SensoLyte® 490 MMP-8 Assay Kit
Fluorimetric

Revision Number:1.1	Last Revised: October 2014
Catalog #	**AS-71133**
Kit Size	500 Assays (96-well) or 1250 Assays (384-well)

- ***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 Ex/Em=340 nm/490 nm upon cleavage	270 µL
Component B	EDANS, fluorescence reference standard Ex/Em=340 nm/490 nm	1 mM, 10 µL
Component C	APMA, 4-aminophenylmercuric acetate Caution: Toxic! Handle with care.	1 M, 100 µL
Component D	Assay buffer	60 mL
Component E	Stop solution	30 mL

Other Materials Required (but not provided)

- Recombinant MMP-8: AnaSpec Cat#72008.
- 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 490 nm with excitation at 340 nm.

Storage and Handling

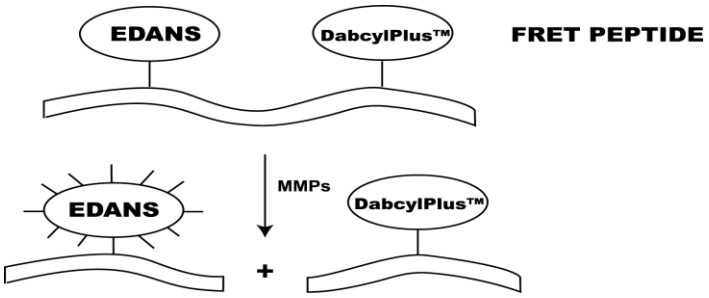
- Store all components at -20°C
- Protect Components A and B from light
- If used frequently, Components D and E can be stored at 4°C for convenience.

Introduction

Matrix metalloproteinases (MMPs) belong to a family of secreted or membrane-associated zinc endopeptidases capable of digesting extra cellular 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® 490 MMP-8 Assay Kit provides a convenient assay for high throughput screening of MMP-8 inducers and inhibitors. It can detect MMP-8 activity in a variety of biological samples using an EDANS/ DabcylPlus™ fluorescence resonance energy transfer (FRET)[1] peptide. In the intact FRET peptide, DabcylPlus™ quenches the fluorescence of EDANS. Upon MMP-8 cleavage of the FRET peptide into two separate fragments (**Scheme 1**), the fluorescence of EDANS is recovered, and can be monitored at excitation/emission = 340 nm/490 nm. DabcylPlus™ is a quencher that is more water-soluble than Dabcyl.

The assays are performed in a convenient 96-well or 384-well microplate format.



Scheme 1. Proteolytic cleavage of EDANS/ DabcylPlus™ FRET peptide by MMPs

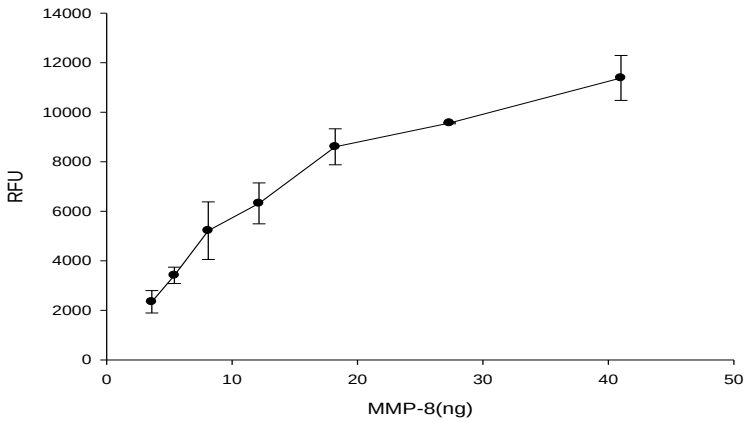


Figure 1.Sensitivity of SensoLyte® 490 MMP-8 assay kit
Active recombinant MMP-8 was serially diluted in assay buffer containing substrate, and the fluorescence was recorded at Ex/Em=340 nm/ 490 nm. (Flexstation 384II, Molecular Devices)

Note: the sensitivity also depends on the endogenous activity of MMP-8 in different preparation. MMP-8 from different sources might vary in its endogenous activity. (Mean ± S.D., n=3).

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 hr at 37°C. Activate pro-MMP-8 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 organic mercury. Handle with care! Dispose it according to appropriate regulations.
Note 3: Activate zymogen by APMA at higher protein concentration. After activation, the enzyme may be further diluted.

2. Prepare working solutions.

Note: Warm all kit components 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).

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 appropriate concentration in assay buffer (Component D).

3. Set up enzymatic reaction.

3.1 Add test compounds and MMP-8 diluent into microplate. The suggested total volume of MMP-8 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-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 auto fluorescence and may give false results.
- Substrate control contains assay buffer only.

Note: Use assay buffer (Component D) to bring the total volume of all the controls to 50 µL (96-well plate) or 20 µL (384-well).

4. Pre-incubation.

4.1 Incubate the plate at the desired temperature for enzymatic reaction (e.g. 25°C or 37°C) for 10-15 min. In the mean time, 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 into the wells of a 96-well plate. Or add 20 µL into the wells of a 384-well plate. Mix the reagents completely by shaking the plate gently for 30-60 second.

5.2 Measure fluorescence signal:

For kinetic reading: Immediately start measuring fluorescence intensity at Ex/Em=340±30 nm/490±30 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/well stop solution (Component E) to 96-well plate or 20 µL/well to 384-well plate. Mix the reagents. Then measure fluorescence intensity at Ex/Em=340±30 nm/490±30 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-2, 12, 13, and 14. 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 immuno-affinity purification or other methods before measuring its specific activity using 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 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 1 h 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 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-8 substrate solution: Dilute MMP-8 substrate (Component A) 1: 100 in assay buffer (Component D).

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)	5 mL
Total volume	5 mL

3.2 MMP-8 diluent: If you use purified MMP-8 as 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 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 MMP-8 containing sample to a 96-well plate, or 20 µL/well to a 384-well plate.

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).
- Positive control: contains MMP-8 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-8 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=340±30 nm/490±30 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=340±30 nm/490±30 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.
  - A variety of data analyses can be done, e.g., determining inhibition %, EC_{50}, IC_{50}, etc.

Appendix II: Fluorometer calibration

- EDANS fluorescence reference standard: Dilute 1 mM EDANS (Component B) to 5 μM in deionized water. Perform 2-fold serial dilutions to get concentrations of 2.5, 1.25, 0.625, 0.3125, 0.156, 0.078, and 0 μM. Add 50 μL/well (96-well plate) or 20 μL/well (384-well plate) of the serially diluted EDANS from 5 μM to 0 nM.
- Add 50 μL/well (96-well plate) or 20 μL/well (384-well plate) of MMP-8 substrate solution (refer to Step 3 of the Standard Operation Protocol for preparing substrate solution).

  Note: The MMP-8 substrate solution should be added to the EDANS reference standard to correct 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 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.
- Plot EDANS fluorescence reference standard as RFU (relative fluorescence unit) versus concentration as in **Figure 2**.

  Note: The final concentrations of EDANS 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-8 enzymatic reaction final product.

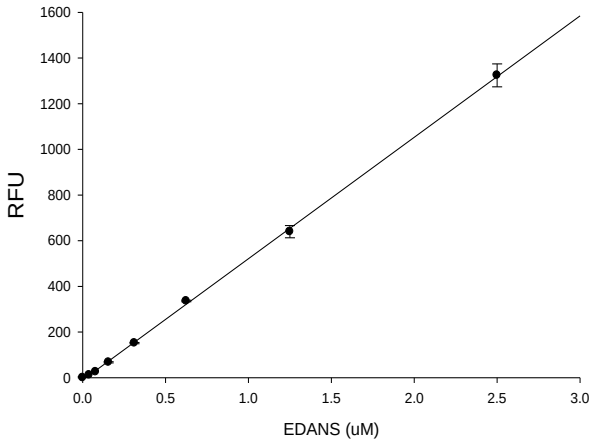


Figure 2. EDANS reference standard curve. EDANS was diluted in assay buffer containing MMP-8 substrate and fluorescence was recorded at Ex/Em=340 nm/490 nm (FLx800, Bio-Tek Instruments)

References

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