optimized Performance:*** Optimized to detect aggrecanase-1 activity
- ***Enhanced Value:*** Ample reagents to perform 100 assays in a 96-well format
- ***High Speed:*** Entire process can be completed in one hour
- ***Assured Reliability:*** Detailed protocol and references provided

Kit Components, Storage and Handling

Component	Description	Quantity
Component A	5-FAM/5-TAMRA, Aggrecanase-1 substrate, Ex/Em=490nm/520 nm	60 µL
Component B	5-FAM, fluorescence reference standard, Ex/Em=490nm/520nm	1mM, 10 µL
Component C	Assay Buffer	20 mL
Component D	Control Inhibitor	100 µM, 10 µL
Component E	Stop Solution	10 mL

Other Materials Required (but not provided)

- Aggrecanase-1 source: Human recombinant enzyme (Millipore, Cat#CC1028).
- 96-well microplate: Black, flat-bottom, non-binding 96-well plate.
- Fluorescence microplate reader: Capable of detecting emission at 520 nm with excitation at 490nm.

Storage and Handling

- Store all kit components at -20°C.
- Protect Components A and B from light and from moisture.
- Components C and E can be stored at room temperature for convenience.

Introduction

Aggrecanases are proteolytic enzymes that belong to ADAMTS (A disintegrin and metalloprotease with thrombospondin motif) family of proteases. Aggrecanases cleave aggrecan, the major structural component of cartilage. Proteolysis of this core protein of cartilage alters normal mechanical properties of compressibility and resilience during joint loading, and contributes to various symptoms of arthritis.[1, 2] ADAMTS-4 (aggrecanase-1) is a major aggrecanase in human osteoarthritic cartilage.[3]

The SensoLyte® 520 Aggrecanase-1 Assay Kit is optimized to detect activity of ADAMTS-4 and for screening of inhibitors. This kit contains a novel internally quenched 5-FAM/TAMRA FRET substrate for aggrecanase-1 (km~5µM). Active ADAMTS-4 cleaves the FRET substrate into two separate fragments resulting in an increase of 5-FAM fluorescence which can be monitored at excitation/emission= 490 nm/520 nm. The cleavage of the FRET ADAMTS-4 substrate by other members of aggrecanase family, such as ADAMTS-1 and ADAMTS-5, is negligible. The assay can detect as low as 0.1 ng/mL active aggrecanase-1.

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 aggrecanase inhibitors using purified enzyme.

1. Prepare working solutions.

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

1.1 Aggrecanase substrate solution: Dilute aggrecanase-1 substrate (Component A) 1:100 in assay buffer (Component C). For each experiment, prepare fresh substrate solution.

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

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

1.2 Aggrecanase diluent: Dilute the enzyme to an appropriate concentration in assay buffer (Component C).

1.3 Control inhibitor (TAPI-0, Peptides International Louisville, KY): Dilute the 100 µM inhibitor solution (Component D) to 10µM in assay buffer (Component C). Add 10 µl of the 10µM 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 enzyme without test compound.
- Inhibitor control contains aggrecanase and TAPI-O inhibitor.

- ➢ Vehicle control contains aggrecanase enzyme and vehicle used in delivering test compound (e.g. DMSO, concentration not to exceed 1%).
- ➢ Test compound control contains assay buffer (Component C) and test compound.
- ➢ Substrate control contains assay buffer (Component C).

2.3 Using the assay buffer (Component C), 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 aggrecanase substrate solution from Step 1.1 into each well. For best accuracy, it is advisable to have the aggrecanase 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 (Component E) 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.

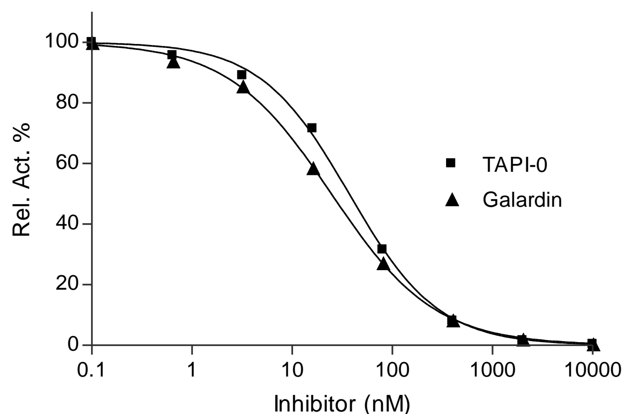

Figure 1. Inhibition of aggrecanase-1 activity by matrix metalloproteinases inhibitors was measured using SensoLyte® 520 Aggrecanase-1 Assay Kit.

Protocol B. Measuring aggrecanase activity in biological samples.

1. Prepare aggrecanase containing biological samples.

1.1 Prepare synovial fluids or cell media samples:

- Collect synovial fluids or supernatant of cell culture media (e.g. stimulated chondrocytes) and centrifuge for 10 - 15 min at 1,000X g, 4°C. Collect the supernatant and store at -70°C until use.
- Centrifuge samples for 10 - 15 min at 1,000X g, 4°C.
- Collect the supernatant and store at -70°C until use.

1.2 Prepare tissue samples:

- Homogenize tissue samples in assay buffer (Component C) containing 0.1% (v/v) Triton-X 100.
- Incubate for 15 min. at 4°C.
- Centrifuge for 15 min. at 2,000xg at 4°C and collect the supernatant. Store at -70°C until use.

1.3 Prepare cell lysates:

- Wash cells with PBS.
- Add an appropriate amount of assay buffer (Component C) containing 0.1% (v/v) Triton-X 100 to cells or cell pellet. Collect the cell suspension in a microcentrifuge tube.
- Incubate the cell suspension at 4°C for 10 minutes.
- Centrifuge the cell suspension for 10 minutes at 2,500X g, 4°C. Collect the supernatant and store at -70°C until use.

Note: Triton-X 100 and PBS are not provided.

2. Prepare working solutions.

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

2.1 Aggrecanase substrate solution: Dilute aggrecanase-1 substrate (Component A) 1:100 in assay buffer (Component C). For each experiment, prepare fresh substrate solution.

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

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

2.2 Aggrecanase diluent: If purified aggrecanase is used as a positive control, then dilute the enzyme to an appropriate concentration in assay buffer (Component C).

3. Set up enzymatic reaction.

3.1 Add 50 μL of aggrecanase containing biological sample.

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

- Positive control contains purified active aggrecanase.
- Substrate control contains assay buffer.

3.3 Using the assay buffer (Component C), 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 aggrecanase substrate solution into each well. For best accuracy, it is advisable to have the aggrecanase 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 E) 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 1mM 5-FAM (Component B) to 10µM (1:100) with assay buffer (Component C). Do 2-fold serial dilutions to get concentrations of 2500, 1250, 625, 312.5, 156, 78 nM, include an assay buffer blank. Add 50 µL/well of these serially diluted 5-FAM reference solutions.

- Add 50 µL/well of the diluted aggrecanase substrate solution (refer to Protocol A, step 1.1 for preparation).

  Note: The aggrecanase 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.

- 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 5000, 2500, 1250, 625, 312.5, 156, 78, and 0 nM. 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.

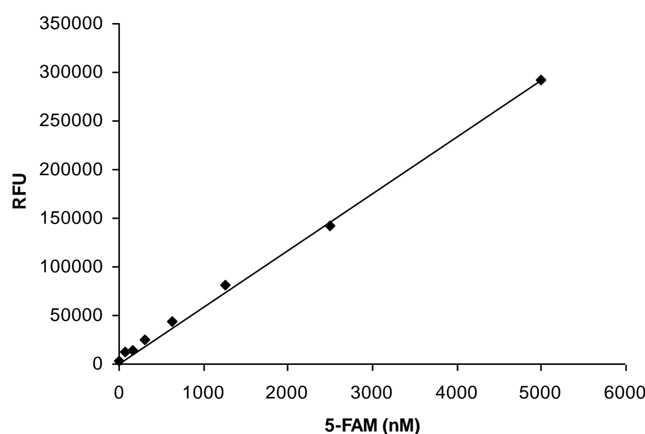

Figure 2. 5-FAM reference standard. 5-FAM was serially diluted in assay buffer containing substrate, and the fluorescence recorded at Ex/Em=490 nm/ 520 nm. (Flexstation 384II, Molecular Devices)

References

1. Tortorella, MD. *Science* 284, 1663 (1999).
2. Hardingham, T. *Curr. Rheumatol. Rep.* 10(1), 30 (2008).
3. Naito, S, et al. *Pathol. Int.* 57, 703 (2007).