Product Manual

MMP-9 Fluorometric Drug Discovery Kit, RED

Designed to screen MMP-9 inhibitors using a quenched fluorogenic peptide.

Instruction Manual

BML-AK306

For research use only

✦MMP-9 Fluorometric Drug Discovery Kit, RED – BML-AK306✦

BACKGROUND

Matrix metalloproteinase-9 (MMP-9, gelatinase B, 92kDa type IV 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 specificities[1-3]. Targets of MMP-9 include native and denatured collagens, fibronectin, elastin, laminin, pro-TNF-α, and interleukins and their receptors[1-4]. MMP-9 is secreted as a 92kDa proenzyme (as measured by SDS-PAGE), and activated by cleavage to forms 82kDa and smaller[5]. MMP-9 is an important target for inhibitor screening due to its involvement in diseases such as alopecia[6], cancer, angiogenesis, and metastasis[3,6,7].

The *MMP-9 Fluorometric Drug Discovery Kit, RED* is a complete assay system designed to screen MMP-9 inhibitors using a quenched fluorogenic substrate OmniMMP™ RED: TQ3-GABA-Pro-Cha-Abu-Smc-His-Ala-Dab(6-TAMRA)-Ala-Lys-NH_2 [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 (576nm after excitation at 545nm) 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_ms near their limits of solubility, making enzyme and inhibitor kinetics difficult. MMP K_ms for OmniMMP™ RED substrate are well below its solubility limit. 3) OmniMMP™ RED is avidly cleaved by MMPs, with k_{cat}/K_ms in the range of ~10^4-10^6 $M^{-1}sec^{-1}$. 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-9, a potential therapeutic target. The compound NNGH[8] 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. *A novel role of metalloproteinase in cancer-mediated immunosuppression*: B.-C. Sheu *et al.*; Cancer Res. **61**, 237 (2001)
5. *Analysis of the role of the COOH-terminal domain in the activation, proteolytic activity, and tissue inhibitor of metalloproteinase interactions of gelatinase B*: J. P. O'Connell *et al.*; J. Biol. Chem. **269**, 14967 (1994)
6. *Identification of clustered cells in human hair follicle responsible for MMP-9 gelatinolytic activity: consequences for the regulation of hair growth*: F. Jarrousse *et al.*; Int. J. Dermatol. **40**, 385 (2001)
7. *Matrix metalloproteinase-9 triggers the angiogenic switch during carcinogenesis*: G. Bergers *et al.*; Nat. Cell Biol. **2**, 737 (2000)
8. *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 A WHITE PLATE DUE TO PLATE AUTOFLUORESCENCE AT THE WAVELENGTHS USED****

COMPONENTS OF BML-AK306 KIT

BML-SE360-9090 MMP-9 ENZYME (HUMAN, RECOMBINANT)

FORM: *E. coli.* Recombinant human MMP-9 catalytic domain (calculated MW 39kDa) 2.68U/µl

UNIT DEFINITION: One unit is defined as the amount of enzyme that will hydrolyze 100µM thiopeptide Ac-PLG-[2-mercapto-4-methyl-pentanoyl]-LG-OC_2H_5 (Cat. # BML-P125) at 100 pmol/min @ 37°C.

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

QUANTITY: 130 U

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

FORM: 250µM in DMSO

STORAGE: -70°C

QUANTITY: 40µl

BML-KI582-0010 6-TAMRA CALIBRATION STANDARD

FORM: 10µM in DMSO; MW=430.4

STORAGE: -70°C

QUANTITY: 20µl

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

FORM: 1.3 mM in DMSO

STORAGE: -70°C

QUANTITY: 50µl

BML-KI175-0020 ASSAY BUFFER

50mM HEPES, 10mM $CaCl_2$, 0.05% Brij-35, pH 7.5

FORM: Liquid in screw-cap plastic bottle

STORAGE: -20°C

QUANTITY: 20ml

80-2409 96-WELL BLACK NBS MICROPLATE

STORAGE: Room temperature.

OTHER MATERIALS REQUIRED

- Fluorescent microplate reader capable of excitation at 545nm and emission at 576nm. The following Ex/Em has also been used: 530/590.
- Pipetmen or multi-channel pipetmen capable of pipetting 1-100µl 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 microtiter plate at -70°C for the highest stability. Components with storage temperatures other than -70°C can be stored at the temperature listed OR at -70°C without any deleterious effects. The MMP-9 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-9 enzyme is stable for at least 5 freeze/thaw cycles. To minimize the number of freeze/thaw cycles, aliquot the MMP-9 into separate tubes and store at -70°C. When setting up the assay, do not maintain diluted components at reaction temperature (e.g. 37°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 (NNGH, BML-PI115-9090) 1/200 in assay buffer BML-KI582 as follows. Add 1µl inhibitor into 200µl assay buffer, in a separate tube. Warm to reaction temperature (e.g. 37°C).
3. Thaw the DMSO stock vial of substrate BML-P277-9090 and dilute sufficient volume to 5µM in assay buffer (10µl needed per well). Warm to reaction temperature (e.g. 37°C).
4. Dilute MMP-9 enzyme to 45mU/µl in assay buffer to required total volume (20µl are needed per well). Warm to reaction temperature (e.g. 37°C) shortly before assay.
5. Pipet assay buffer into each desired well of the black microplate as follows:

   Calibration = 80µl in 3 wells (see step 11)

   Control (no inhibitor) = 70µl

   Inhibitor NNGH = 50µl

   Test inhibitor = varies (see Table 1)

   Example of plate:

well#	sample
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°C).
7. Add 20µl MMP-9 (diluted in step 4) to the control, inhibitor NNGH, and test inhibitor wells. Final amount of MMP-9 will be 0.9U per well (9mU/µl).
8. Add 20µl NNGH inhibitor (diluted in step 2) to the inhibitor NNGH wells only. Final inhibitor concentration = 1.3µM**.** Note: 1.3µM NNGH will inhibit MMP-9 by > 96% under these conditions (see **Figure 1**).
9. Add desired volume of test inhibitor to appropriate wells. See **Table 1**. Final DMSO concentrations above 3% have been shown to decrease the rate of MMP-9 in this assay (**Figure 3**).

10. Incubate plate for 30-60 minutes at reaction temperature (e.g. 37°C) to allow inhibitor/enzyme interaction.
11. In the meantime, calibrate the fluorescent microplate reader, using Ex/Em=545/576nm, with cutoff set at 570nm: Prewarm 80µl assay buffer to reaction temperature in 3 wells in the microplate, then to each add 10µl BML-P277-9090 substrate peptide to give the concentration to be used in the assay (e.g., for 0.5µM final add 10µl 5µM) 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 50nm) 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 10µl BML-P277-9090 substrate (diluted and equilibrated to reaction temperature in step 3). Final substrate concentration = 0.5µM.
13. Continuously read plates in the fluorescent microplate reader, using Ex/Em=545/576nm, with cutoff set at 570nm. 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-9 (45mU/µl)	Inhibitor (6.5µM)	Substrate (5µM)	Total Volume
Control	70µl	20µl	0	10µl	100µl
Inhibitor NNGH	50µl	20µl	20µl	10µl	100µl
Test inhibitor*	Xµl	20µl	Yµl	10µl	100µl

*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 100µl.

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:

Inhibitor % activity remaining = (**V** inhibitor / **V** control) x 100

See **Figure 1** for example of results.

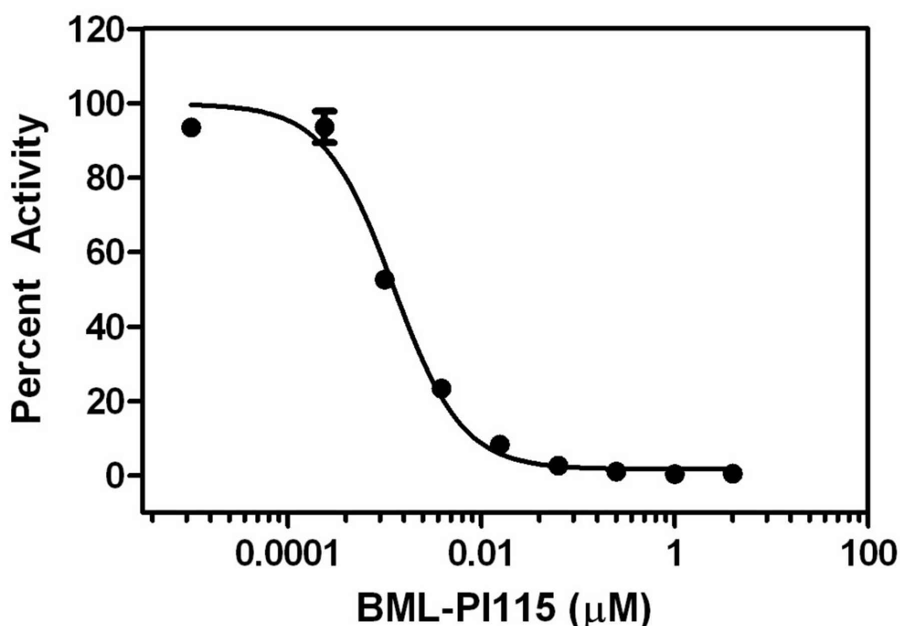

Figure 1. Inhibitor dose response curve: NNGH was pre-incubated with MMP-9 enzyme before reactions were started by the addition of substrate. Final concentrations of reagents were 9 mU/µl MMP-9, 500nm OmniMMP™ Red, 1% DMSO. IC_{50} was calculated as 1.2nm. Data are presented as mean of duplicate wells ± SEM.

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

X pmoles substrate/min = 1/CF x **V** x 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).

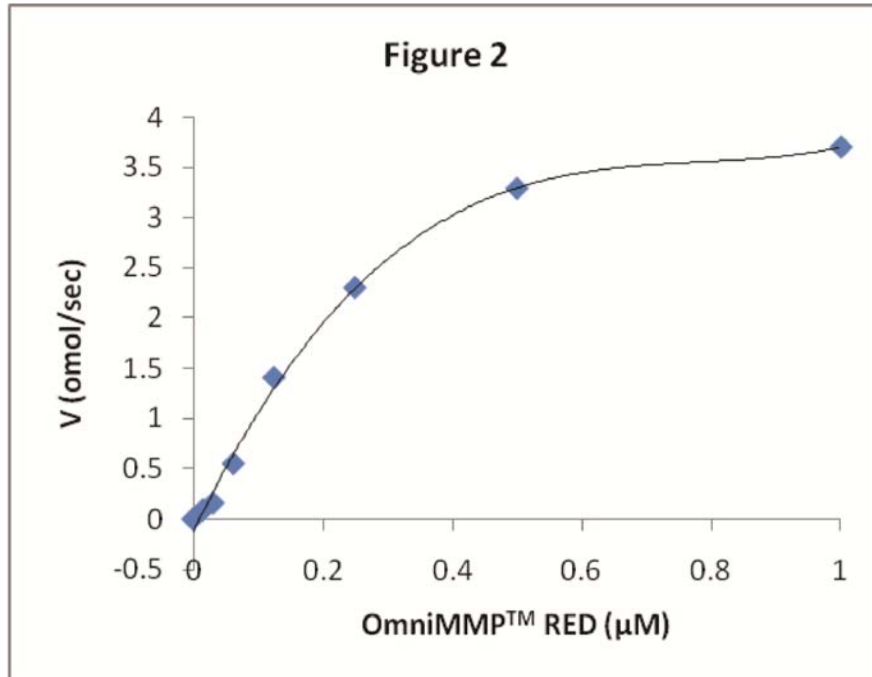

Figure 2. Example graph for k_{cat}/K_m determination for MMP-9. K_m = 0.35 µM, k_{cat}/K_m = 2.3 x 10^7 $M^{-1}sec^{-1}$.

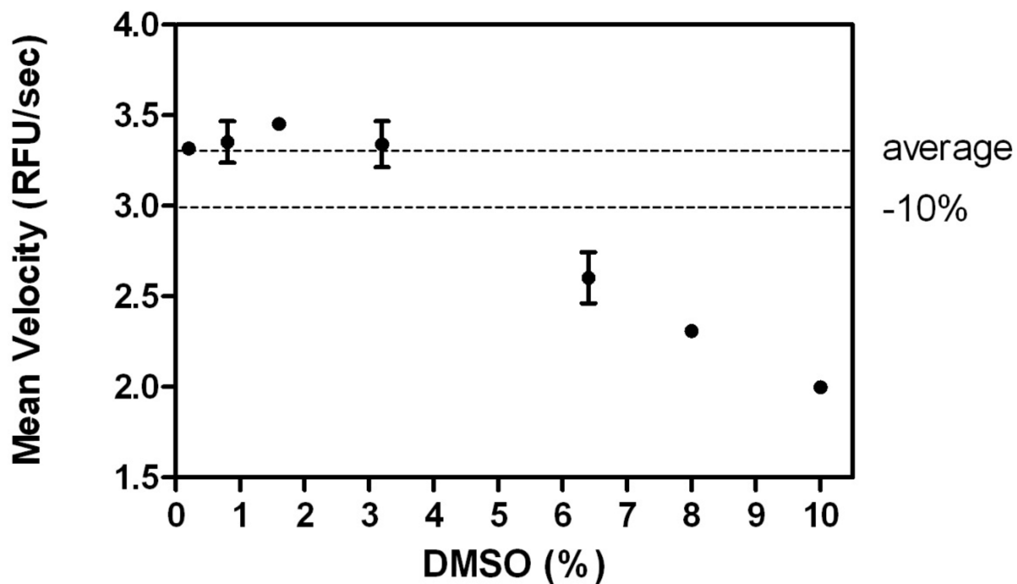

Figure 3. DMSO Sensitivity: Final concentrations of reagents were 9 mU/µl MMP-9 enzyme, 500nm OmniMMP™ Red, variable DMSO concentration. Data are presented as mean of duplicate wells ± SEM.

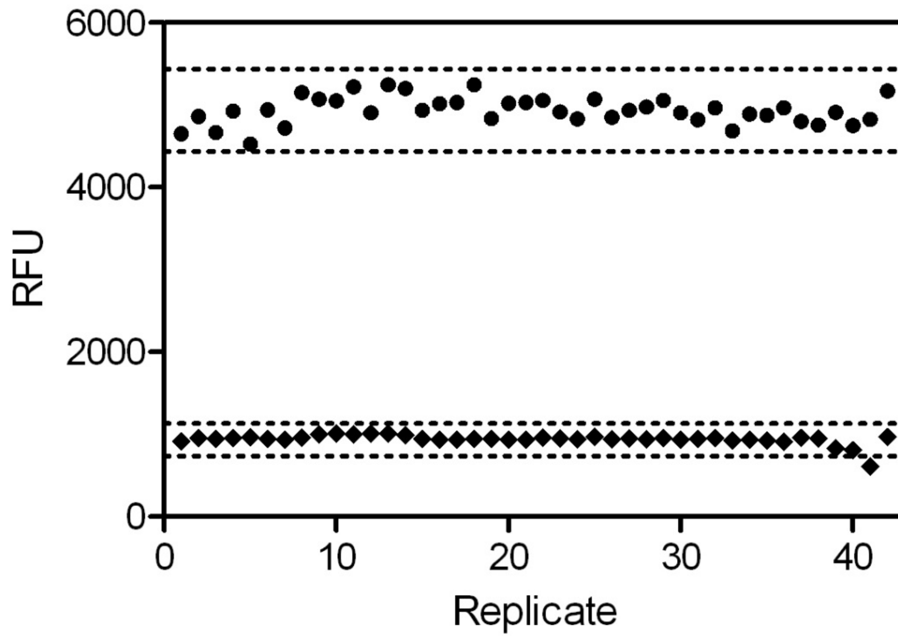

Figure 4. Z' analysis: MMP-9 enzyme was pre-incubated without (●) or with (◆) NNGH Inhibitor for 30 minutes at ~25°C before the addition of OmniMMP™ Red substrate. After the addition of substrate, the reaction proceeded at ~25°C before the fluorescence was measured at 530nm/590nm, 515nm cutoff on a BioTek Synery2 plate reader. Final concentrations were 500nm substrate, 9 mU/µl MMP-9, 1µM NNGH, 0.5% DMSO. The Z' factor for this assay was 0.83, (Z' factor = $1-((3SD^{control} + 3SD^{inhibitor})/(mean^{control} - mean^{inhibitor}))$. Dashed lines indicate 3 * standard deviation.

Notes

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TRADEMARKS AND PATENTS

Several Enzo Life Sciences products and product applications are covered by US and foreign patents and patents pending.

Product Manual

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