SensoLyte® 520 MMP-14 Assay Kit

Fluorimetric

Revision Number: 1.1	*Last updated: October 2014*

Catalog #	**AS-72025**
Kit Size	100 Assays (96-well)

- ***Optimized Performance:*** Optimal conditions for the detection of MMP-14 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-14 substrate 5-FAM/QXL™520 FRET peptide Ex/Em=490nm/520nm 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)

- Recombinant MMP-14: AnaSpec Cat# 72068.
- 96-well: Black microplate provides better signal to noise ratio.
- Fluorescence microplate reader: Capable of detecting emission at 520 nm with excitation at 490nm.

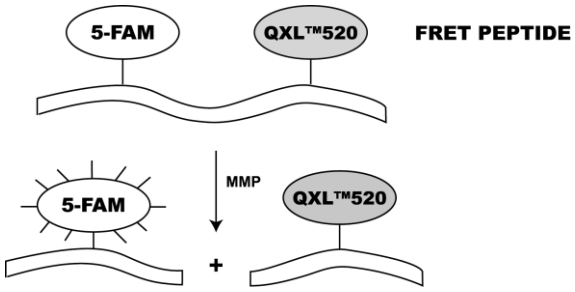
Storage and Handling

- Store all kit components at -20°C.
- Keep Components A and B from light.
- 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] MMP-14 (MT1-MMP), membrane-type MMP, and plays an important role in tumor invasion. MMP-14 is expressed on the surface of invasive tumor cells,[3] in stromal cells of human colon, breast, and head and neck carcinomas.[4] Human MT1-MMP consists of 559 amino acid residues with a calculated Mr of ~63000 kDa.[3] MMP-14 is secreted as a zymogen with a prodomain, a catalytic domain, a hinge region, a hemopexin-like domain, and a transmembrane domain. It can activate pro-MMP-2, [3] pro-MMP-13,[5] and degrade a variety of substrates, including fibrillar collagens I, II, III, fibronectin, vitronectin and laminin-1.[6, 7]

The SensoLyte® 520 MMP-14 Assay Kit provides a convenient assay for high throughput screening of MMP-14 inducers and inhibitors. It detects MMP-14 activity in a variety of biological samples using a 5-FAM/QXL™520 fluorescence resonance energy transfer (FRET)[8] peptide. In the intact FRET peptide, QXL™520 quenches the fluorescence of 5-FAM. Upon cleavage into two separate fragments by MMP-14 (**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. The assays are performed in a convenient 96-well microplate format.



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

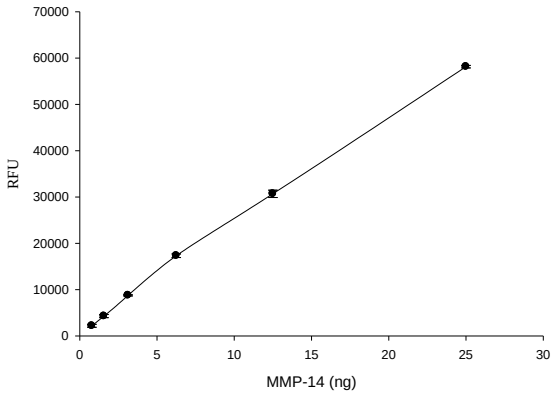


Figure 1. Sensitivity of the SensoLyte® 520 MMP-14 assay kit.
Activated MMP-14 was serially diluted in assay buffer. Enzyme at each dilution was mixed with MMP-14 FRET substrate and then incubated at 37°C for 60 min. Endpoint fluorescence signal was recorded at Ex/Em=490nm/520nm with cut off at 515nm. (FlexStation 384II, Molecular Devices). The assay is able to detect as low as 0.2 ng of active MMP-14.

Protocol

Note 1: For fluorometer 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. Screening protease inhibitors using purified or recombinant MMP-14

1. Activate pro-MMP-14.

1.1 Incubate pro-MMP-14 with 1 mM APMA (diluted Component C) for 2-3 hours at 37°C. Activate pro-MMP-14 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 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-14 substrate solution: Dilute MMP-14 substrate (Component A) 1: 100 in assay buffer (Component D) according to Table 1.

Table 1. MMP-14 substrate solution for one 96-well plate (100 assays).

Components	Volume
MMP-14 substrate (Component A)	50 µL
Assay buffer (Component D)	4.95 mL
Total volume	5 mL

2.2 MMP-14 diluent: Dilute activated MMP-14 to an appropriate concentration in assay buffer (Component D).

3. Set up enzymatic reaction.

3.1 Add test compounds and MMP-14 diluent into a microplate. For one well of a 96-well plate, the suggested volume of enzyme solution is 40 µL and 10 µL of test compound.

3.2 Simultaneously set up the following controls:

- Positive control contains MMP-14 diluent without test compound.
- Inhibitor control contains MMP-14 diluent and a known MMP-14 inhibitor.
- Vehicle control contains MMP-14 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.

3.3 Use assay buffer (Component D) to bring the total volume of all the controls to 50 µL.

4. Pre-incubation.

4.1 Incubate plate at the desired temperature for enzymatic reaction (e.g. 25°C or 37°C) for 10-15 min. Also incubate MMP-14 substrate solution at the same temperature.

5. Initiate the enzymatic reaction.

5.1 Add 50 µL of MMP-14 substrate solution to each well

5.2 Mix the reagents completely by shaking the plate gently for 30-60 sec.

5.3 Measure fluorescence signal:

- For kinetic reading: Immediately start measuring fluorescence intensity at Ex/Em=490 +20 nm/520+20 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 per well of stop solution (Component E). Mix the reagents. Then measure fluorescence intensity at Ex/Em=490 +20 nm/520+20 nm.

6. Data analysis: Refer to Appendix I.

Protocol B. Measuring MMP-14 activity in biological samples

Note: The FRET substrate in this kit can also be cleaved by MMP- 1, 2, 7, 8, 12, and 13. If several MMPs are coexisting in your samples and you want to measure the activity of MMP-14, then MMP-14 must first be isolated by immuno-affinity purification or other methods before measuring its specific activity using the current assay kit.

1. Prepare MMP-14 containing biological samples.

1.1 Prepare cell lysates:

- Wash cells with PBS
- Add an appropriate amount of assay buffer (Component D) containing 0.1% (v/v) Triton-X 100 to cells or cell pellet. Collect the cell suspension in a microcentrifuge tube.
- Incubate the cell suspension at 4°C for 10 min.
- Centrifuge the cell suspension for 10 min at 2,500X g, 4°C. Collect the supernatant and store at - 70°C until use.
- Tissue samples should be homogenized in assay buffer (Component D) containing 0.1% (v/v) Triton-X 100, and then incubated for 15 min at 4°C. Centrifuge for 15 min at 2,000xg at 4°C and collect the supernatant. Store at -70°C until use.

Note 1: Triton-X 100 is not provided.
Note 2: To detect MMP-14 activity in cell culture the assay may need optimization. For example the cells could be incubated with substrate in PBS or serum free media.

2. Activate pro-MMPs.

2.1 Incubate the MMP containing-samples with APMA (Component C) at a final concentration of 1 mM for 2-3 hours 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.
Note 3: Pro-MMP-14 needs to be activated by APMA at higher protein concentration, and then be diluted to a working concentration in assay buffer. Avoid vigorous vortexing of enzyme.

3. Prepare working solutions.

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

3.1 MMP-14 substrate solution: Dilute MMP-14 substrate (Component A) 100-fold in assay buffer (Component D) according to Table 1.

Table 1. MMP-14 substrate solution for one 96-well plate (100 assays)

Components	Volume
MMP-14 substrate (Component A)	50 µL
Assay buffer (Component D)	4.95 mL
Total volume	5 mL

3.2 MMP-14 diluent: If you use purified MMP-14 as a positive control, then dilute MMP-14 to an appropriate concentration in assay buffer (Component D).

4. Set up the enzymatic reaction.

4.1 Add 50 µL/well of MMP-14 containing sample.

4.2 Set up the following control:

- Substrate control contains assay buffer (50 µL/well).
- Positive control contains MMP-14 diluent (50 µL/well).

5. Initiate the enzymatic reaction.

5.1 Add 50 μL/well of MMP-14 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 nm+20 /520+20 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+20 nm/520+20 nm

6. 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 the 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 will 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
  - A variety of data analyses can be done, e.g., determining inhibition %, EC_{50}, IC_{50}, etc.

Appendix II. Fluorometer Calibration

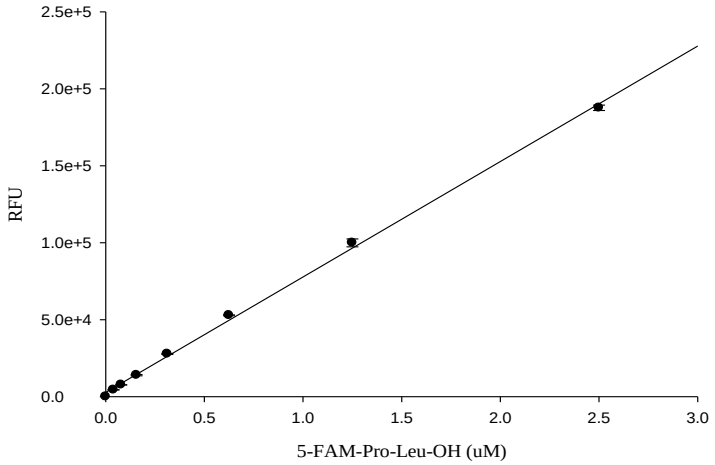
- 5-FAM fluorescence reference standard: Dilute 1 mM 5-FAM-Pro-Leu-OH (Component B) to 5 μM in assay buffer. Do 2-fold serial dilutions to get concentrations of 2.5, 1.25, 0.625, 0.3125, 0.156, and 0.078, include a water blank. Add 50 μL/well of the serially diluted 5-FAM-Pro-Leu-OH from 5 μM to 0 nM.
- Add 50 μL/well of MMP-14 substrate solution (refer to standard operation protocol step 3 for preparation).

Note: MMP-14 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.

- Optional: 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.
- Plot 5-FAM fluorescent reference standard as RFU (relative fluorescence unit) versus concentration as in **Figure 2**.

Note: The final concentration of 5-FAM-Pro-Leu-OH reference standard is 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-14 enzymatic reaction.

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



5-FAM-Pro-Leu-OH was diluted in assay buffer containing MMP-14 substrate. Fluorescence signal was measured at Ex/Em=490nm/520nm with cut off 515nm (FlexStation 384II, Molecular Devices).

References:

1. Woessner, JF. Jr. and CJ. Taplin, *J Biol Chem* **263**, 16918 (1988).
2. Woessner, JF. Jr. *FASEB J* **5**, 2145 (1991).
3. Sato, T. et al., *Nature* **370**, 61 (1994).
4. Okada, A. et al., *PNAS* **92**, 2730 (1995).
5. Ohuchi, E. et al. *J Biol Chem* **272**, 2446 (1997).
6. Pei, D. and SJ. Weiss. *J Biol Chem* **271**, 9135 (1996).
7. Knight, CG. et al. *FEBS Lett* **296**, 263 (1992).
8. Stryer, L. *Annu Rev Biochem* **47**, 819 (1978).