SensoLyte® 520 MMP-13 Assay Kit
Fluorimetric

Revision number: 1.2	*Last updated: October 2014*
Catalog #	**AS-71156**
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 MMP-13 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-13 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 *Caution: Organic mercury. Handle with care! Do not dispose into sink!*	1 M, 20 µL
Component D	Assay buffer	20 mL
Component E	Stop solution	10 mL

Other Materials Required (but not provided)

- Purified MMP-13: AnaSpec Cat#72011.
- 96-well or 384-well microplate: Black, flat-bottom 96-well or 384-well plate with non-binding surface.
- Fluorescence microplate reader: Capable of detecting emission at 520 nm with excitation at 490 nm

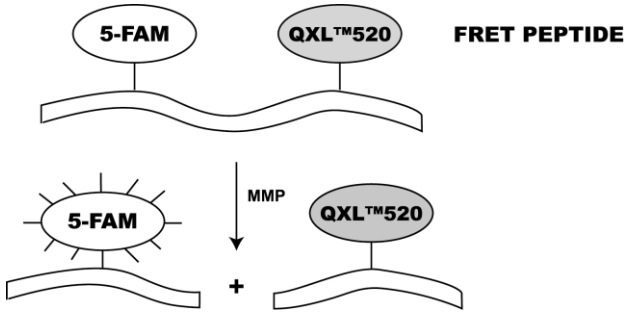
Storage and Handling

- Store all kit 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

The matrix metalloproteinases (MMPs) constitute a family of zinc-dependent endopeptidases that function within the extracellular matrix. These enzymes are responsible for the breakdown of connective tissues and are important in bone remodeling, menstrual cycle and the repair of tissue damage. MMP-13 (collagenase-3)[1] is a member of the MMP family of extracellular proteases. Targets of MMP-13 include collagen, gelatin, aggrecan, plasminogen and CXCL12. MMP-13 is secreted as a 60-kDa proenzyme (as measured by SDS-PAGE), and activated by cleavage to a mature 48-kDa MMP-13. MMP-13 is an important target for inhibitor screening due to its involvement in diseases such as cancer and arthritis.

The SensoLyte® 520 MMP-13 Assay Kit provides a convenient assay for high throughput screening of MMP-13 inducers and inhibitors. It also detects MMP-13 activity in a variety of biological samples using a 5-FAM/QXL™520 fluorescence resonance energy transfer (FRET)[2] peptide. In the intact FRET peptide, the fluorescence of 5-FAM is quenched by QXL™520. Upon cleavage into two separate fragments by MMP-13 (**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 auto fluorescence of test compounds and cellular components. This 5-FAM/QXL™520 substrate can detect the activity of nanogram of MMP-13 (**Figure 1**).



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

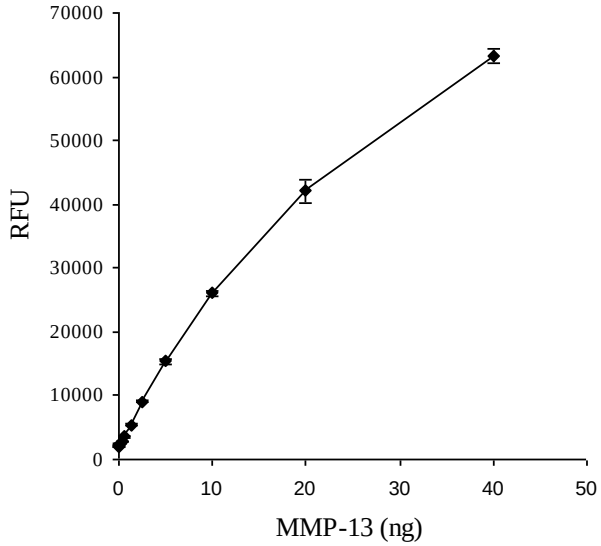


Figure 1. Sensitivity of the SensoLyte® 520 MMP-13 assay kit

Purified MMP-13 was first activated with 1mM APMA and then serially diluted in assay buffer. The enzyme at each dilution was mixed with MMP-13 FRET substrate and then incubated at 37ºC for 60 min. The endpoint fluorescence signal was recorded at Ex/Em=490 nm/520 nm with cut off 515 nm on FlexStation 384II (Molecular Device, CA). The assay is able to detect as low as 0.4 ng/mL of MMP-13 (mean ± S.D., n=3). Note: the sensitivity also depends on the endogenous activity of MMP-13 in different preparations. MMP-13 from different sources might vary in its endogenous activity.

Protocol

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

Protocol A. Screening protease inhibitors using purified or recombinant MMP-13.

1. Activate pro-MMP-13.

1.1 Incubate pro-MMP-13 with 1 mM APMA (diluted Component C) for 40 min at 37°C. Activate pro-MMP-13 immediately before the experiment.
Note 1: Keep activated enzyme on ice. Avoid vigorous vortex of 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 and must be handled 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-13 substrate solution: Dilute MMP-13 substrate (Component A) 1: 100 in assay buffer (Component D).

Table 1. MMP-13 substrate solution for one 96-well plate (100 assays)

Components	Volume
MMP-13 substrate (100X, Component A)	50 µL
Assay buffer (Component D)	4.95 mL
Total volume	5 mL

2.2 MMP-13 diluent: Dilute activated MMP-13 to an appropriate concentration in assay buffer (Component D).

3. Set up enzymatic reaction.

3.1 Add test compounds and MMP-13 diluent into microplate. The suggested total volume of MMP-13 diluent and test compound is 50 µL (96-well plate) or 20 µL (384-well plate).

3.2 Simultaneously set up the following controls:

- Positive control contains MMP-13 diluent without test compound.
- Inhibitor control contains MMP-13 diluent and a known MMP-13 inhibitor.
- Vehicle control contains MMP-13 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 (96-well plate) or 20 µL (384-well plate).

4. Pre-incubation.

4.1 Incubate the plate at the desired temperature for enzymatic reaction for 10-15 min. Also incubate MMP-13 substrate solution at the same temperature.

5. Initiate the enzymatic reaction.

5.1 50 µL (96-well plate) or 20 µL (384-well plate) of MMP-13 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 (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=490/520 nm.

5.3 Data analysis: Refer to Appendix I.

Protocol B. Measuring MMP-13 activity in biological samples.

Note: According to our data, the cleavage rate of MMP-13 on the FRET substrate in this kit is 5-50 fold faster rate than those of MMP-1, 2, 3, 7, 8, 9, 12, and 14. If several MMPs are coexisting in your samples and you want to specifically measure MMP-13's activity, please choose SensoLytePlus® MMP-13 assay kit (AnaSpec Cat# 72019). Alternatively, MMP-13 can be isolated by immuno-affinity purification or other methods first before measuring its specific activity using the current assay kit.

1. Prepare MMP-13 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 in the assay buffer (Component D) for 40 min at 37°C. Activate MMP immediately before the experiment.

Note 1: Keep activated enzyme on ice. Avoid vigorous vortex of 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-13 substrate solution: Dilute MMP-13 substrate (Component A) 1: 100 in assay buffer (Component D).

Table 1. MMP-13 substrate solution for one 96-well plate (100 assays)

Components	Volume
MMP-13 substrate (100X, Component A)	50 µL
Assay buffer (Component D)	4.95 mL
Total volume	5 mL

3.2 MMP-13 diluent: If you use purified MMP-13 as a positive control, then dilute MMP-13 to an appropriate concentration in assay buffer (Component D).

Note: Pro-MMP-13 needs to be activated by APMA at higher protein concentration, and then diluted to a working concentration in assay buffer. Please refer to Step 2. Avoid vigorous vortex 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-13 containing sample.

4.2 Set up the following control:

- Substrate control contains assay buffer [50 µL/well (96-well plate) or 20 µL/well (384-well plate)].
- Positive control contains MMP-13 diluent [50 µL/well (96-well plate) or 20 µL/well (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-13 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 /520nm 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 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 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 represents the background fluorescence. Subtract this background reading from the readings of the other wells to get the relative fluorescence unit (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. 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 (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-13 substrate solution (refer to Protocol A, step 2.1 for preparation).

  Note: The MMP-13 substrate solution should be added to the 5-FAM reference standard to correct the absorptive quenching by the FRET peptide. If multiple concentrations of substrate are used, this step must be performed for each concentration.
- 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 in **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-13 enzymatic reaction final product.

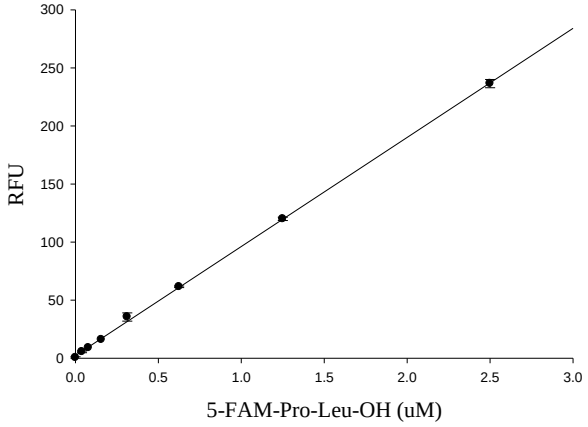


Figure 2. . 5-FAM-Pro-Leu-OH reference standard curve

5-FAM-Pro-Leu-OH was serially diluted in assay buffer containing 520 MMP-13 substrate and fluorescence was recorded at Ex/Em=490 /520 nm (FLx800, Bio-Tek Instruments). Mean ± S.D., n=2.

References

1. Freije, JM. et al. *J. Biol. Chem.* **269**, 16766 (1994).
2. Stryer, L. et al. *Annu. Rev. Biochem.* **47,** 819 (1978).