SensoLyte® 390 Generic MMP Assay Kit
Fluorimetric

Revision#1.3	*Last Updated: May 2019*
Catalog #	**AS-72202**
Kit Size	100 Assays (96-well plate)

- ***Optimized Performance:*** Optimal conditions for detection of generic MMP activity
- ***Enhanced Value:*** It provides ample reagents to perform 100 assays in a 96-well plate format.
- ***High Speed:*** Minimal hands-on time
- ***Assured Reliability:*** Detailed protocol and references are provided

Kit Components, Storage and Handling

Catalog #	Description	Quantity
Component A	Mca/Dnp, MMP substrate, Ex/Em=330 nm /390 nm upon cleavage	50 µl
Component B	Mca fluorescence reference standard, Ex/Em=330nm /390 nm	1 mM, 10 µL
Component C	APMA, 4-aminophenylmercuric acetate	1M, 20 µL
Component D	Assay buffer	20 mL
Component E	Stop Solution	10 mL

Other Materials Required (but not provided)

- MMPs source: Pro-enzymes, validated in MMPs assays, can be ordered from AnaSpec: MMP-1 (Cat#72004), MMP-2 (Cat#72005), MMP-7 (Cat#72007), MMP-8 (Cat#72008), and MMP-14 (Cat#72068).
  Catalytic domain MMPs, validated in MMPs assays, can be ordered from AnaSpec: MMP-1 (Cat#55575-1, 55575-10), MMP-9(Cat##55576-1, 55576-10, 55576-50), MMP-12 (Cat#55525-1, 55525-10, 55525-50) and MMP-13 (Cat#72257).
- 96-well microplate: Black, flat-bottom, 96-well plate with non-binding surface.
- Microplate reader: Capable of detecting emission at 390 nm with excitation at 330 nm.

Storage and Handling

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

Introduction

Matrix metalloproteinases (MMPs) belong to a family of secreted or membrane-associated zinc endopeptidases capable of digesting extracellular matrix components.[1, 2] MMPs are involved in tumor development and metastasis,[3,4] rheumatoid arthritis[5] and other diseases. They are proposed as therapeutic targets for these diseases.

The SensoLyte® 390 Generic MMP Assay Kit can detect the activity of several MMPs such as MMP-1, 2, 7, 8, 9, 12, 13, 14, 15, 16 and 24 (Figure 1). This kit can be used for detection of generic MMP activity in biological samples or for high throughput screening (HTS) of MMP inducers and inhibitors using purified MMPs.

This kit provides a Mca/Dnp fluorescence resonance energy transfer (FRET) peptide as a MMP substrate. In the intact FRET peptide, the fluorescence of Mca is quenched by Dnp. Upon cleavage into two separate fragments by MMPs, the fluorescence of Mca is recovered, and can be monitored at excitation/emission wavelengths = 330 nm/390 nm.

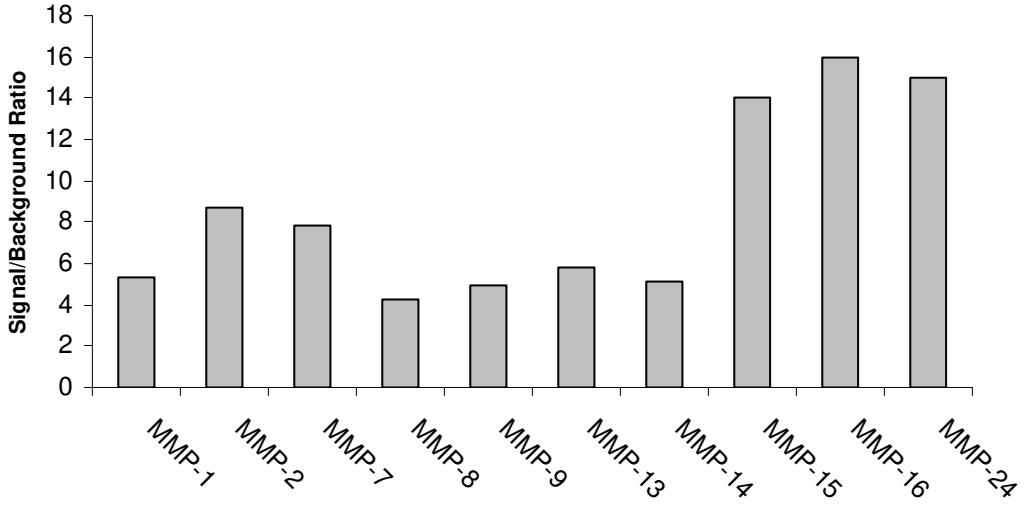

Figure 1. Detection of MMP activities using the SensoLyte® 390 generic MMP assay kit. MMPs enzymes, 30 ng each, were mixed with Mca/Dnp FRET peptide substrate and fluorescence was monitored for 1 hr. Endpoint signals were compared to background (FRET substrate without enzyme) to demonstrate MMP activities. The FRET peptide can detect the activity of nanogram range of all MMPs. (FlexStation 384II, Molecular Devices; Ex/Em=330nm/390 nm)

PROTOCOL

Note 1: For standard curve, please refer to Appendix II (optional).
Note 2: Please follow protocol A or B based on your needs.

Protocol A. Screening protease inhibitors using purified or recombinant MMPs.

1. Activate pro-MMPs.

Note: Activation is required for all pro-MMPs. If you use only catalytic domain of MMPs, this APMA activation step can be omitted.

1.1 Incubate MMPs: Incubate pro-MMPs with 1 mM APMA (diluted Component C). Refer to Appendix III for incubation time. Activate pro-MMPs immediately before the experiment.

Note 1: Keep activated enzyme on ice. Avoid vigorously vortexing the enzyme. Prolonged storage of activated enzyme will further de-activate the enzyme.

Note 2: APMA can be diluted with assay buffer (Component D). APMA belongs to the organic mercury class of compounds. Handle with care! Dispose of it according to appropriate regulations.

Note 3: APMA activation of zymogen at a higher protein concentration is recommended. After activation, the enzyme can be further diluted for the experiment.

2. Prepare working solutions.

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

2.1 Generic MMP substrate solution: Dilute generic MMP substrate (Component A) 1: 100 in assay buffer (Component D). Prepare enough diluted substrate solution for one experiment and store the rest as undiluted at -20°C. Refer to Table 1.

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

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

2.2 MMP diluent: Dilute activated MMPs to an appropriate concentration in assay buffer (Component D).

3. Set up enzymatic reaction.

3.1 Add test compounds and MMP diluent into microplate. The suggested volume of enzyme solution for a 96-well plate is 40 µL/well and test compound is 10 µL/well.

3.2 Set up the following controls at the same time:

- Positive control contains MMP diluent without test compound.
- Inhibitor control contains MMP diluent and known MMPs inhibitor.
- Vehicle control contains MMP diluent and vehicle used to deliver test compound (e.g. DMSO).
- 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 only.

3.3 Using the assay buffer (Component E), 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. Initiate the enzymatic reaction.

4.1 Add 50 µL of MMP substrate solution into the wells of a 96-well plate. Mix the reagents completely by shaking the plate gently for 30-60 sec.

4.2 Measure fluorescence signal:

- For kinetic reading: Immediately start measuring fluorescence intensity at Ex/Em=330 nm/390 nm continuously and record data every 5 min. for 30 to 60 min.

- For end-point reading: Incubate the reaction at room temperature for 30 to 60 min. Keep the 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=330 nm/390 nm.

4.3 Data analysis: Refer to Appendix I.

Protocol B. Measuring MMPs activity in biological samples.

1. Prepare MMP containing biological samples

1.1 Prepare samples of synovial fluids or cells supernatants:

- Collect synovial fluids or supernatant of cell culture media (e.g. stimulated fibroblast) and centrifuge 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:

- Tissue samples should be homogenized in assay buffer (Component D) containing 0.1% (v/v) Triton-X 100, and then centrifuged for 15 min. at 10,000x g at 4°C. Collect the supernatant and store at -70°C until use.

Note: Triton-X 100 is not provided.

2. Activate pro-MMP

2.1 Incubate MMPs: Incubate the MMP containing-samples or purified pro-MMPs with 1 mM APMA (diluted Component C). Refer to Appendix III for incubation time. Activate MMP immediately before the experiment.

Note 1: If you want to measure endogenous active MMPs only or if you use already active catalytic domain of MMPs, this APMA activation step can be omitted.

Note 2: Keep enzyme-containing sample on ice. Avoid vigorously vortexing the enzyme. Prolonged storage of activated enzyme will de-activate the enzyme.

Note 3: APMA can be diluted with assay buffer (Component D). APMA belongs to **the organic mercury class of compounds.** Handle with care! Dispose it according to appropriate regulations.

3. Prepare working solutions

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

3.1 MMP substrate solution: Dilute generic MMP substrate (Component A) 1:100 in assay buffer (Component D). Prepare enough diluted substrate solution for one experiment and store the rest as undiluted at -20°C.

3.2 MMP diluent: If you use purified MMP, dilute MMP to an appropriate concentration in assay buffer (Component D).

4. Set up the enzymatic reaction

4.1 Add 50 µL of MMP containing biological sample.

4.2 Set up the following control:

- Substrate control contains assay buffer
- Positive control contains purified active MMP

4.3 Using the assay buffer (Component E), bring the total volume of all controls to 50 µL.

4.4 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.

5. Initiate the enzymatic reaction.

5.1 Add 50 µL/well of MMP substrate solutions to the sample and control wells. Mix the reagents by shaking the plate gently for 30 seconds.

5.2 Measure fluorescence signal:

- For kinetic reading: Immediately start measuring fluorescence intensity at Ex/Em= 330 nm/390 nm continuously and record data every 5 min. for 30 to 60 min.
- For end-point reading: Incubate the reaction at room temperature for 30 to 60 min. Keep the plate away 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=330 nm/390 nm.

5.3 Data analysis: Refer to Appendix I.

Appendix I. Data Analysis

- The fluorescence reading from the substrate control well is the background fluorescence. This background reading has to be subtracted from the readings of the other wells. Fluorescence reading is expressed as relative fluorescence unit (RFU).
- For kinetics analysis:
  - Plot data as RFU versus time for each sample. To convert RFU to concentration of the product of enzymatic reaction, please refer to Appendix II for setting up the fluorescence reference standard.
  - Determine the range of initial time points during which the reaction is linear. 10-15% conversion appears to be the optimal range.
  - Obtain the initial reaction velocity (Vo) in RFU/min. Determine 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 or enzyme concentration.
  - A variety of data analyses can be done, e.g., determining inhibition %, EC_{50}, IC_{50}, etc.

Appendix II. Instrument Calibration

- Mca fluorescence reference standard: Dilute 1mM Mca (Component B) to 20 µM in assay buffer (Component D). Perform 2-fold serial dilutions to obtain 10, 5, 2.5, 1.25, 0.625, and 0.313 µM Mca solutions, include an assay buffer blank. Add 50 µL/well of these serially diluted 5-Mca reference solutions.

- Add 50 µL/well of the diluted MMP substrate solution (refer to Protocol A, step 2 for preparation).

  Note: The MMP substrate solution is added to the Mca 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.

- Mix the reagents by gently shaking the plate for 3 to 5 seconds.
- Measure the fluorescence intensity of the reference standard and substrate control wells at Ex/Em=330 nm/390 nm. Use the same setting of sensitivity as used in the enzyme reaction.
- Plot the Mca fluorescent reference standard curve as RFU (relative fluorescent unit) versus concentration as shown in Figure 2.

  Note: The final concentrations of Mca reference standard solutions are 10, 5, 2.5, 1.25, 0.625, 0.313, 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 enzymatic reaction final product.

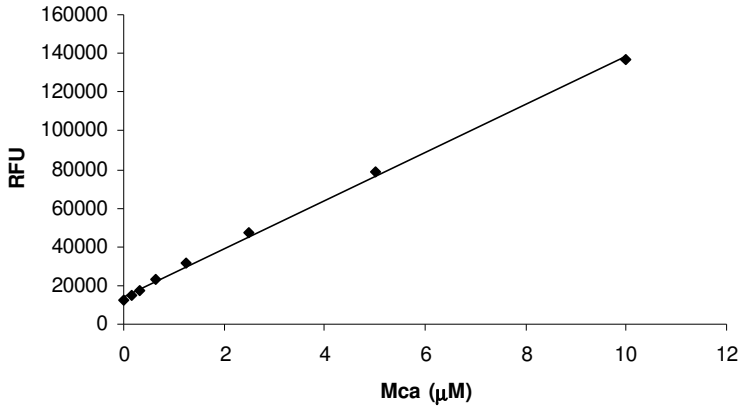

Figure 2. Mca standard. Mca was serially diluted in assay buffer containing MMP generic substrate (FlexStation 384II, Molecular Devices; Ex/Em=330nm/390 nm).

Appendix III. MMPs Activation Time

Protocols for pro-MMP activation.

MMPs	Activated by treating with
MMP-1 (collagenase)	1 mM APMA (diluted component C) at 37°C for 3 h.
MMP-2 (gelatinase)	1 mM APMA (diluted component C) at 37°C for 1 h.
MMP-3 (stromelysin)	1 mM APMA (diluted component C) at 37°C for 6 h.
MMP-7 (matrilysin, PUMP-1)	1 mM APMA (diluted component C) at 37°C for 1 h.
MMP-8 (neutrophil collagenase)	1 mM APMA (diluted component C) at 37°C for 1 h.
MMP-9 (92 kDa gelatinase)	1 mM APMA (diluted component C) at 37°C for 2 h.
MMP-10 (stromelysin 2)	1 mM APMA (diluted component C) at 37°C for 2 h.
MMP-12 (macrophage elastase)	1 mM APMA (diluted component C) at 37°C for 2 h.
MMP-13 (collagenase-3)	1 mM APMA (diluted component C) at 37°C for 40 min.
MMP-14	1 mM APMA (diluted component C) at 37°C for 2-3 h.

MMP-15	1 mM Mersalylic acid at 37°C for 30 min

References

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