SensoLyte® 490 MMP-12 Assay Kit
Fluorimetric

Revision#1.2	*Last updated: July 2021*
Catalog #	**AS-71137**
Kit Size	500 Assays (96-well plate)

- ***Convenient Format:*** All essential assay components are included.
- ***Optimized Performance:*** Optimal conditions for the detection of MMP-12 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-12 substrate EDANS/DabcylPlus™ FRET peptide 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)

- MMP-12: AnaSpec Cat##55525-1, 55525-10, 55525-50
- 96-well microplate: Black, flat-bottom plates with non-binding surface.
- Fluorescence microplate reader: Capable of detecting emission at 490 nm with excitation at 340 nm.

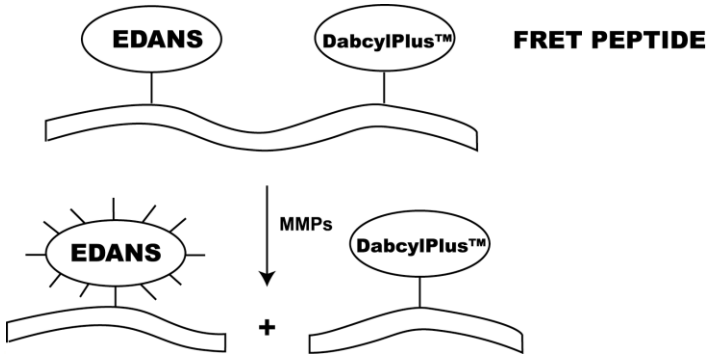
Storage and Handling

- Store all components at -20°C
- Protect Components A and B from light 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.[1,2]. MMP-12 (macrophage elastase) is involved in smoke-induced emphysema,[3], tumor,[4] and other diseases. MMP-12 is secreted as a 54 kDa zymogen and becomes 45 kDa active form after proteolytic cleavage.

The SensoLyte® 490 MMP-12 Assay Kit provides a convenient assay for high throughput screening of MMP-12 inducers and inhibitors. It detects MMP-12 activity in a variety of biological samples using a EDANS/DabcylPlus™ fluorescence resonance energy transfer (FRET) peptide.[5] In the intact FRET peptide the fluorescence of EDANS is quenched by DabcylPlus™. Upon cleavage into two separate fragments by MMP-12 (**Scheme 1**), the fluorescence of EDANS is recovered, and can be monitored at excitation/emission wavelengths = 340 nm/490 nm.



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

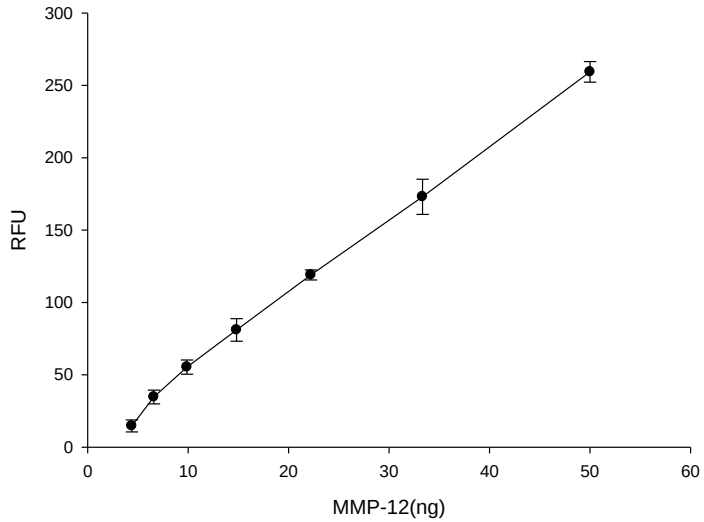


Figure 1. Sensitivity of SensoLyte® 490 MMP-12 assay kit.

Active recombinant MMP-12 was serially diluted in assay buffer. The enzyme at each dilution was mixed with MMP-12 FRET substrate and then incubated at RT for 30 min. The endpoint fluorescence signal was recorded at Ex/Em=360±40 nm/460±40 nm on Flex800 (Bio-Tek, VT). The assay is able to detect as low as 4.3 ng of active MMP-12.

Note: The sensitivity also depends on the endogenous activity of MMP-12 in different preparation. MMP-12 from different sources might vary in its endogenous activity. (mean±S.D., n=3).

Protocol

Note 1: For fluorimeter calibration, please refer to Appendix II (recommended for the 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-12.

1. Activate pro-MMP-12.

1.1 Incubate pro-MMP-12 with 1 mM APMA (diluted Component C) for 2 h at 37°C. Activate pro-MMP-12 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 organic mercury. Handle with care! Dispose it according to appropriate regulations.
Note 3: Activation of zymogen by APMA at higher protein concentration is recommended. After activation, the enzyme may be further diluted.
Note 4: AnaSpec MMP-12 (Cat##55525-1, 55525-10, 55525-50) is catalytic domain enzyme which does not require pre-activation.

2. Prepare working solutions.

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

2.1 MMP-12 substrate solution: Dilute MMP-12 substrate (Component A) 1: 100 in assay buffer (Component D).

Table 1. MMP-12 substrate solution for one 96-well plate (100 assays).

Components	Volume
MMP-12 substrate (100X, Component A)	50 μL
Assay buffer (Component D)	5 mL
Total volume	5 mL

2.2 MMP-12 diluent: Dilute activated MMP-12 to appropriate concentration in assay buffer (Component D).

3. Set up enzymatic reaction.

3.1 Add test compounds and MMP-12 diluent into microplate. The suggested total volume of MMP-12 diluent and test compound is 50 μL/well.

3.2 Simultaneously set up the following controls.

- Positive control contains MMP-12 diluent without test compound.
- Inhibitor control contains MMP-12 diluent and a known MMP-12 inhibitor.
- Vehicle control contains MMP-12 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.

  Note: Use assay buffer (Component D) to bring the total volume of all the controls to 50 μL/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-12 substrate solution at the same temperature.

5. Initiate the enzymatic reaction.

5.1 Add 50 µL/well of MMP-12 substrate solution. 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). 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-12 activity in biological samples.

Note: The FRET substrate in this kit can also be cleaved by MMP-1, 2, 3, 8, and 13. If several MMPs are coexisting in your samples and you want to specifically measure MMP-12's activity, then MMP-12 must first be isolated by immuno-affinity purification or other methods before measuring its specific activity using current assay kit.

1. Prepare MMP-12 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 Tissues samples should be homogenized in assay buffer (Component D) containing 0.1% 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 the final concentration of 1 mM in the assay buffer (Component D) for 2 h at 37°C. Activate MMP right 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 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-12 substrate solution: Dilute MMP-12 substrate (Component A) 1: 100 in assay buffer (Component D).

Table 1. MMP-12 substrate solution for one 96-well plate (100 assays).

Components	Volume
MMP-12 substrate (100X, Component A)	50 μL
Assay buffer (Component D)	5 mL
Total volume	5 mL

3.2 MMP-12 diluent: If you use purified MMP-12 as positive control, then dilute MMP-12 to an appropriate concentration in assay buffer (Component D).

Note: Pro-MMP12 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 vortexing of enzyme.

4. Set up the enzymatic reaction.

4.1 Add 50 μL/well of MMP-12 containing sample.

4.2 Set up the following control:

- Substrate control contains assay buffer (50 μL/well)
- Positive control contains MMP-1 diluent (50 μL/well)

5. Initiate the enzymatic reaction.

5.1 Add 50 μL/well of MMP-12 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 from direct light. Optional: Add 50 μL/well 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 is the background fluorescence. This background reading has to be subtracted from the readings of the other wells. This reading is the relative fluorescence unit (RFU).
- For kinetic reading:

- Plot data as RFU versus time for each sample. If you want to convert the RFU to the concentration of the product in the enzymatic reaction, please refer to Appendix II for setting up 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 by determining 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 the concentration of test compounds or enzymes.
  - 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. Do 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 of serially diluted EDANS from 5 µM to 0 nM.
- Add 50 µL/well MMP-12 substrate solution (refer to Protocol A, step 2.1 for preparation)

  Note: MMP-12 substrate solution is added to the EDANS 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 into the enzymatic reaction before taking the end-point reading, the same volume of stop solution should be added to reference standard wells for better comparison.
- Measure the fluorescence intensity of the reference standard wells at Ex/Em=340/490 nm. Use the same setting of sensitivity as used in the enzyme reaction.
- Plot EDANS fluorescent reference standard as RFU (relative fluorescent unit) versus concentration as in **Figure 2**.

  Note: The final concentration of EDANS 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 different batch of experiments. It is also an indicator of the amount of final product of the MMP-12 enzymatic reaction.

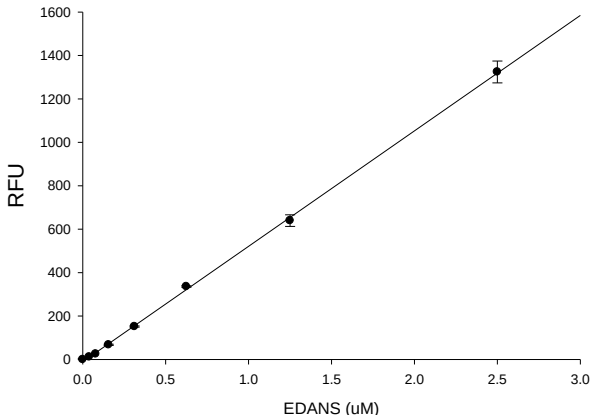


Figure 2. The EDANS reference standard calibration curve.

EDANS was diluted in assay buffer containing MMP-12 substrate. The fluorescence signal was measured by a fluorescence microplate reader (FLx800, Bio-Tek Instruments) with a filter set of Ex/Em=360±40 nm/460±40 nm. (Samples were done in duplicates).

References

1. Woessner, JF. et al. *J. Biol. Chem.* **263**, 16918 (1988).
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3. Hautamaki, RD. et al. *Science*. **277**, 2002 (1997).
4. Dong, Z. et al. *Cell*. **88**, 801 (1997).
5. Stryer, L. et al. *Annu.Rev. Biochem.* **47**, 819 (1978).