



MMP-1 Fluorometric Drug Discovery Kit

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

**Instruction Manual
BML-AK405**

For research use only

✦ MMP-1 Fluorometric Drug Discovery Kit - BML-AK405 ✦

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 cancer³.

The *MMP-1 Fluorescent Drug Discovery Kit* is a complete assay system designed to screen MMP-1 inhibitors using a quenched fluorogenic peptide: OmniMMP™ fluorogenic substrate Mca-Pro-Leu-Gly-Leu-Dpa-Ala-Arg-NH₂ [Mca=(7-methoxycoumarin-4-yl)-acetyl; Dpa=N-3-(2,4-dinitrophenyl)-L- α - β -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-1, a potential therapeutic target. The compound NNGH⁹ 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.

COMPONENTS OF BML-AK405 KIT

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

FORM: *E. coli* recombinant human MMP-1 catalytic domain (calculated MW 19.9 kDa), 30.6 U/μL. Purity >95% by SDS-PAGE.

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-OC₂H₅ (Cat. # BML-P125) at 100 pmol/min@ 37°C.

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

QUANTITY: 2000U

PRESENTATION: 66 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 CaCl₂, 0.05% Brij-35, pH 7.5

FORM: Liquid in screw-cap plastic bottle

STORAGE: Room temperature

QUANTITY: 20 mL

80-2406 96-WELL WHITE NBS MICROPLATE

STORAGE: Room temperature.

(Sold Separately as BML-KI571)

OTHER MATERIALS REQUIRED

Fluorescent microplate reader capable of excitation at 328 nm and emission at 420nm. The following Ex/Em have also been used: 320,340/393,400,405.
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°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°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 μ L inhibitor into 200 μ L 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 μ M in assay buffer (10 μ L needed per well). Warm to reaction temperature (e.g. 37°C).
4. Dilute MMP-1 enzyme 1/40 in assay buffer to required total volume (20 μ L 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 μ L in 3 wells (see step 11)
Control (no inhibitor) = 70 μ L
Inhibitor NNGH = 50 μ 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°C).
7. Add 20 μL 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 (153 mU/ μL). Remember to **not** add MMP-1 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 . Note: 1 μM 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°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 40 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 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. For example, record data at 1 minute time intervals for 10 minutes.
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 (40μM)	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

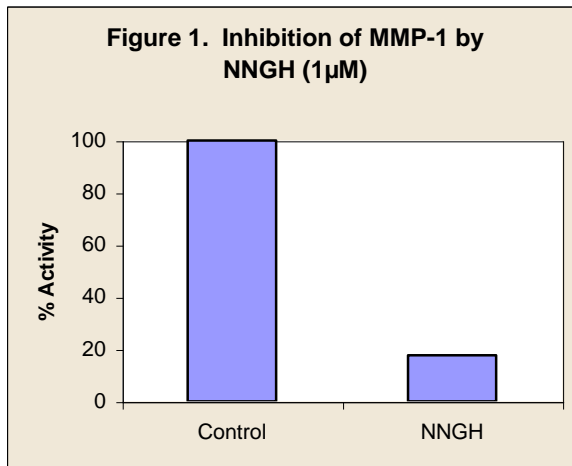
Plotting

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:

Inhibitor % activity remaining = $(V \text{ inhibitor} / V \text{ control}) \times 100$
See Figure 1 for example of results.



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).

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