Enabling Discovery in Life Science®

MMP-2 Fluorometric Drug Discovery Kit, RED

Designed to screen MMP-2 inhibitors using a quenched fluorogenic peptide.

Instruction Manual
BML-AK302

For research use only

BACKGROUND

Matrix metalloproteinase-2 (MMP-2, gelatinase A, 72 kDa 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-2 include native and denatured collagens, fibronectin, elastin, laminin-5, pro-TNF-α, and neurocan[1-6]. MMP-2 is secreted as a 72 kDa proenzyme (as measured by SDS-PAGE), and activated by cleavage to 62 and 59 kDa[7]. MMP-2 is an important target for inhibitor screening due to its involvement in diseases such as atherosclerosis[8], and cancer growth, angiogenesis, and metastasis[3].

The *MMP-2 Fluorometric Drug Discovery Kit, RED* is a complete assay system designed to screen MMP-2 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 (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_ms near their limits of solubility, making enzyme and inhibitor kinetics difficult. MMP K_ms for OmniMMP™ RED substrate are below its solubility limit. 3) In addition to the efficient binding as exhibited by low K_ms, 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-2, a potential therapeutic target. The compound NNGH[9] 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)
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4. *Induction of cell migration by matrix metalloproteinase-2 cleavage of laminin-5*: G. Giannelli *et al.*; Science **277**, 225 (1997)
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6. *Determination of protease cleavage site motifs using mixture-based oriented peptide libraries*: B.E. Turk *et al.*; Nat. Biotechnol. **19**, 661 (2001)
7. *Intermolecular autolytic cleavage can contribute to the activation of progelatinase A by cell membranes*: S.J. Atkinson *et al.*; J. Biol. Chem. **270**, 30479 (1995)
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9. *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-AK302 KIT

BML-SE237-9090 MMP-2 ENZYME (HUMAN, RECOMBINANT)
FORM: *E. coli* recombinant human MMP-2 catalytic domain (calculated MW 40 kDa). Purity >95% by SDS-PAGE.
UNIT DEFINITION: One unit is defined as the amount of enzyme that will hydrolyze 100 μM 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: 150 U
PRESENTATION: Liquid in screw-cap microfuge vial.

BML-P277-9090 SUBSTRATE (OmniMMP™ RED fluorogenic substrate peptide; MW=1910.7)
FORM: 250 μM in DMSO
STORAGE: -70 °C
PRESENTATION: 40 μl in amber screw-cap microfuge vial.

BML-KI582 6-TAMRA CALIBRATION STANDARD
FORM: 10 μM in DMSO; MW=430.4
STORAGE: -70 °C
PRESENTATION: 20 μl 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 μl in screw-cap microfuge vial.

BML-KI175 ASSAY BUFFER
50 mM HEPES, 10 mM $CaCl_2$, 0.05% Brij-35, pH 7.5
FORM: Liquid in screw-cap plastic bottle
STORAGE: Room temperature
QUANTITY: 20 ml

80-2409 96-WELL 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 µl 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 °C for the highest stability. The MMP-2 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-2 enzyme is stable for at least 5 freeze/thaw cycles. To minimize the number of freeze/thaw cycles, aliquot the MMP-2 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 (BML-PI115-9090) 1/200 in assay buffer BML-KI175 as follows. Add 1 µl inhibitor into 200 µl assay buffer, in a separate tube. Warm to reaction temperature (e.g. 37 °C).
3. Thaw the substrate (BML-P277-9090) and dilute sufficient volume to 5 µM in assay buffer (10 µl needed per well). Warm to reaction temperature (e.g. 37 °C).
4. Dilute MMP-2 enzyme to 58 mU/µl in assay buffer to required total volume (20 µl 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 1/2 volume microplate as follows:
   Calibration = 80 µl in 3 wells (see step 11)
   Control (no inhibitor) = 70 µl
   Inhibitor NNGH = 50 µl
   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 20 µl MMP-2 (diluted in step 4) to the control, inhibitor NNGH, and test inhibitor wells. Final amount of MMP-2 will be 1.16 U per well.
8. Add 20 µl NNGH inhibitor (diluted in step 2) to the inhibitor NNGH wells only. Final inhibitor concentration = 1.3 µM**.** Note: 1.3 µM NNGH will inhibit MMP-2 by ~90% 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 °C) to allow inhibitor/enzyme interaction.
11. In the meantime, calibrate the fluorescent microplate reader, using Ex/Em=545/576 nm, with cutoff set at 570 nm: Pre-warm 80µl assay buffer to reaction temperature in 3 wells in the microplate, then to each add 10 µl BML-P277-9090 substrate peptide to give the concentration to be used in the assay (e.g., for 0.5 µM final add 10 µl 5 µM) 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 10 µl (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-2 (58 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

<u>To determine inhibitor % remaining activity</u>:

Inhibitor % activity remaining = (**V** inhibitor / **V** control) x 100
See Figure 1 for example of results.

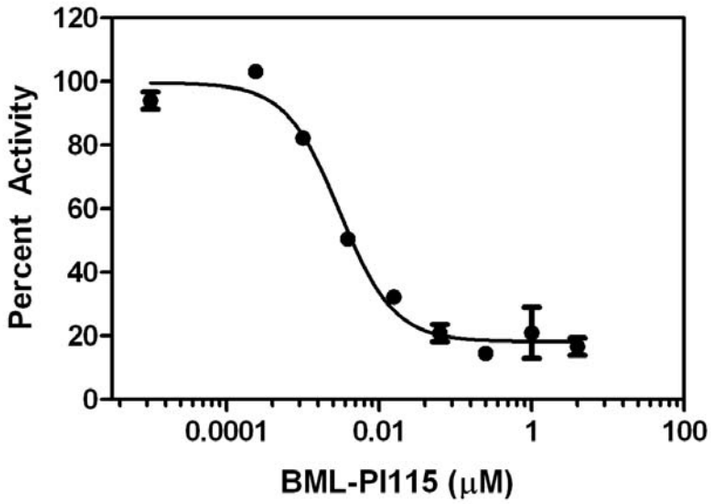

Figure 1. Inhibitor dose response curve: NNGH was pre-incubated with MMP-2 enzyme before reactions were started by the addition of substrate. Final concentrations of reagents were 11.6 mU/µl MMP-2, 500 nM OmniMMP™ Red, 0.5% DMSO. IC_{50} ~ 3 nM. The dose-response curve was derived from a fit to a four parameter Hill-Slope model, Y=Bottom + (Top-Bottom)/(1+10^((LogIC_{50}-X)*HillSlope)) using Graphpad Prism software. Data are presented as mean of duplicate wells ± SEM.

<u>To determine the activity of the samples expressed as picomoles substrate hydrolyzed per minute</u>:

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)

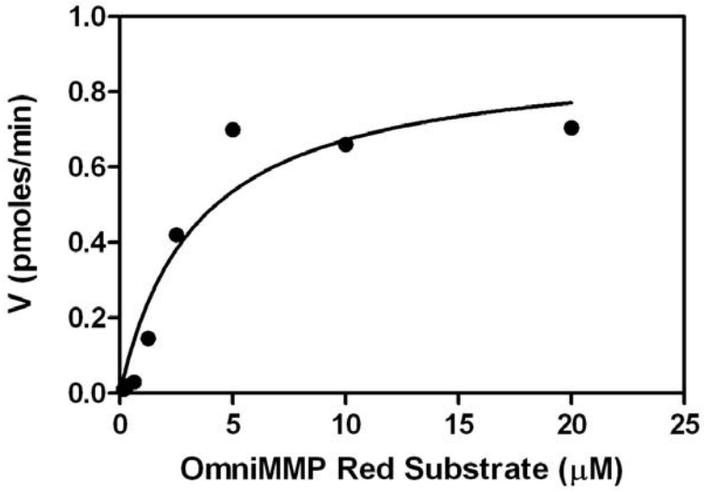

Figure 2. Example graph for k_{cat}/K_m determination for MMP-2. K_m = 3.4 µM, k_{cat}/K_m = 3.1 x 10^6 $M^{-1}sec^{-1}$. Data shown are the mean of duplicate wells ± SEM.

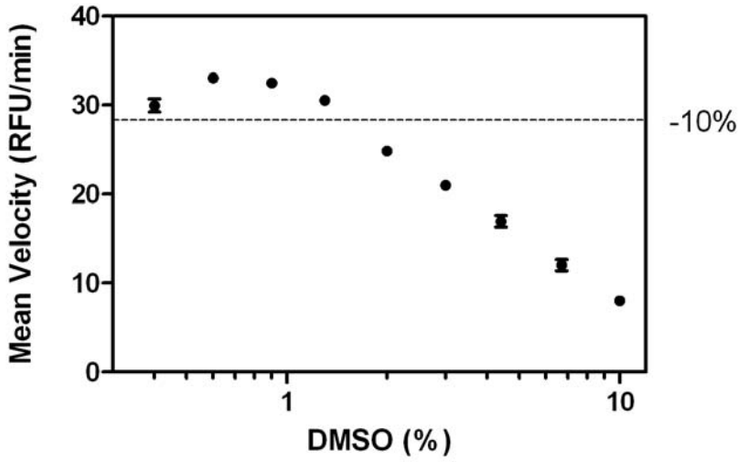

Figure 3. DMSO Sensitivity: Final concentrations of reagents were 11.6 mU/ul MMP-2 enzyme, 1 µM OmniMMP™ Red, variable DMSO concentration. Data are presented as mean of duplicate wells ± SEM.

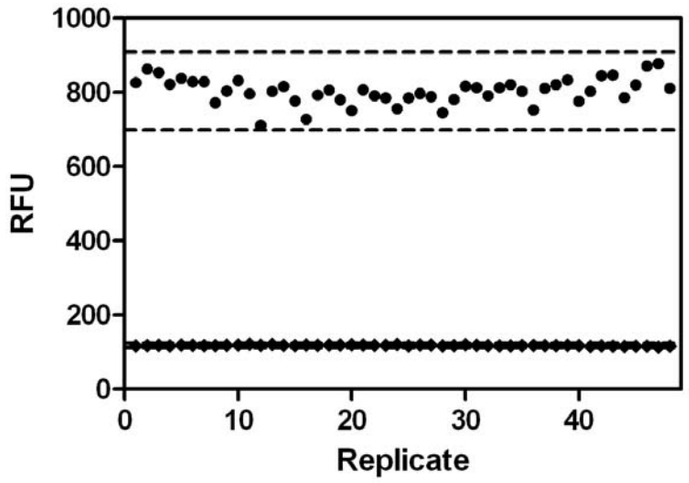

Figure 4. Z factor analysis: MMP-2 enzyme (●) or KI175 buffer (◆) was preincubated to 37 °C before the addition of OmniMMP™ Red substrate. After the addition of substrate, the reaction proceeded at ~37 °C for 45 minutes before the fluorescence was measured at 530 nm/590 nm, on a BioTek Synergy2 plate reader. Final concentrations were 500 nM substrate, 11.6 mU/µl MMP-2, 0.5 % DMSO. The Z factor for this assay was 0.84, (Z factor = $1-((3SD^{control} + 3SD^{no\ enzyme})/(mean^{control} - mean^{no\ enzyme}))$. Dashed lines indicate 3 * standard deviation.

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