



MMP-1 Fluorometric Drug Discovery Kit, RED

Designed to screen MMP-1 inhibitors using a quenched fluorogenic peptide.

**Instruction Manual
BML-AK301**

For research use only

BACKGROUND

Matrix metalloproteinase-1 (MMP-1, interstitial collagenase, fibroblast collagenase) is a member of the MMP family of extracellular proteases. These enzymes play a role in many normal and disease states by virtue of their broad substrate specificities¹. Targets of MMP-1 include collagen, gelatin, entactin, pro-TNF- α , and the chemokine SDF-1¹⁻⁴. MMP-1 is secreted as a 52-56 kDa glycosylated proenzyme (as measured by SDS-PAGE), and activated by cleavage to forms of 22-46 kDa⁵. MMP-1 is an important target for inhibitor screening due to its involvement in diseases such as cancer^{3,6}.

The *MMP-1 Fluorometric Drug Discovery Kit, RED* is a complete assay system designed to screen MMP-1 inhibitors using a quenched fluorogenic substrate OmniMMP™ RED: TQ3-GABA-Pro-Cha-Abu-Smc-His-Ala-Dab(6-TAMRA)-Ala-Lys-NH₂ [TQ3=quencher; GABA=4-aminobutyric acid; Cha=L-cyclohexylalanine; Abu=2-aminobutyric acid; Smc=S-methyl-L-cysteine; Dab=2,4-diaminobutyric acid; 6-TAMRA=6-tetramethylrhodamine]. TAMRA fluorescence is thoroughly quenched by the TQ3 group until cleavage by MMPs separates the two moieties.

The OmniMMP™ RED substrate offers key advantages over other MMP substrates. 1) Emission at the red end of the spectrum (576 nm after excitation at 545 nm) avoids the interference at lower wavelengths often exhibited by screening compounds, and by substances commonly found in biological samples and tissue culture medium. 2) MMP substrate peptides display poor aqueous solubility, often with K_m s near their limits of solubility, making enzyme and inhibitor kinetics difficult. MMP K_m s for OmniMMP™ RED substrate are below its solubility limit. 3) OmniMMP™ RED is avidly cleaved by MMPs, with k_{cat}/K_m s in the range of 10^4 - 10^6 M⁻¹sec⁻¹. 4) The ultra-strong fluorescence of OmniMMP™ RED allows for substrate concentrations much lower than the K_m , a condition generally desirable in inhibitor screening assays.

The assays are performed in a convenient 96-well microplate format. The kit is useful to screen inhibitors of MMP-1, a potential therapeutic target. The compound NNGH⁷ is also included as a prototypic control inhibitor.

Please contact Enzo Life Sciences for kit components in bulk.

REFERENCES:

1. *Matrix metalloproteinases: they're not just for matrix anymore!*: L.J. McCawley and L.M. Matrisian; *Curr. Opin. Cell Biol.* **13**, 534 (2001)
2. *Updated biological roles for matrix metalloproteinases and new "intracellular" substrates revealed by degradomics*: G.S. Butler and C.M. Overall; *Biochemistry* **48**, 10830 (2009)
3. *Matrix metalloproteinases: regulators of the tumor microenvironment*: K. Kessenbrock and Z. Werb; *Cell* **141**, 52 (2010)
4. *Matrix metalloproteinase activity inactivates the CXC chemokine stromal cell-derived factor-1*: G.A. McQuibban *et al.*; *J. Biol. Chem.* **276**, 43503 (2001)
5. *Mechanisms of activation of tissue procollagenase by matrix metalloproteinase 3 (stromelysin)*: K. Suzuki *et al.*; *Biochemistry* **29**, 10261 (1990)
6. *Identification of MMP-1 as a putative breast cancer predictive marker by global gene expression analysis*: I. Poola *et al.*; *Nat. Med.* **11**, 481 (2005)
7. *Discovery of CGS 27023A, a non-peptidic, potent, and orally active stromelysin inhibitor that blocks cartilage degradation in rabbits*: L.J. MacPherson *et al.*; *J. Med. Chem.* **40**, 2525 (1997)

PLEASE READ ENTIRE BOOKLET BEFORE PROCEEDING WITH THE ASSAY. CAREFULLY NOTE THE HANDLING AND STORAGE CONDITIONS OF EACH KIT COMPONENT. PLEASE CONTACT ENZO LIFE SCIENCES TECHNICAL SERVICES FOR ASSISTANCE IF NECESSARY. **DO NOT PERFORM THIS ASSAY IN WHITE PLATE DUE TO PLATE AUTOFLUORESCENCE AT THE WAVELENGTHS USED**

COMPONENTS OF BML-AK301 KIT

BML-SE180-9090 MMP-1 ENZYME (HUMAN, RECOMBINANT)

FORM: *E. coli* recombinant human MMP-1 catalytic domain (calculated MW 19.9 kDa) Purity >95% by SDS-PAGE.

UNIT DEFINITION: One unit is defined as the amount of enzyme that will hydrolyze 100 μ M thiopeptolide Ac-PLG-[2-mercapto-4-methyl-pentanoyl]-LG-OC₂H₅ (Cat. # BML-P125) at 100 pmol/min@ 37°C.

STORAGE: -70 °C; Avoid freeze/thaw cycles

QUANTITY: 2000 U

PRESENTATION: Liquid in screw-cap microfuge vial.

BML-P277-9090 SUBSTRATE (OmniMMP™ RED fluorogenic substrate peptide; MW=1910.7)

FORM: 250 μ M in DMSO

STORAGE: -70 °C

PRESENTATION: 40 μ l in amber screw-cap microfuge vial.

BML-KI582-0010 6-TAMRA CALIBRATION STANDARD

FORM: 10 μ M in DMSO; MW=430.4

STORAGE: -70 °C

PRESENTATION: 20 μ l in amber screw-cap microfuge vial.

BML-PI115-9090 INHIBITOR (NNGH; MW=316.4)

FORM: 1.3 mM in DMSO

STORAGE: -70 °C

PRESENTATION: 50 μ l in screw-cap microfuge vial.

BML-KI175-0020 ASSAY BUFFER

50 mM HEPES, 10 mM CaCl₂, 0.05% Brij-35, pH 7.5

FORM: Liquid

STORAGE: Room Temperature

Presentation: 20 ml in screw-cap plastic bottle

80-2409 96-WELL 1/2 Volume Black NBS Microplate

STORAGE: Room Temperature.

OTHER MATERIALS REQUIRED

- Fluorescent microplate reader capable of excitation at 545 nm and emission at 576 nm. An Ex/Em of 540/590 has also been used.
- Pipetmen or multi-channel pipetmen capable of pipetting 1-100 μ l accurately.
- Ice bucket to keep reagents cold until use.
- Water bath or incubator for component temperature equilibration.

EXPERIMENTAL METHODS

Note on storage: Store all components except the microplate and assay buffer (room temperature) at $-70\text{ }^{\circ}\text{C}$ for the highest stability. The MMP-1 enzyme should be handled carefully in order to retain maximal enzymatic activity. It is stable, in diluted or concentrated form, for several hours on ice. As supplied, MMP-1 enzyme is stable for at least 4 freeze/thaw cycles. To minimize the number of freeze/thaw cycles, aliquot the MMP-1 into separate tubes and store at $-70\text{ }^{\circ}\text{C}$. When setting up the assay, do not maintain diluted components at reaction temperature (e.g. $37\text{ }^{\circ}\text{C}$) for an extended period of time prior to running the assay.

To start assay:

1. Briefly warm kit components BML-P277-9090, BML-KI582, and BML-PI115-9090 to RT to thaw DMSO.
2. Dilute inhibitor (BML-PI115-9090) 1/200 in assay buffer BML-KI175 as follows: Add $1\text{ }\mu\text{l}$ inhibitor into $200\text{ }\mu\text{l}$ assay buffer, in a separate tube. Warm to reaction temperature (e.g. $37\text{ }^{\circ}\text{C}$).
3. Thaw the substrate (BML-P277-9090) and dilute sufficient volume to $5\text{ }\mu\text{M}$ in assay buffer ($10\text{ }\mu\text{l}$ needed per well). Warm to reaction temperature (e.g. $37\text{ }^{\circ}\text{C}$).
4. Dilute MMP-1 enzyme to $765\text{ mU}/\mu\text{l}$ in assay buffer to required total volume ($20\text{ }\mu\text{l}$ are needed per well). Warm to reaction temperature (e.g. $37\text{ }^{\circ}\text{C}$) shortly before assay.
5. Pipet assay buffer into each desired well of the 1/2 volume microplate as follows:
 - Calibration = $80\text{ }\mu\text{l}$ in 3 wells (see step 11)
 - Control (no inhibitor) = $70\text{ }\mu\text{l}$
 - Inhibitor NNGH = $50\text{ }\mu\text{l}$
 - Test inhibitor = varies (see Table 1)

Example of plate:

<u>well#</u>	<u>sample</u>
A1	Calibration
B1	Calibration
C1	Calibration
D1	Control
E1	Control
F1	Inhibitor NNGH
G1	Inhibitor NNGH
H1	Test inhibitor
A2...	Test inhibitor...

6. Allow microplate to equilibrate to assay temperature (e.g. $37\text{ }^{\circ}\text{C}$).
7. Add $20\text{ }\mu\text{l}$ MMP-1 (diluted in step 4) to the control, inhibitor NNGH, and test inhibitor wells. Final amount of MMP-1 will be 15.3 U per well.
8. Add $20\text{ }\mu\text{l}$ NNGH inhibitor (diluted in step 2) to the inhibitor NNGH wells only. Final inhibitor concentration = $1.3\text{ }\mu\text{M}$. Note: $1\text{ }\mu\text{M}$ NNGH will not completely inhibit MMP-1 under these conditions (see Figure 1).
9. Add desired volume of test inhibitor to appropriate wells. See Table 1.
10. Incubate plate for 30-60 minutes at reaction temperature (e.g. $37\text{ }^{\circ}\text{C}$) to allow inhibitor/enzyme interaction.
11. In the meantime, calibrate the fluorescent microplate reader, using Ex/Em= $545/576\text{ nm}$, with cutoff set at 570 nm : Pre-warm $80\mu\text{l}$ assay buffer to reaction temperature in 3 wells in the microplate, then to each add $10\mu\text{l}$ substrate peptide (BML-P277-9090) to give the concentration to be used in the assay (e.g., for $0.5\text{ }\mu\text{M}$ final add $10\mu\text{l}$ $5\mu\text{M}$) and mix. When the fluorescent

signal is constant, use this reading as the zero (Blank) value in arbitrary fluorescence units (RFUs). Using the same wells, with their mixtures of substrate peptide and buffer, add 10µl (diluted in assay buffer) calibration standard BML-KI582 to give 3 different final molar concentrations ranging between 2 and 10% of the substrate peptide molar concentration (e.g., 10, 25, and 50 nM) and measure their fluorescence. Use these values to build a standard curve relating micromolar BML-KI582 concentration (x axis) to RFUs (y axis). The slope of the line is the conversion factor (CF). If multiple concentrations of substrate peptide are used, such as in kinetic determinations, step 11 must be performed for each concentration, due to absorptive quenching by the substrate peptide. Note: this calibration can be done at any time.

12. Start reactions by the addition of 10 µl BML-P277-9090 substrate (diluted and equilibrated to reaction temperature in step 3). Final substrate concentration = 0.5 µM.
13. Continuously read plates in the fluorescent microplate reader, using Ex/Em=545/576 nm, with cutoff set at 570 nm. For example, record data at 1 minute time intervals for 10 minutes at set reaction temperature (e.g. 37°C).
14. Perform data analysis (see below).

NOTE: Retain microplate for future use of unused wells.

TABLE 1. Example of Samples.

Sample	Assay buffer	MMP-1 (765 mU/µl)	Inhibitor (6.5 µM)	Substrate (5µM)	Total Volume
Control	70 µl	20 µl	0	10 µl	100 µl
Inhibitor NNGH	50 µl	20 µl	20 µl	10 µl	100 µl
Test inhibitor*	X µl	20 µl	Y µl	10 µl	100 µl

*Test inhibitor is the experimental inhibitor. Dissolve/dilute inhibitor into assay buffer and add to appropriate wells at desired volume “Y”. Adjust volume “X” to bring the total volume to 100 µl.

DATA ANALYSIS

Plotting

15. Plot data as RFUs (minus Blank RFU value determined during calibration, step 11) versus time for each sample.
16. Determine the range of initial time points during which the reaction is linear.
17. Obtain the initial reaction velocity (**V**) in RFUs/min: determine the slope of a line fit to the initial linear portion of the data plot using an appropriate routine.
18. It is best to use a range of inhibitor concentrations, each in duplicate. Average the slopes of duplicate samples.

Data Reduction

To determine inhibitor % remaining activity:

$$\text{Inhibitor \% activity remaining} = (\mathbf{V} \text{ inhibitor} / \mathbf{V} \text{ control}) \times 100$$

See Figure 1 for example of results.

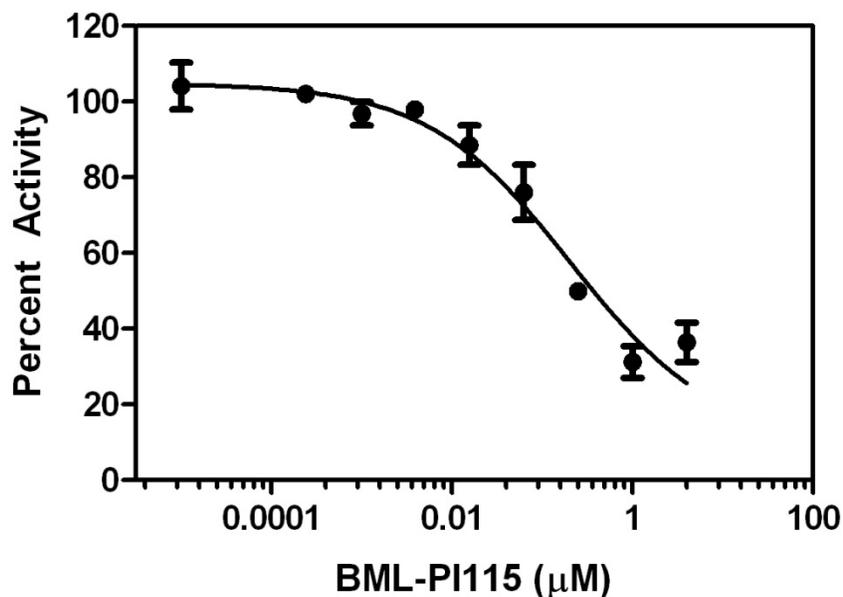


Figure 1. Inhibitor dose response curve: NNGH was pre-incubated with MMP-1 enzyme before reactions were started by the addition of substrate. Final concentrations of reagents were 153 mU/μl MMP-1, 500 nM OmniMMP™ Red, 0.5% DMSO. $IC_{50} = 300$ nM. The dose-response curve was derived from a fit to a four parameter Hill-Slope model, $Y = \text{Bottom} + (\text{Top} - \text{Bottom}) / (1 + 10^{((\text{Log}IC_{50} - X) * \text{HillSlope}))})$ using Graphpad Prism software where the bottom was constrained to 0. Data are presented as mean of duplicate wells \pm SEM.

To determine the activity of the samples expressed as picomoles substrate hydrolyzed per minute:

$$X \text{ pmoles substrate/min} = 1/CF \times V \times \text{vol}$$

Where CF is the conversion factor (micromolar concentration/RFUs, from step 11), V is initial reaction velocity (RFUs/min, from step 17), and vol is the reaction volume in microliters (100).

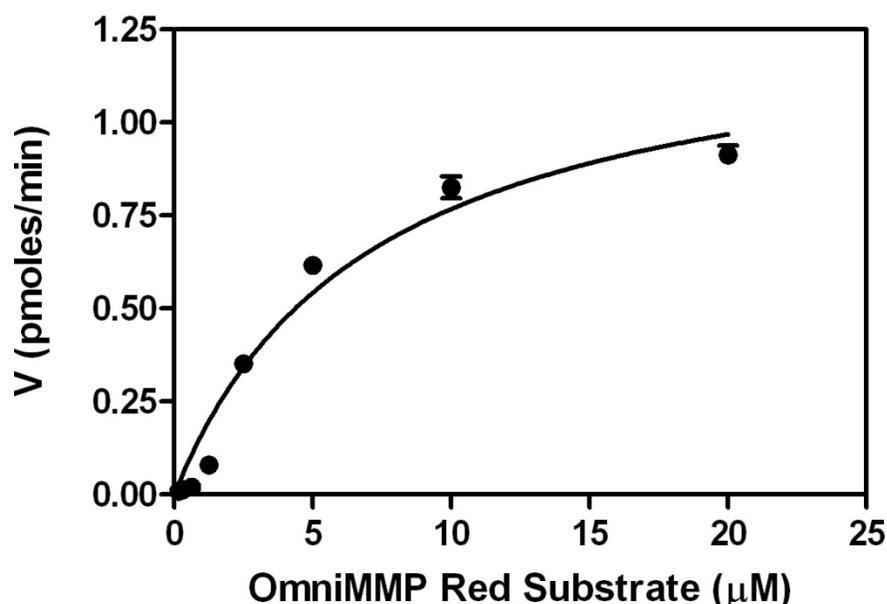


Figure 2. Example graph for k_{cat}/K_m determination for MMP-1. $K_m = 7.1$ μM, $k_{cat}/K_m = 3 \times 10^6$ M⁻¹sec⁻¹. Data shown are the mean of duplicate wells \pm SEM.

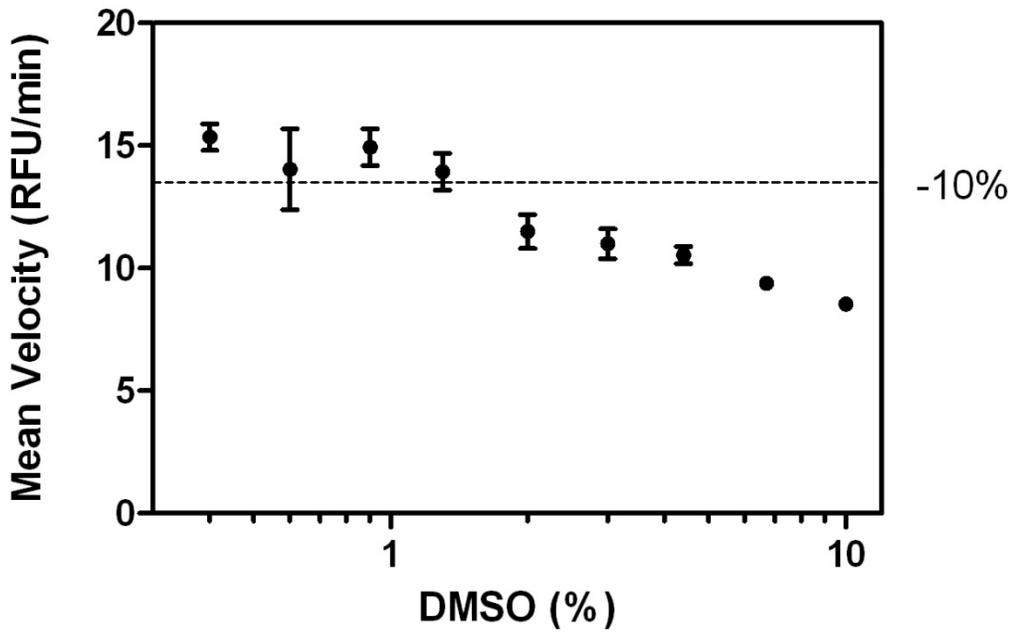


Figure 3. DMSO Sensitivity: Final concentrations of reagents were 153 mU/ul MMP-1 enzyme, 1 μ M OmniMMP™ Red, variable DMSO concentration. Data are presented as mean of duplicate wells \pm SEM.

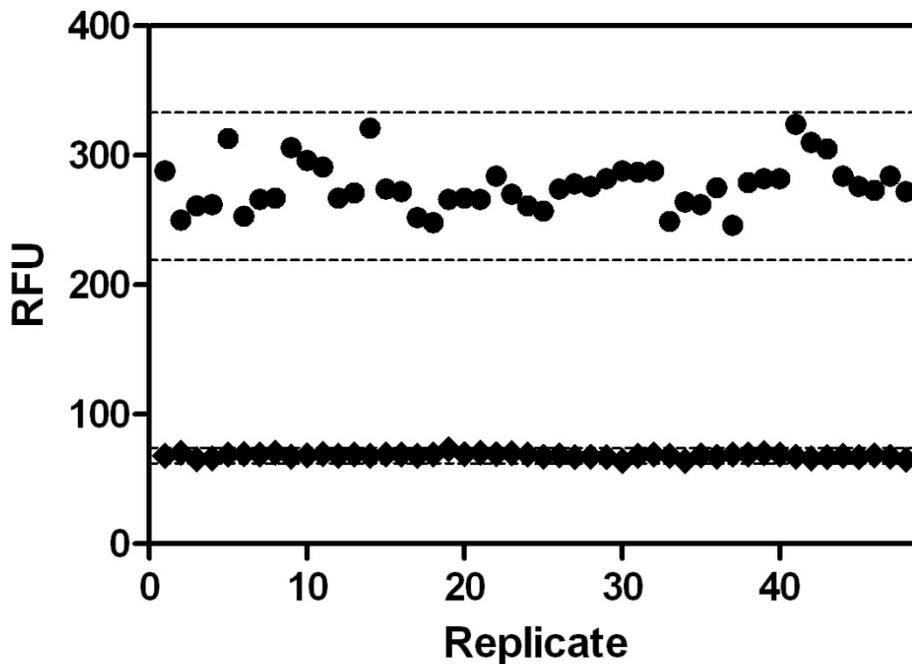


Figure 4. Z' analysis: MMP-1 enzyme was pre-incubated without (●) or with (◆) NNGH Inhibitor for 30 minutes at \sim 25 $^{\circ}$ C before the addition of OmniMMP™ Red substrate. After the addition of substrate, the reaction proceeded at \sim 25 $^{\circ}$ C for 45 minutes before the fluorescence was measured at 530 nm/590 nm, on a BioTek Synergy2 plate reader. Final concentrations were 500 nM substrate, 153 mU/ μ l MMP-1, 1 μ M NNGH, 1.0 % DMSO. The Z' factor for this assay was 0.7, (Z' factor = $1 - ((3SD^{\text{control}} + 3SD^{\text{inhibitor}}) / (\text{mean}^{\text{control}} - \text{mean}^{\text{inhibitor}}))$). Dashed lines indicate 3 * standard deviation.



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