Product Manual

MMP-3 Fluorometric Drug Discovery Kit, GREEN

Designed to screen MMP-3 inhibitors using a quenched fluorogenic peptide.

Instruction Manual

BML-AK303

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BACKGROUND

Matrix metalloproteinase-3 (MMP-3, stromelysin-1, transin-1) 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 specificities1-3. Targets of MMP-3 include collagens, fibronectin, and laminin, plasminogen, HB-EGF, E-cadherin, and other MMPs1-5. MMP-3 is secreted as a 55-59 kDa glycosylated proenzyme (measured by SDS-PAGE), and activated by cleavage to forms of 21-48 kDa. It is unique from other MMPs in that its pH optimum is 5.9, rather than around 7.06.

The MMP-3 Fluorometric Drug Discovery Kit, GREEN is a complete assay system designed to screen MMP-3 inhibitors using a quenched fluorogenic MMP-3 substrate: 5-FAM-Arg-Pro-Lys-Pro-Val-Glu-Nva-Trp-Arg-Lys(TQ2W)-NH2 [5-FAM=5-carboxyfluorescein; Nva=norvaline; TQ2W=quencher]. FAM fluorescence is thoroughly quenched by the TQ2W group until cleavage by MMPs separates the two moieties.

The MMP-3 Fluorogenic Substrate offers key advantages over other MMP substrates. 1) Emission at the green end of the spectrum avoids the interference at lower wavelengths often exhibited by screening compounds, and by substances commonly found in biological samples and tissue culture medium. 2) The ultra-strong fluorescence of this substrate allow for substrate concentrations much lower than the Km, a condition generally desirable in inhibitor screening/kinetics assays.

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 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 degradation of extracellular matrix: biological consequences: S.D. Shapiro; Curr. Opin. Cell Biol. 10, 602 (1998)
5. Release of an invasion promoter E cadherin fragment by matrilysin and stromelysin-1: V. Noë et al.; J. Cell Sci. 114, 111 (2001)
6. A rationalization of the acidic pH dependence for stromelysin-1 (matrix metalloproteinase-3) catalysis and inhibition: L.L. Johnson et al.; J. Biol. Chem. 275, 11026 (2000)
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.

Reagants require separate storage conditions.

MATERIALS SUPPLIED

BML-SE109-9090 MMP-3 ENZYME (HUMAN, RECOMBINANT)

FORM: *E. coli* recombinant human MMP-3 catalytic domain (calculated MW 19.5 kDa), 10 U/µl. Purity >95% by SDS-PAGE.

UNIT DEFINITION: One unit is defined as the amount of enzyme that will hydrolyze 100µM thiopeptolide 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: 300U

PRESENTATION: 30 µl in screw-cap microfuge vial.

BML-P278-9090 SUBSTRATE

(MPP-3 Fluorogenic Substrate peptide; MW=2156.9)

FORM: 250 µM in DMSO (dimethylsulfoxide)
STORAGE: -70°C
PRESENTATION: 40 µl in amber screw-cap microfuge vial.

BML-KI583-0010 5-FAM CALIBRATION STANDARD
FORM: 10 µM in DMSO; MW=376.3
STORAGE: -70°C
PRESENTATION: 20 µl in amber screw-cap microfuge vial.

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

FORM: 1.3mM in DMSO
STORAGE: -20°C
PRESENTATION: 50µl in screw-cap microfuge vial.

BML-KI127-0020 ASSAY BUFFER

50mM MES, 10mM $CaCl_2$, 0.05% Brij-35, pH 6.0

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 NEEDED

1. Fluorescent microplate reader capable of excitation at 494 nm and emission at 521nm.
2. Pipetmen or multi-channel pipetmen capable of pipetting 1-100µl accurately
3. Ice bucket to keep reagents cold until use.
4. Water bath or incubator for component temperature equilibration Phenylmethylsulfonyl fluoride (PMSF), Sigma #P7626 or equivalent.

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-3 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-3 enzyme is stable for 4 freeze/thaw cycles. To minimize the number of freeze/thaw cycles, aliquot the MMP-3 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-P278-9090, BML-KI583, and BML-PI115-9090 to RT to thaw DMSO.
2. Dilute inhibitor (NNGH, BML-PI115-9090) 1/200 in assay buffer BML-KI583 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-P278-9090 and dilute sufficient volume to 5 µM in assay buffer (10µl needed per well). Warm to reaction temperature (e.g. 37°C).
4. Dilute MMP-3 enzyme to 100 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 = 80µl 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-3 (diluted in step 4) to the control, inhibitor NNGH, and test inhibitor wells. Final amount of MMP-3 will be 2 U per well (20 mU/□l).
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 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=494/521: Prewarm 80 µl assay buffer to reaction temperature in 3 wells in the microplate, then to each add 10µl BML-P278-9090 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 calibration standard peptide BML-KI583 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-KI583 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-P278-9090 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=494/521 nm, with cutoff set at 515 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-3 (100mU/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, 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.

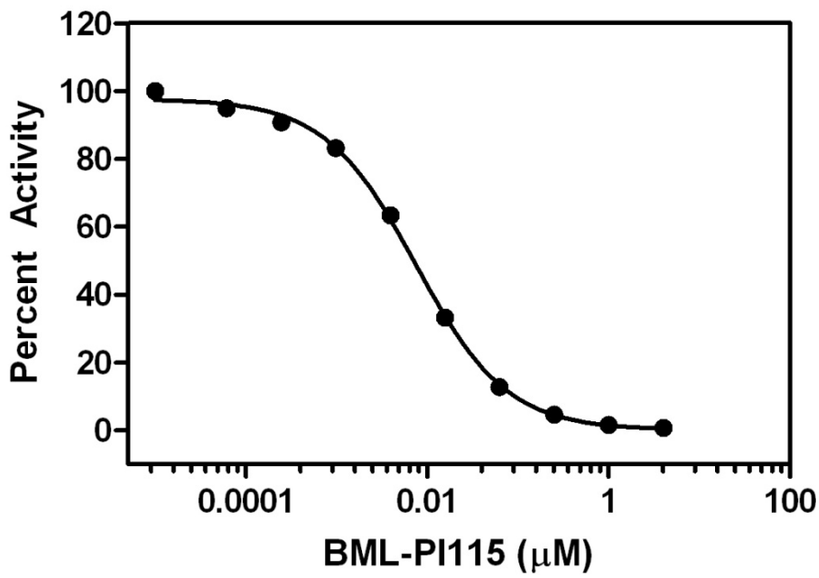

Figure 1. Inhibitor dose response curve: NNGH was pre-incubated with MMP-3 enzyme before reactions were started by the addition of substrate. Final concentrations of reagents were 20 mU/µl MMP-3, 500 nM MMP-3 fluorogenic substrate, 0.5 % DMSO. IC_{50} was calculated as 7.5 nM. Data are presented as mean 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).

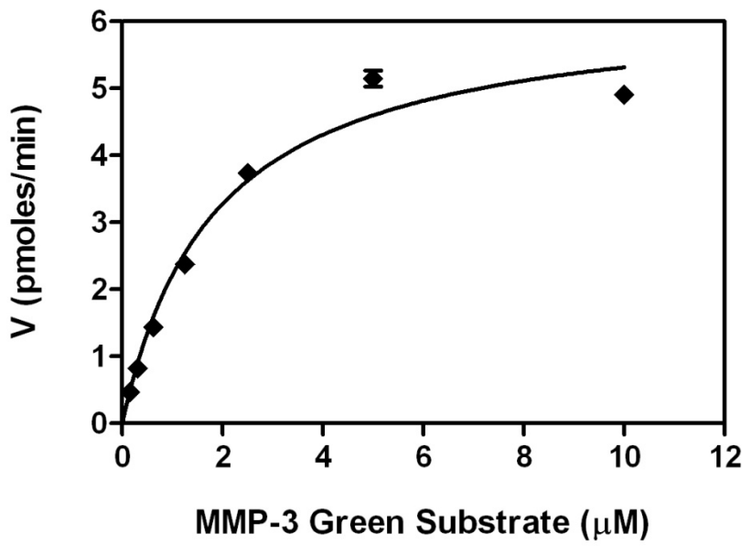

Figure 2. Example graph for k_{cat}/K_m determination for MMP-3. *Km* = 1.8 µM, k_{cat}/K_m = 2.2 x 10^4 $M^{-1}sec^{-1}$.

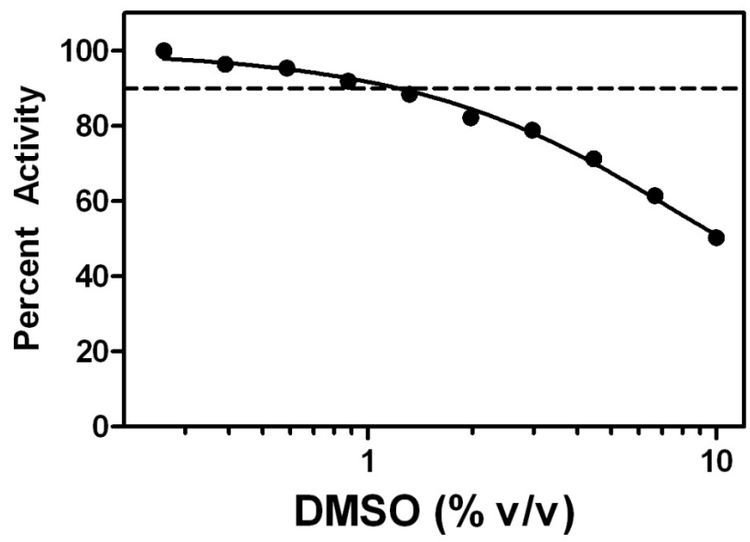

Figure 3. DMSO Sensitivity: Final concentrations of reagents were 20 mU/µl MMP-3 enzyme, 500 nM MMP-3 fluorogenic substrate, variable DMSO concentration. Data are presented as mean of duplicate wells ± SEM.

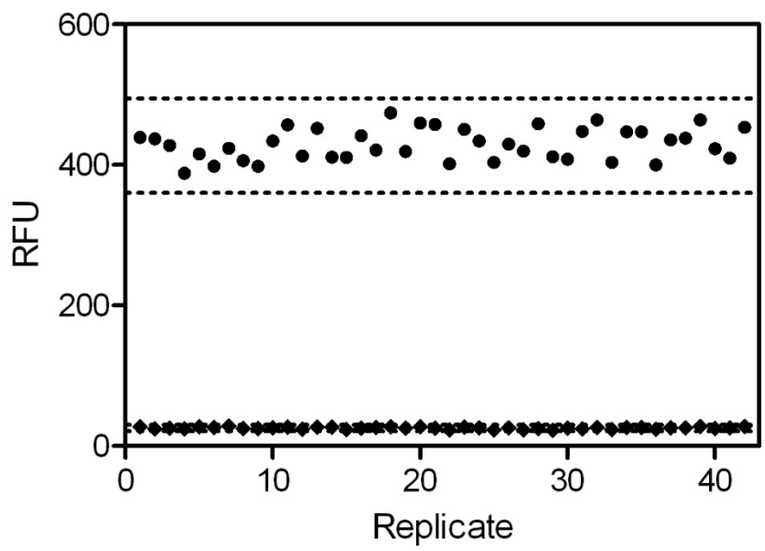

Figure 4. Z' analysis: MMP-3 enzyme was pre-incubated without (●) or with (◆) NNGH Inhibitor for 30 minutes at ~25 °C before the addition of MMP-3 Green substrate. After the addition of substrate, the reaction proceeded at ~25 °C before the fluorescence was measured at 485 nm/521 nm 515 nm cutoff on a Spectramax Gemini plate reader. Final concentrations were 500 nM substrate, 20 mU/µl MMP-3, 1 µM NNGH, 0.5% DMSO. The Z' factor for this assay was 0.82, (Z' factor = 1-((3SD^{control} + 3SD^{inhibitor})/(mean^{control} –mean^{inhibitor})). Dashed lines indicate the 3 Standard deviation range.

NOTES

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