SensoLyte® Generic MMP Assay Kit
Colorimetric

Revision Number: 1.1	*Last updated: October 2014*
Catalog #	**AS-72095**
Kit Size	100 Assays (96-well plate)

- ***Optimized Performance:*** This kit is optimized to detect MMP activity and screen for MMP inhibitors
- ***Enhanced Value:*** It provides enough reagents to perform 100 assays in a 96-well format.
- ***High Speed:*** The entire process can be completed in minimal time/
- ***Assured Reliability:*** Detailed protocol and references are provided.

Kit Components, Storage and Handling

Component	Description	Quantity
Component A	MMP colorimetric substrate	10 mM, 100 µL
Component B	Reference standard	10 mM, 10 µL
Component C	Assay Buffer	20 mL
Component D	MMP inhibitor	2 mM, 10 µL
Component E	Trypsin	1 mg/mL, 100 µL
Component F	Trypsin inhibitor	10 mg/mL, 100 µL
Component G	Stop Solution	5 mL

Other Materials Required (but not provided)

- MMPs source: Enzymes, validated in MMPs assays, can be ordered from AnaSpec: MMP-1 (Cat#72004), MMP-2 (Cat#72005), MMP-3 (Cat#72006), MMP-7 (Cat#72007), MMP-8 (Cat#72008), MMP-9 (Cat#72009), mMMP-9 (Cat#72069), MMP-10 (Cat#72067), MMP-12 (Cat#72010), MMP-13 (Cat#72011), and MMP-14 (Cat#72068).
- 96-well microplate: Clear microplates provide better signal for absorbance reading.
- Microplate reader: Capable of detecting absorbance at 412 nm.

Storage and Handling

- Store all kit components at -20°C.
- Aliquot Components E and F as needed to avoid freeze-thaw cycles.
- Store Component C at 4°C in the dark.
- Component G 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 and processing bioactive mediators.[1,2] Based on their role in normal and pathological processes, including embryogenesis, wound healing, inflammation, arthritis,[3] and cancer.[4,5] MMPs have been chosen as therapeutic targets for the treatment of many diseases.

The SensoLyte® Generic MMP Assay Kit can be used for the continuous spectrophotometric assay of MMPs activity, including MMP-1, 2, 3, 7, 8, 9, 12, 13, and 14 and for high throughput screening of MMP inducers and inhibitors. The chromogenic substrate is a thiopeptolide that is cleaved by the MMPs, releasing a sulfhydryl group. The sulfhydryl group reacts with Ellman's Reagent (5,5'-dithiobis(2-nitrobenzoic acid). The final product of this reaction, 2-nitro-5-thiobenzoic acid (TNB), can be detected at 412 nm using a microplate reader. The assays are performed in a convenient 96-well microplate format and can detect as low as nanogram level of active MMPs.

Protocol. Screening MMP inhibitors using purified enzymes

Note: For standard curve, please refer to Appendix II (optional).

1. Activate pro-MMPs.

1.1 Incubate pro-MMPs with 10μg/mL trypsin (Component E). Refer to Appendix III for information on incubation time. Activate pro-MMPs immediately before the experiment.

1.2 Add trypsin inhibitor (Component F) at a final concentration of 100 μg/mL.

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, thiol inhibitors, reducing agents (DTT, β-mercaptoethanol) will interfere with the assay. If APMA was used for MMPs activation it should be dialyzed out.

Note 3: It is preferable that the zymogen is activated by trypsin at higher protein concentration. After activation, you may dilute the enzyme for further experiment.

2. Prepare working solutions.

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

2.1 MMP substrate solution: Dilute MMP substrate (Component A) 1:50 in assay buffer (Component C). For each experiment prepare fresh substrate solution.

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

Components	Volume
MMP substrate (50X, Component A)	100 μL
Assay buffer (Component C)	4.9 mL
Total volume	5 mL

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

2.3 MMP inhibitor (GM6001): Dilute 2 mM inhibitor solution (Component D) to 20 μM in assay buffer (Component C). Add 10 μl of the 20 μM inhibitor solution into each of the inhibitor control well of a 96-well plate.

3. Set up the enzymatic reaction.

3.1 Add test compounds and diluted enzyme solution to the microplate wells. For one well of

96-well plate, the suggested volume of enzyme solution is 40 μL and 10 μL of test compound.

3.2 Simultaneously establish the following control wells, as deemed necessary:

- Positive control contains MMP enzyme without test compound.
- Inhibitor control contains MMP enzyme and a known MMP inhibitor.
- Vehicle control contains MMP 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).

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 MMP substrate solution into each well. For best accuracy, it is advisable to have the MMP substrate solution equilibrated to the assay temperature. Mix the reagents completely by shaking the plate gently for 30 sec.

4.2 Measure absorbance signal:

- For kinetic reading: Immediately start measuring absorbance reading at 412 nm continuously and record data every 10 min. for 60 min.
- For end-point reading: Incubate the reaction for 60 min. Optional: Add 50 μL of stop solution (Component G) to each well. Mix the reagents and measure absorbance at 412 nm.

4.3 For methods of data analysis: Refer to Appendix I.

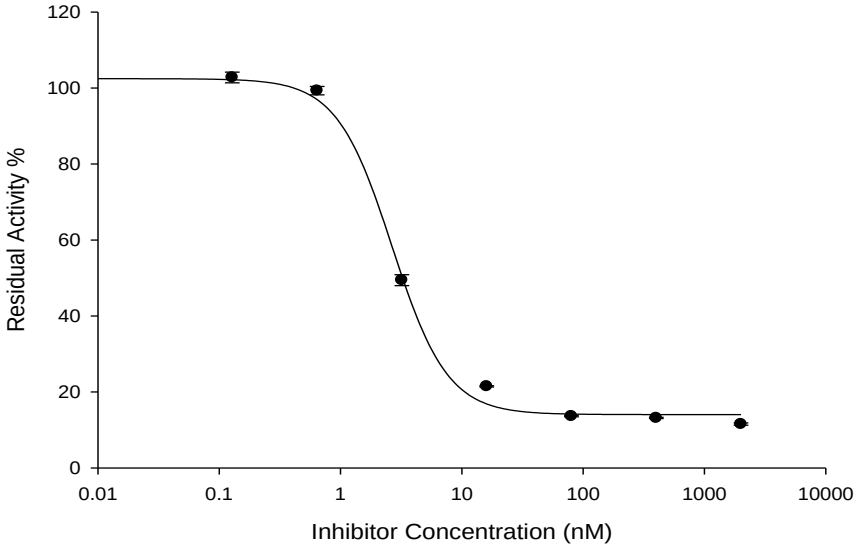

Figure 1. GM6001 inhibition of MMP-9 activity measured with SensoLyte® Generic MMP Assay Kit.

Appendix I. Data Analysis

- The absorbance reading from the substrate control well is used as the background absorbance. This background reading should be subtracted from the readings of the other wells containing substrate.
- For kinetics analysis:
  - Plot data as Abs (absorbance units) versus time for each sample. To convert absorbance to the concentration of the product of the enzymatic reaction, please refer to Appendix II for establishing a 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 Abs/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 Abs (absorbance units) 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

- Glutathione reference standard: Dilute 10 mM of reduced glutathione (Component B) to 200 μM in assay buffer (Component C). Do 2-fold serial dilutions to get concentrations of 100, 50, 25, 12.5, 6.25, 3.12 μM, include an assay buffer blank. Add 50 μL/well of these serially diluted reference solutions.

  Note 1: Store Component B at –20°C. Avoid freeze-thaw.

  Note 2: Reduced glutathione (GSH) is a tripeptide (g-glutamylcysteinylglycine) with a free thiol group, which reacts with Ellman's reagent and produces a yellow color. GSH can be used as a reference standard to calculate the concentration of SH groups in the sample.
- Add 50 μL/well of MMP substrate solution (refer to Step 2.1 for preparation).
- Measure absorbance at 412 nm.
- Plot Abs versus concentration as shown in Figure 2.
- The final concentrations of reference standard are 100, 50, 25, 12.5, 6.25, 3.12, 1.56, and 0 μM. This reference standard is used to calibrate the variation of different instruments and different experiments. It is also an indicator of final amount of SH groups in the enzymatic reaction.

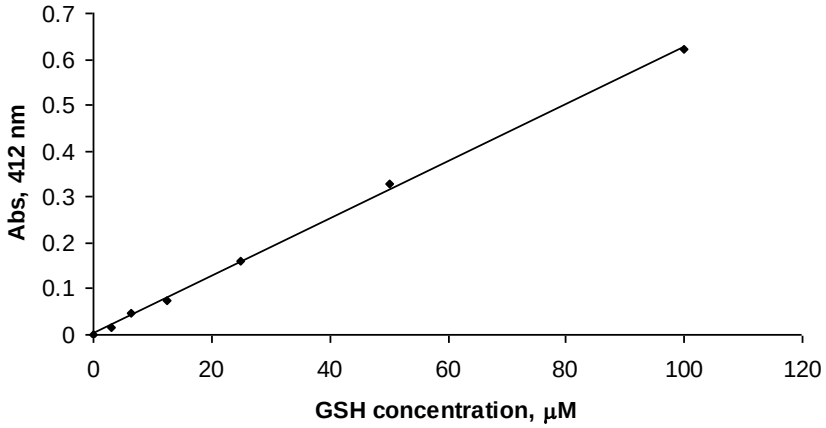

Figure 2. GSH reference standard, GSH was serially diluted in assay buffer, and the absorbance was measured at 412 nm (Ultra Microplate Reader EL808, Bio-Tek Instruments, Inc).

Appendix III.

Table 1. Activation of pro-MMPs

MMPs	Activation
MMP-1 (Interstitial collagenase)	Trypsin, 10 μg/mL, 20 min at room temperature
MMP-2 (Gelatinase A)	1 mM APMA, 1 hour at 37°C
MMP-3 (Stromelysin-1)	Trypsin, 10 μg/mL, 30 min at 37°C
MMP-7 (Matrilysin)	Trypsin, 10 μg/mL, 2 hours at 37°C
MMP-8 (Neutrophil collagenase)	Trypsin, 10 μg/mL, slow activation at 37°C
MMP-9 (Gelatinase B)	Trypsin, 10 μg/mL, 2 hours at 37°C
MMP-10 (Stromelysin-2)	Trypsin, 10 μg/mL, 30 min at 37°C
MMP-12 (Macrophage elastase)	Autolytic, refolding in assay buffer
MMP-13 (Collagenase-3)	Trypsin, 10 μg/mL, 30 min at 37°C
MMP-14 (MT1-MMP)	Trypsin, 5 μg/mL, 60 min at room temperature

References

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