SensoLyte® 520 MMP Substrate Sampler Kit

Fluorimetric

Revision Number: 1.1	*Last updated: October 2014*
Catalog #	**AS-71170**
Kit Size	16 X 20 assays

- ***Convenient Format:*** All essential assay components are included.
- ***Optimized Performance:*** Optimal conditions for the screening of the best peptide substrate for MMPs.
- ***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	16 different MMP substrates Refer to Appendix I, Table 1 for peptide sequence. Ex/Em=490 nm/520 nm upon cleavage	100 µM, 150 µL each
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, 100 µL
Component D	Assay buffer	50 mL
Component E	Stop solution	30 mL

Other Materials Required (but not provided)

- MMPs: Purified enzymes can be ordered from AnaSpec. MMP-1 (Cat##55575-1, 55575-10, 55575-50, 72004), MMP-2 (Cat#72005), MMP-3 (Cat#72006), MMP-7 (Cat#72007), MMP-8 (Cat#72008), MMP-9 (Cat##55576-1, 55576-10, 55576-50), MMP-12 (Cat##55525-1, 55525-10, 55525-50, 72010), MMP-13 (Cat#72011).
- Microplate: Black, flat-bottom, 96-well plate with non-binding surface.
- 96-well microplate: Black microplate provides better signal to noise ratio.

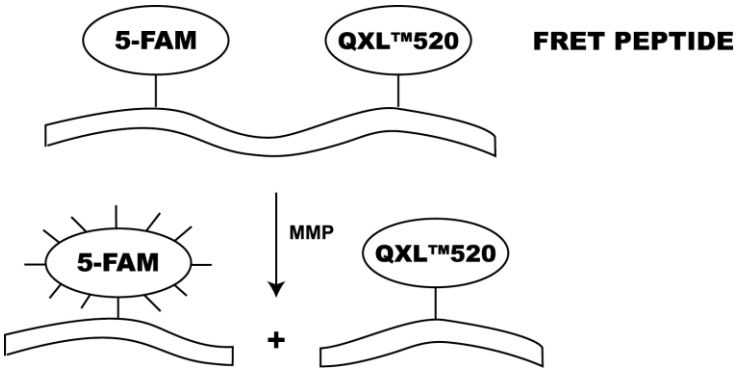
Storage and Handling

- Store all components at –20°C
- Keep Components A and B from light

Introduction

Matrix Metalloproteinases (MMPs) belong to a family of secreted or membrane-associated zinc endopeptidases capable of digesting extracellular matrix components[1;2]. These enzymes are responsible for connective tissues breakdown, bone remodeling, and damaged tissue repair. They are also involved in a number of diseases, such as tumor development and metastasis[3-6] as well as in rheumatoid arthritis[7;8]. They are proposed as the therapeutic target for these diseases.

The SensoLyte® MMP Substrate Sampler Kit provides 16 literature-documented FRET[9] peptide substrates (Appendix II, Table 1). It provides a convenient platform for profiling substrate specificity of MMPs and optimizing assay condition for MMPs. 5-FAM and QXL™520, a pair of optimal fluorophore and quencher, are used in these FRET peptides. In an intact FRET peptide, the fluorescence of 5-FAM is quenched by QXL™520. Upon MMP cleavage of the FRET peptides into two separate fragments, the fluorescence of 5-FAM is recovered, and can be monitored at excitation/emission = 490 nm/520 nm (Scheme 1). With excellent fluorescence quantum yield and longer excitation and emission wavelength, the fluorescence signal of 5-FAM is less interfered by the autofluorescence of test compounds and cellular components, thus providing better assay sensitivity.



Scheme 1. Proteolytic cleavage of the 5-FAM and QXL™520 paired FRET peptide.

Protocol

Note 1: Activate MMP (zymogen) by treating with 1 mM APMA (diluted Component C) in assay buffer (Component D). For detailed procedures, please refer to Appendix III, Table 1. Prepare freshly activated MMP and keep them on ice before each experiment. APMA belongs to organic mercury. Handle with care! Do not dump into sink!

Note 2: Warm all the kit components until thawed to room temperature before starting the experiments.

1. Prepare working solutions.

1.1 MMP substrate solution: Dilute MMP substrate (Component A) 1:10 in assay buffer (Component D).

1.2 Fluorescence reference standard: Dilute the 1 mM 5-FAM-Pro-leu-OH (Component B) to 2 μM in assay buffer (Component D). Do 1:2 serial dilutions to get concentrations of 1, 0.5, 0.25, 0.125, 0.0625, 0.031, and 0.016 μM.

1.3 Prepare MMPs:

If you use purified MMPs: Dilute activated MMP to appropriate concentration in assay buffer (Component D). Each well in a 96-well microplate will need 50 μL of diluted enzyme. Calculate an appropriate amount of MMP diluent for all your wells.

If you use MMP-containing biological sample: Each well in a 96-well microplate will need 50 μL of sample.

Note: The MMP-containing biological samples may need pre-activation by 1 mM APMA (diluted Component C). For detailed procedures, please refer to Appendix III, Table 1

2. Add reagents prepared in Step 1 into a black 96-well microplate according to Tables 2 and 3.

Table 2. The layout of the samples in a 96-well microplate.

	1	2	3	4	5	6	7	8	9	10	11	12
A	RS1	RS1	SC 1	SC 1	SP 1	SP 1						
B	RS2	RS2	SC 2	SC 2	SP2	SP2						
C	RS3	RS3	SC 3	SC3	SP3	SP3						
D	RS4	RS4	…	…	…	…						
E	RS5	RS5										
F	RS6	RS6										
G	RS7	RS7										
H	RS8	RS8										

Note 1: RS=Reference standard, SC= substrate control, SP=sample.

Note 2: We suggest performing at least two parallel assays for each sample.

Table 3. Reagent composition for each well.

Reference Standard		**Substrate Control****		**Sample**	
5-FAM-Pro-leu-OH (Serially diluted)*	100 μL	MMP substrate solution	50 μL	MMP substrate solution	50 μL
		Assay buffer	50 μL		
Total volume	100 μL	Total volume	100 μL	Total volume	50 μL

Note: *Add the serially diluted 5-FAM-Pro-leu-OH from 2 μM to 0.016 μM into wells RS1 through to RS8.

** For each MMP substrate used, a substrate control containing only the substrate without any enzyme added should be set up.

3. Calibrate the fluorescence microplate reader.

3.1 Measure the fluorescence intensity of the reference standard (RS) at Ex/Em=490 nm/520 nm. Adjust the sensitivity of the microplate reader until satisfactory signals can be read. Use the same setting of sensitivity in the subsequent enzymatic reaction.

4. Initiate the enzymatic reaction.

4.1 Add 50 µL MMP-containing samples to each sample well (SP), but not in reference standard (RS) and substrate control (SC) wells. Mix the reagents by shaking the plate gently for 30 seconds.

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 minutes.
- For end-point reading: Incubate the reaction for 30 to 60 minutes, and keep from light. Optional: Add 50 µL of stop solution (Component E) per well. Measure fluorescence intensity at Ex/Em=490 nm/520 nm.

Note: If you do end-point reading, 50 µL of stop solution should be added into the reference standard (RS), and re-read the fluorescence signal of the reference standard to get a better standard curve.

4.3 Data analysis: Refer to the Appendix I for data analysis section.

Appendix I. Data Analysis

- The fluorescence reading from the substrate control well is the background fluorescence. The reading from sample wells should be subtracted with this background fluorescence reading.
- Plot 5-FAM-Pro-Leu-OH fluorescent reference standard as RFU (relative fluorescence unit) versus concentration (Figure 1).

  Note: The final concentration of 5-FAM-Pro-Leu-OH reference standard solutions are 2, 1, 0.5, 0.25, 0.125, 0.0625, 0.031, and 0.016 µM. If you add 50 µL stop solution, the final concentration should be adjusted accordingly. This reference standard is used to calibrate for the variation of different instruments and different batches of experiments. It is also an indicator of the amount of MMP enzymatic reaction final product.
- Plot data as RFU versus time for each sample.
- 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 (V_0) in RFU/min or µM/min. Determine the slope of the linear portion of the data plot.
- A variety of data analyses can be done, e.g., determining inhibition %, IC_{50}, K_m, K_i, etc.

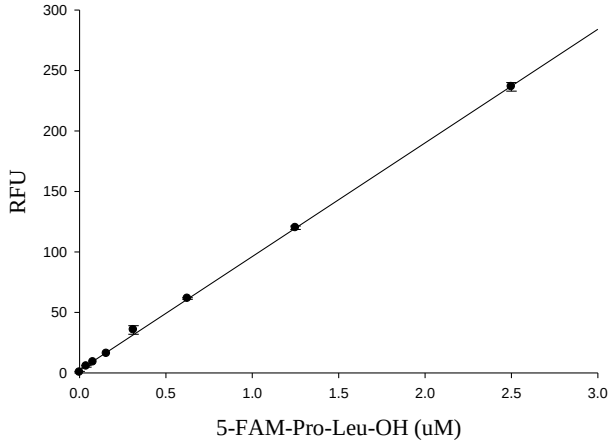


Figure 1. 5-FAM-Pro-Leu-OH reference standard. 5-FAM-Pro-Leu-OH was serially diluted in assay buffer according to the protocol. The fluorescence was monitored by a fluorescence microplate reader (Bio-Tek FLx800) with a filter set of Excitation/Emission=485±20 nm/528±20 nm. (mean±S.D., n=2).

Appendix II: Sequence of MMP substrates

Table 1: MMP substrate sequences.

Substrate No.	Catalog Number	Cleavage Enzyme	FRET Peptide Sequence
SB1	Cat# 60568	MMP-13	QXL520™-Pro-Leu-Gly-Leu-Trp-Ala-D-Arg-Lys(5-FAM)-NH_2 [10-12],
SB2	Cat# 60569	MMP-1/7/8/12/13	QXL520™ -Pro-Leu-Ala-Leu-Trp-Ala-Arg-Lys(5-FAM)-NH_2 [13-15],
SB3	Cat# 60570	MMP-1/2/8/9/12/13	QXL520 ™ -Pro-Leu-Gly-Cys(Me)-His-Ala-D-Arg-Lys(5-FAM)-NH_2 [16]
SB4	Cat# 60571	MMP-1/2/7/8/12/13	5-FAM-Pro-Leu-Ala-Nva-Dap(QXL520 ™)-Ala-Arg-NH_2 [17-19]
SB5	Cat# 60572	MMP-1/2/7/8/12/13	5-FAM-Pro-Leu-Gly-Leu-Dap(QXL520 ™)-Ala-Arg-NH_2 [20;21]
SB6	Cat#60573	MMP-2/13	QXL520 ™ -Pro-Leu-Gly-Met-Trp-Ser-Arg-Lys(5-FAM)-NH_2 [22;23]
SB7	Cat#60574	MMP-7/12/13	QXL520 ™ -Pro-Tyr-Ala-Tyr-Trp-Met-Arg-Lys(5-FAM)-NH_2 [24;25]
SB8	Cat#60575	MMP-7/12/13	QXL520 ™ -Arg-Pro-Lys-Pro-Leu-Ala-Nva-Trp-Lys(5-FAM)-NH_2 [24;26]
SB9	Cat#60576	MMP-1/2/7/8/12/13	QXL520 ™ -Arg-Pro-Leu-Ala-Leu-Trp-Arg-Lys(5-FAM)-NH_2 [27-29]
SB10	Cat#60577	MMP-13	QXL520 ™ -Pro-Leu-Ala-Tyr-Trp-Ala-Arg-Lys(5-FAM)-NH_2 [30;31]
SB11	Cat#60578	MMP-1/2/8/12/13	5-FAM-Pro-Cha-Gly-Nva-His-Ala-Dap(QXL™520)-NH_2 [32;33]
SB12	Cat#60579	MMP-1/2/3/12/13	5-FAM-Arg-Pro-Lys-Pro-Tyr-Ala-Nva-Trp-Met-Lys(QXL520 ™)-NH_2 [34;35]
SB13	Cat#60580	MMP-3/12	5-FAM-Arg-Pro-Lys-Pro-Val-Glu-Nva-Trp-Arg-Lys(QXL520 ™)-NH_2 [36;37]
SB14	Cat# 60581	MMP-1/2/3/7/8/9/12/13	QXL520 ™ -γ-Abu-Pro-Cha-Abu-Smc-His-Ala-Dab(5-FAM)-Ala-Lys-NH_2 [38] (Smc=S-Methyl-L-cysteine)
SB15	Cat#60582	MMP-1/2/7/8/12/13	QXL520 ™ -γ-Abu-Pro-Gln-Gly-Leu-Dab(5-FAM)-Ala-Lys-NH_2 [39]
SB16	Cat#60583	MMP-12/13	QXL520 ™ -Arg-Pro-Lys-Pro-Gln-Gln-Phe-Trp-Lys((5-FAM)-NH_2 [40]

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 0-20 min.
MMP-3 (stromelysin)	1 mM APMA (diluted Component C) at 37°C for 24 h.
MMP-7 (matrilysin, PUMP-1)	1 mM APMA (diluted Component C) at 37°C for 20 min-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 24 h.
MMP-11 (stromelysin-3)	Already in active form. No APMA treatment is necessary.
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.

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