SensoLyte® 520 Generic MMP Assay Kit *Fluorimetric*

Revision# 1.5	*Last Updated: November 2024*
Catalog #	**AS-71158**
Kit Size	100 Assays (96-well) or 250 Assays (384-well)

- ***Convenient Format:*** All essential assay components are included.
- ***Optimized Performance:*** Optimal conditions for the detection of generic MMP activity.
- ***Enhanced Value:*** Less expensive than the sum of individual components.
- ***High Speed:*** Minimal hands-on time.
- ***Assured Reliability:*** Detailed protocol and references are provided.

Kit Components, Storage and Handling

Component	Description	Quantity
Component A	Generic MMP substrate Ex/Em=490 nm/520 nm upon cleavage	60 µL
Component B	5-FAM-Pro-Leu-OH, fluorescence reference standard Ex/Em=490 nm/520 nm	1 mM, 10 µL
Component C	APMA, 4-aminophenylmercuric acetate	1 M, 20 µL
Component D	Assay buffer	20 mL
Component E	Stop solution	10 mL

Other Materials Required (but not provided)

- MMP Pro-enzymes & Catalytic domains:

  Note: *please, check AnaSpec website for details and select appropriate purified enzyme for your application.*
  MMP-1 (AS-72004), MMP-1 (AS-55575-1, 55575-10), MMP-2 (AS-72005), MMP-3 (AS-72006), MMP-7 (AS-72007), MMP-8 (AS-72008), MMP-9 (AS-55576-1, 55576-10, 55576-50), MMP-10 (AS-72067), MMP-12 (AS-55525-1, 55525-10, 55525-50), MMP-13 (AS-72257), and MMP-14 (AS-72068).
- 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

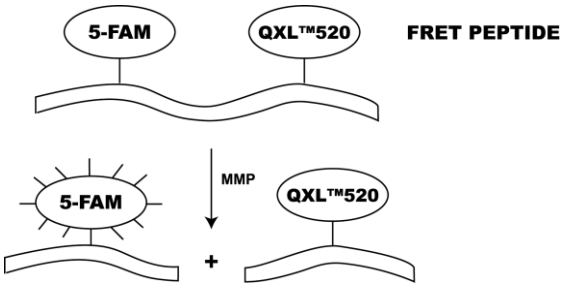
- Store all components at -20°C
- Protect Components A and B from light
- Components D and E can be stored at 4°C for convenience

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® 520 Generic MMP Assay Kit can detect the activity of several MMPs such as MMP-1, 2, 3, 7, 8, 9, 12, 13, and 14 (Figure 1). This kit is ideal for detecting generic MMP activity in biological samples or for high throughput screening of MMPs' inducers and inhibitors using purified MMPs.

This kit uses a 5-FAM/QXL™520 fluorescence resonance energy transfer (FRET)[6] peptide as a MMP substrate. In the intact FRET peptide, the fluorescence of 5-FAM is quenched by QXL™520. Upon cleavage into two separate fragments by MMPs (Scheme 1), the fluorescence of 5-FAM is recovered, and can be monitored at excitation/emission wavelengths = 490 nm/520 nm. With excellent fluorescence quantum yield and longer wavelength, 5-FAM shows less interference from autofluorescence of test compounds and cellular components and is much more sensitive than an EDANS/Dabcyl FRET substrate. The assay can detect as low as sub-nanogram level of active MMPs.



Scheme 1. Proteolytic cleavage of 5-FAM/QXL™ 520 FRET peptide by MMPs.

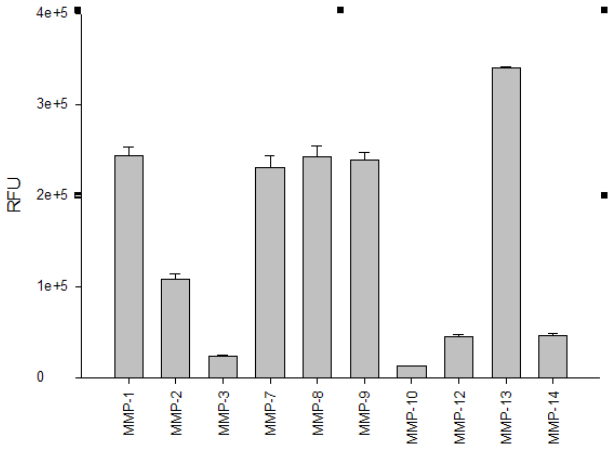


Figure 1. Detect the activity of MMPs using SensoLyte® 520 Generic MMP assay kit. The APMA-activated MMPs, 30 ng each, were mixed with 5-FAM/QXL™520 FRET peptide substrate. After 1 hr incubation, fluorescence signal was measured at Ex/Em = 490/520 nm (FlexStation 384II).

Protocol

Note 1: For instrument calibration, please refer to Appendix II (recommended for first time users).
Note 2: Please use protocol A or B based on your needs.

Protocol A. Screen 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 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 organic mercury. Handle with care! Dispose it according to appropriate regulations.

Note 3: It is preferred that the zymogen is activated by APMA 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 Generic MMP substrate solution: Dilute generic MMP substrate (Component A) 1:100 in assay buffer (Component D).

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 appropriate concentration in assay buffer (Component D).

3. Set up enzymatic reaction.

3.1 Add test compounds and MMP diluent into microplate. The suggested total volume of MMP diluent and test compound is 50 µL (96-well plate) or 20 µL (384-well plate)..

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 D), bring the total volume of all controls to 50 µL (96-well plate) or 20 µL (384-well plate).

3.4 Incubate the plate at the desired temperature for enzymatic reaction for 10-15 min. In the mean time, also incubate MMP-7 substrate solution at the same temperature.

4. Initiate the enzymatic reaction.

4.1 Add 50 µL (96-well plate) or 20 µL (384-well plate) of MMP substrate solution to each well. Mix the reagents completely by shaking the plate gently for 30-60 second.

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 minutes for 30 to 60 minutes.
- For end-point reading: Incubate the reaction at room temperature for 30 to 60 minutes. Keep the plate from direct light. Optional: Add 50 µL (96-well plate) or 20 µL (384-well plate) per well of stop solution (Component E). Mix the reagents. Then measure fluorescence intensity at Ex/Em=490 nm/520 nm.

4.3 Data analysis: Refer to Appendix I.

Protocol B. Measure MMPs activity in biological samples.

1. Prepare MMP containing biological samples

1.1 Collect synovial fluids or supernatant of cell culture media (e.g. stimulated fibroblast) and centrifuge for 10 - 15 min at 1000X g, 4°C. Collect the supernatant and store at -70°C until use.

1.2 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 10000x g at 4°C. Collect the supernatant and store at -70°C until use.

Note: *Triton-X 100 is not provided.*

2. Activate pro-MMPs

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

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

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

3. Prepare working solutions

Note: Warm 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).

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

Note: Pro-MMP needs to be activated before use, refer to Step 2.1. Avoid vigorous vortexing of enzyme.

4. Set up the enzymatic reaction

4.1 Add 50 µL/well (96-well plate) or 20 µL/well (384-well plate) of MMP containing sample.

4.2 Set up the following control:

- Substrate control contains assay buffer (50 µL/well for a 96-well plate or 20 µL/well for a 384-well plate)

5. Initiate the enzymatic reaction.

5.1 Add 50 µL/well (96-well plate) or 20 µL/well (384-well plate) of MMP substrate solution 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=490 nm/520 nm continuously and record data every 5 minutes for 30 to 60 minutes.
- For end-point reading: Incubate the reaction at room temperature for 30 to 60 minutes. Keep the plate away from direct light. Optional: Add 50 µL/well (96-well plate) or 20 µL/well (384-well plate) of stop solution (Component E). Mix the reagents, then measure fluorescence intensity at Ex/Em=490 nm/520 nm.

5.3 Data analysis: Refer to Appendix I.

Appendix I. Data Analysis

- The fluorescence reading from the substrate control well represents the background fluorescence. Subtract this background reading from the readings of the other wells to get the relative fluorescence unit (RFU).
- For kinetics reading:
  - 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 reading:
  - 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

- 5-FAM fluorescence reference standard: Dilute 1 mM 5-FAM-Pro-Leu-OH (Component B) to 5 μM in assay buffer (Component D). Perform 2-fold serial dilutions to get concentrations of 2.5, 1.25, 0.625, 0.3125, 0.156 and 0.078 μM, include an assay buffer blank. Add 50 μL/well (96-well plate) or 20 μL/well (384-well plate) of these serially diluted 5-FAM-Pro-Leu-OH reference solutions.
- Add 50 μL/well (96-well plate) or 20 μL/well (384-well plate) of MMP substrate solution (refer to Protocol A, step 2.1 for preparation).

  Note: The MMP substrate solution should be added to the 5-FAM reference standard to correct for the fluorescence inner filter effect.
- Optional: If the stop solution (Component E) was added into the enzymatic reaction before taking the end-point reading, the same volume of stop solution should be added to the reference standard wells to obtain a better comparison.
- Measure the fluorescence intensity of the reference standard wells at Ex/Em=490 nm/520 nm. Use the same setting of sensitivity as used in the enzyme reaction.
- Plot 5-FAM fluorescence reference standard as RFU (relative fluorescence unit) versus concentration as Figure 2.

  Note: The final concentrations of the 5-FAM-Pro-Leu-OH reference standard solutions are 2.5, 1.25, 0.625, 0.3125, 0.156, 0.078, 0.039, and 0 μM. This reference standard curve is used to calibrate for the variation of different instruments and for different batches of experiments. It is also an indicator of the amount of MMP enzymatic reaction final product.

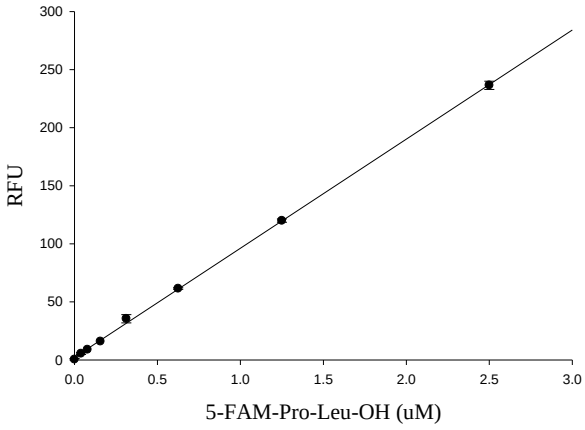


Figure 2. The reference standard curve
5-FAM-Pro-Leu-OH was serially diluted in assay buffer containing 520 MMP generic substrate and fluorescence was recorded at Ex/Em=490 /520 nm (FLx800, Bio-Tek Instruments). Mean ± S.D., n=2.

Appendix III: MMPs activation chart

Table 1: 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 3-6 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.

References

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