Product Manual

MMP-12 Fluorometric Drug Discovery Kit

Catalog # BML-AK403

Designed to screen MMP-12 inhibitors using a quenched fluorogenic peptide.

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Product Manual

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 Support if necessary.

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BACKGROUND

Matrix metalloproteinase-12 (MMP-12, metalloelastase, macrophage elastase, commonly confused with neutrophil elastase) 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]. Targets of MMP-12 include elastin, fibronectin, laminin, plasminogen, u-PAR, and tissue factor pathway inhibitor[1-4]. MMP-12 is secreted as a 53 kDa proenzyme (as measured by SDS-PAGE), and activated by cleavage to forms of 45-22 kDa[5]. MMP-12 is an important target for inhibitor screening due to its involvement in diseases such as cancer and emphysema[2].

The MMP-12 Fluorometric Drug Discovery Kit is a complete assay system designed to screen MMP-12 inhibitors using a quenched fluorogenic peptide: OmniMMP™ fluorogenic substrate Mca-Pro-Leu-Gly-Leu-Dpa-Ala-Arg-NH_2 [Mca=(7-methoxycoumarin-4-yl)-acetyl;Dpa=N-3-(2,4-dinitrophenyl)-L-α-ß-diaminopropionyl][6]. Mca fluorescence is quenched by the Dpa group until cleavage by MMPs at the Gly-Leu bond separates the two moieties[7,8]. The assays are performed in a convenient 96-well microplate format. The kit is useful to screen inhibitors of MMP-3, a potential therapeutic target. The compound NNGH[9] is also included as a prototypic control inhibitor.

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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.

MATERIALS PROVIDED


Avoid freeze / thaw cycles

BML-SE138-9090 MMP-12 ENZYME (HUMAN, RECOMBINANT)

FORM: *E. coli* recombinant human MMP-12 catalytic domain (calculated MW 20.3 kDa), 10 U/µL.

UNIT DEFINITION: One unit is defined as the amount of enzyme that will hydrolyze 100 µM thiopeptolide Ac-PLG-[2-mercapto-4-methyl-pentanoyl]-LG-OC_2H_5 (Prod. No. BML-P125) at 100 pmol/min @ 37°C.

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

QUANTITY: 140 U

PRESENTATION: 14 µL in screw-cap microfuge vial.

BML-P126-9090 SUBSTRATE (OmniMMP™ fluorogenic substrate peptide; MW=1093.2)

FORM: 400 µM (437 µg/mL) in DMSO (dimethylsulfoxide)

STORAGE: -70 °C

PRESENTATION: 200 µL in amber screw-cap microfuge vial.

BML-P127-9090 CALIBRATION STANDARD (OmniMMP™ fluorogenic control peptide, Mca-Pro-Leu-OH; MW=444.5)

FORM: 40 µM (17.8 µg/mL) in DMSO

STORAGE: -70°C

PRESENTATION: 50 µL in amber screw-cap microfuge vial.

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

FORM: 1.3 mM in DMSO

STORAGE: -20 or -70°C

PRESENTATION: 50 µL in screw-cap microfuge vial.

BML-KI175-0020 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

80-2406 ½ VOLUME 96-WELL WHITE NBS MICROPLATE

STORAGE: Room temperature.

OTHER MATERIALS REQUIRED

1. Fluorescent microplate reader capable of excitation at 328 nm and emission at 420 nm. The following Ex/Em have also been used: 320,340/393,400,405.
2. Pipetman or multi-channel pipetman capable of pipetting 1-100 µL accurately.
3. Ice bucket to keep reagents cold until use.
4. 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-12 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-12 enzyme is stable for at least 4 freeze/thaw cycles. To minimize the number of freeze/thaw cycles, aliquot the MMP-12 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-P126-9090, BML-P127-9090, and BML-PI115-9090 to RT to thaw DMSO.
2. Dilute inhibitor (NNGH, 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 DMSO stock vial of substrate BML-P126-9090 and dilute sufficient volume to 40 µM in assay buffer (10 µL needed per well). Warm to reaction temperature (e.g. 37°C).
4. Dilute MMP-12 enzyme 1/285 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, below)

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-12 (diluted in step 4) to the control, inhibitor NNGH, and test inhibitor wells. Final amount of MMP-12 will be 0.7 U per well (7.0 mU/µL). **Remember**-do not add MMP-12 to the calibration wells!
8. Add 20 µL NNGH inhibitor (diluted in step 2) to the inhibitor NNGH wells only. Final inhibitor concentration = 1.3 µM.
9. Add desired volume of test inhibitor to appropriate wells. See Table 1, below.
10. Incubate plate for 30-60 min at reaction temperature (e.g. 37°C) to allow inhibitor/enzyme interaction.
11. In the meantime, calibrate the fluorescent microplate reader, using Ex/Em=328/420: Prewarm assay buffer to reaction temperature in 3 wells in the microplate, then to each add 10 µL BML-P126-9090 substrate peptide to give the concentration to be used in the assay (e.g., for 4 µM final add 10 µL 40µM) 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 calibration standard peptide BML-P127-9090 to give 3 different final molar concentrations ranging between 2 and 10% of the substrate peptide molar concentration (e.g., 80, 200, and 400 nM) and measure their fluorescence. Use these values to build a standard curve relating micromolar BML-P127-9090 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-P126-9090 substrate (diluted and equilibrated to reaction temperature in step 3). Final substrate concentration = 4 µM.
13. Continuously read plates in the fluorescent microplate reader, using Ex/Em=328/420. **Suggestion-** record data at 1 min time intervals for 10 min.
14. Perform data analysis (see below).

NOTE: Retain microplate for future use of unused wells.

TABLE 1. Example of Samples

Sample	Assay buffer	MMP-12 (35 mU/µL)	Inhibitor (6.5 µM)	Substrate (40 µM)	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

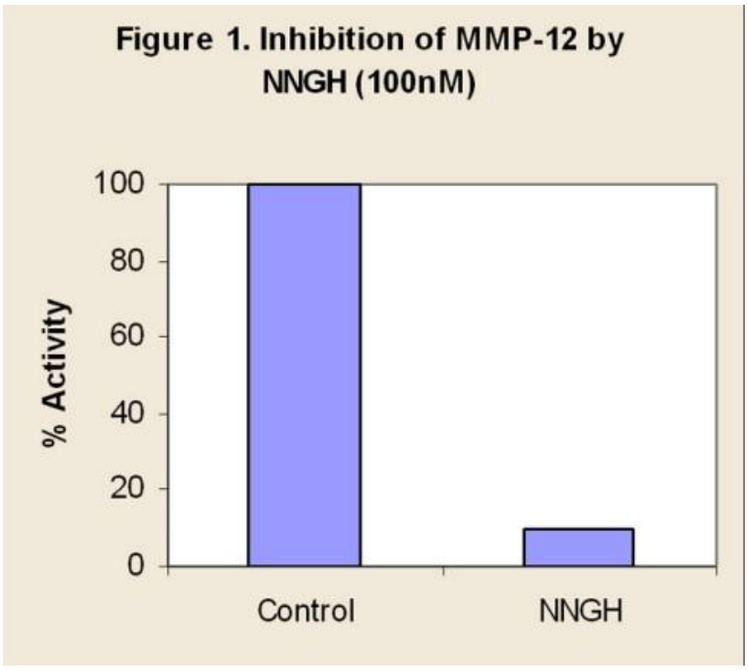
1. Plot data as RFUs (minus Blank RFU value determined during calibration, step 11) versus time for each sample.
2. Determine the range of initial time points during which the reaction is linear.
3. Obtain the initial reaction velocity (V) in RFUs/min: determine the slope of a line fit to the initial linear portion of the data plot using an appropriate routine.
4. 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.



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

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 DATA ANALYSIS step 3), and vol is the reaction volume in microliters (100).

NOTES

NOTES

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