SensoLyte® 520 MMP-8 Assay Kit
Fluorimetric

Revision Number: 1.2	*Last updated: July 2021*
Catalog #	**AS-71154**
Kit Size	100 Assays (96-well plate)

- ***Convenient Format:*** All essential assay components are included.
- ***Optimized Performance:*** Optimal conditions for the detection of MMP-8 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	MMP-8 substrate 5-FAM/QXL™520 FRET peptide 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)

- Recombinant MMP-8: AnaSpec Cat#72008.
- 96-well microplate: Black, flat-bottom plate with non-binding surface.
- Fluorescence microplate reader: Capable of detecting emission at 520 nm with excitation at 490 nm.

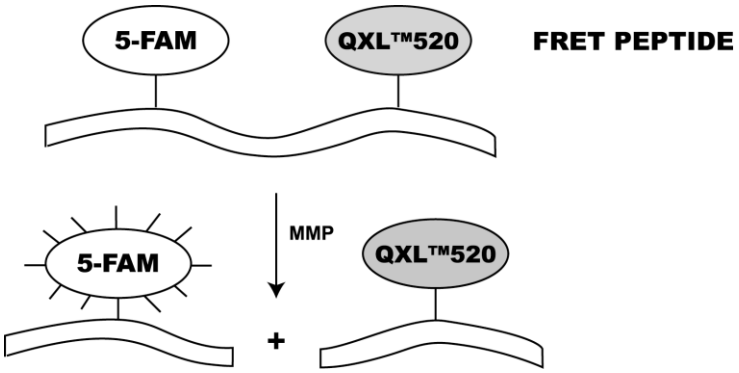
Storage and Handling

- Store all components at -20°C
- Protect Components A and B from light and moisture
- 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. The importance of MMPs in tumor development and invasion as well as other diseases is well known. MMP-8 (neutrophil collagenase) is proposed as a potential anti-cancer drug target.

The SensoLyte® 520 MMP-8 Assay Kit provides a convenient assay for high throughput screening of MMP-8 inducers and inhibitors. It detects MMP-8 activity in a variety of biological samples using a 5-FAM/QXL™520 fluorescence resonance energy transfer (FRET)[1] peptide. In the intact FRET peptide, the fluorescence of 5-FAM is quenched by QXL™520. Upon cleavage into two separate fragments by MMP-8 (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. Compared to an EDANS/DABCYL FRET substrate, this 5-FAM/QXL™520 substrate provides better assay sensitivity.



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

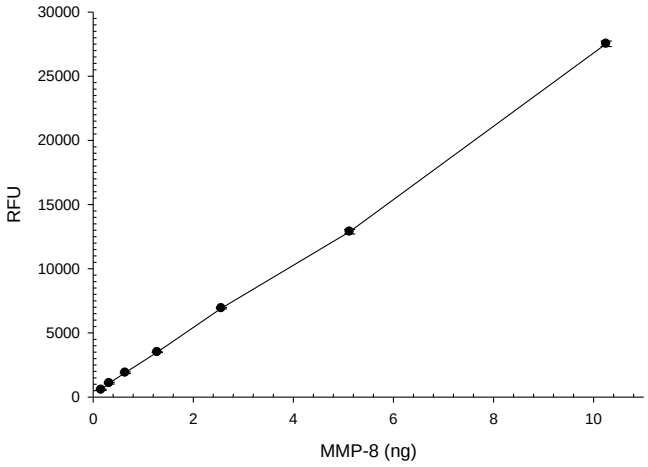


Figure 1. Sensitivity of SensoLyte® 520 MMP-8 assay kit.

Recombinant active MMP-8 was serially diluted in assay buffer. Enzyme at each dilution was mixed with MMP-8 FRET substrate and then incubated at RT for 30 min. Endpoint fluorescence signal was recorded at Ex/Em=490 nm/520 nm with cut off at 515 nm (FlexStation 384II, Molecular Devices). The assay is able to detect as low as 0.2 ng of active MMP-8 (mean±S.D., n=2). Note: the sensitivity also depends on the endogenous activity of MMP-8 from different preparation. MMP-8 from different sources will vary in its endogenous activity.

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 protease inhibitors using purified or recombinant MMP-8

1. Activate pro-MMP-8.

1.1 Incubate pro-MMP-8 with 1 mM APMA (diluted Component C) for 1 h at 37°C. Activate pro-MMP-8 immediately before the experiment.
Note 1: Keep activated enzyme on ice. Avoid vigorously vortexing the enzyme. Prolonged storage of activated enzyme will further deactivate 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 according to appropriate regulations.
Note 3: Activation of zymogen by APMA at higher protein concentration is preferred. After activation, the enzyme may be further diluted.

2. Prepare working solutions.

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

2.1 MMP-8 substrate solution: Dilute MMP-8 substrate (Component A) 1: 100 in assay buffer (Component D) according to Table 1.

Table 1. MMP-8 substrate solution for one 96-well plate (100 assays)

Components	Volume
MMP-8 substrate (100X, Component A)	50 µL
Assay buffer (Component D)	4.95 mL
Total volume	5 mL

2.2 MMP-8 diluent: Dilute activated MMP-8 to an appropriate concentration in assay buffer (Component D).

3. Set up enzymatic reaction.

3.1 Add test compounds and MMP-8 diluent into a microplate. The suggested total volume of MMP-8 diluent and test compound is 50 µL/well.

3.2 Simultaneously set up the following controls:

- Positive control contains MMP-8 diluent without test compound.
- Inhibitor control contains MMP-8 diluent and a known MMP-8 inhibitor.
- Vehicle control contains MMP-8 diluent and vehicle used in delivering 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/well.

4. Pre-incubation.

4.1 Incubate the plate at the desired temperature for enzymatic reaction for 10-15 min. Also incubate MMP-8 substrate solution at the same temperature.

5. Initiate the enzymatic reaction.

5.1 Add 50 µL of MMP-8 substrate solution to each well. Mix the reagents completely by shaking the plate gently for 30-60 sec.

5.2 Measure fluorescence signal:
For kinetic reading: Immediately start measuring fluorescence intensity at Ex/Em=490 /520 nm and continuously record data every 5 min for 30 to 60 min.
For end-point reading: Incubate the reaction at 37°C for 30 to 60 min. Keep plate from direct light. Optional: Add 50 µL/well of stop solution (Component E). Mix the reagents. Then measure fluorescence intensity at Ex/Em=490/520 nm.

5.3 Data analysis: Refer to Appendix I.

Protocol B. Measuring MMP-8 activity in biological samples

Note: The FRET substrate in this kit can also be cleaved by MMP-1, 2, 12, and 13. If several MMPs are coexisting in your samples and you want to specifically measure MMP-8's activity, then MMP-8 must first be isolated by immunoaffinity purification or other methods before measuring its specific activity using the current assay kit.

1. Prepare MMP-8 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 1,000X 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 with APMA (Component C) at a final concentration of 1 mM for 1 h at 37°C. Activate MMP immediately before the experiment.
Note 1: Keep activated enzyme on ice. Avoid vigorously vortexing the enzyme. Prolonged storage 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 and must be handled with care! Dispose 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-8 substrate solution: Dilute MMP-8 substrate (Component A) 1: 100 in assay buffer (Component D) according to Table 1.

Table 1. MMP-8 substrate solution for one 96-well plate (100 assays)

Components	Volume
MMP-8 substrate (100X, Component A)	50 µL
Assay buffer (Component D)	4.95 mL
Total volume	5 mL

3.2 MMP-8 diluent: If you use purified MMP-8 as a positive control, then dilute MMP-8 to an appropriate concentration in assay buffer (Component D).

Note: Pro-MMP-8 needs to be activated by APMA at higher protein concentration, and then be diluted to a working concentration in assay buffer. Please refer to Step 2. Avoid vigorous vortexing of enzyme.

4. Set up the enzymatic reaction.

4.1 Add 50 µL/well of MMP-8 containing sample.

4.2 Set up the following control:
- Substrate control contains assay buffer (50 µL/well)
- Positive control contains MMP-8 diluent (50 µL/well)

5. Initiate the enzymatic reaction.

5.1 Add 50 µL/well of MMP-8 substrate solution to the sample and control wells. Mix the reagents by shaking the plate gently for 30 sec.

5.2 Measure fluorescence signal:
For kinetic reading: Immediately start measuring fluorescence intensity at Ex/Em=490 /520 nm and continuously record data every 5 min for 30 to 60 min.
For end-point reading: Incubate the reaction at 37°C for 30 to 60 min. Keep plate from direct light. Optional: Add 50 µL/well of stop solution (Component E). Mix the reagents and measure fluorescence intensity at Ex/Em=490/520 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 should be subtracted from the readings of the other wells. The fluorescence readings are expressed in relative fluorescence units (RFU).
- For kinetic 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. Typically, 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 (Figure 1).
  - 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. Do 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 of these serially diluted 5-FAM-Pro-Leu-OH reference solutions.

- Add 50 µL/well of MMP-8 substrate solution (refer to Protocol A, step 2.1 for preparation).

  Note: MMP-8 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.
- If the stop solution (Component E) was added to the enzymatic reaction before taking the end-point reading, the same volume of stop solution should be added to reference standard wells for proper 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 in **Figure 2**.

  Note: The final concentrations of 5-FAM-Pro-Leu-OH reference standards are 2.5, 1.25, 0.625, 0.3125, 0.156, 0.078, 0.039, and 0 µM. This reference standard is used to calibrate the variation of different instruments and to account for experimental variability. It is also an indicator of the amount of final product of the MMP-8 enzymatic reaction.

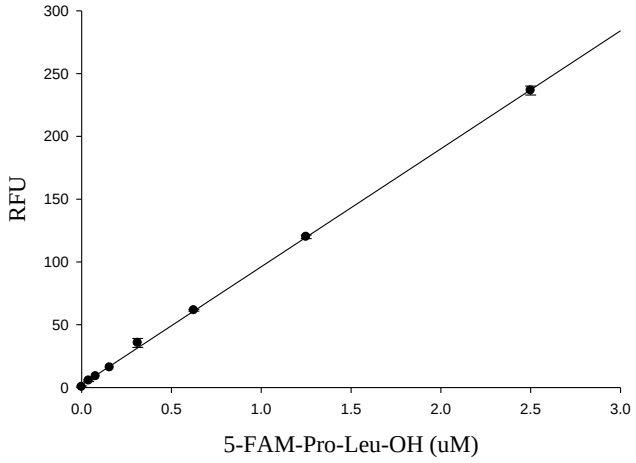


Figure 2. 5-FAM-Pro-Leu-OH reference standard curve

5-FAM-Pro-Leu-OH was serially diluted in assay buffer according to the protocol. The fluorescence was monitored by a fluorescence microplate reader (Bio-Tek FLx800) with a filter set of Ex/Em=485±20 nm/528±20 nm. (mean ±S.D., n=2).

References

1. Stryer, L. *Annu. Rev. Biochem.* **47**, 819 (1978).