Enabling Discovery in Life Science®

MMP-3 Fluorometric Drug Discovery Kit

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

Instruction Manual
BML-AK401

For research use only

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 specificities[1]. Targets of MMP-3 include collagens, fibronectin, and laminin, plasminogen, HB-EGF, E-cadherin, and other MMPs[1,2,3]. 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 7.0[4].

The *MMP-3 Fluorimetric Drug Discovery Kit* is a complete assay system designed to screen MMP-3 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-β-diaminopropionyl][5]. Mca fluorescence is quenched by the Dpa group until cleavage by MMPs at the Gly-Leu bond separates the two moieties[5,6]. 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[7] is also included as a prototypic control inhibitor.

REFERENCES:

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3. V. Noë *et al. J. Cell Sci.* 2000 **114** 111
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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-AK401 KIT

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
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: 300 U
PRESENTATION: 30 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: 1.3mM in DMSO
STORAGE: -20 or -70°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

80-2406 ½ VOLUME 96-WELL WHITE NBS MICROPLATE
STORAGE: Room temperature.
(Sold separately as BML-KI571)

OTHER MATERIALS REQUIRED

Fluorescent microplate reader capable of excitation at 328nm 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 µl accurately
Ice bucket to keep reagents cold until use.
Water bath or incubator for component temperature equilibration

EXPERIMENTAL METHODS

***PLEASE NOTE:* The recommended dilution of substrate BML-P126-9090 in step 3 has been changed so that the volume added to assay wells (in steps 11 and 12) is 10µl instead of 1µl.**

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-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-KI127 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 40µM in assay buffer (10µl needed per well). Warm to reaction temperature (e.g. 37°C).
4. Dilute MMP-3 enzyme 1/100 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
I1…	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 (0.02U/µl). Remember to **not** add MMP-3 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 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 10µl BML-P126-9090 substrate peptide to give the concentration to be used in the assay (e.g., for 4 µM final add 10µl 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 10µl 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-3 (0.1U/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

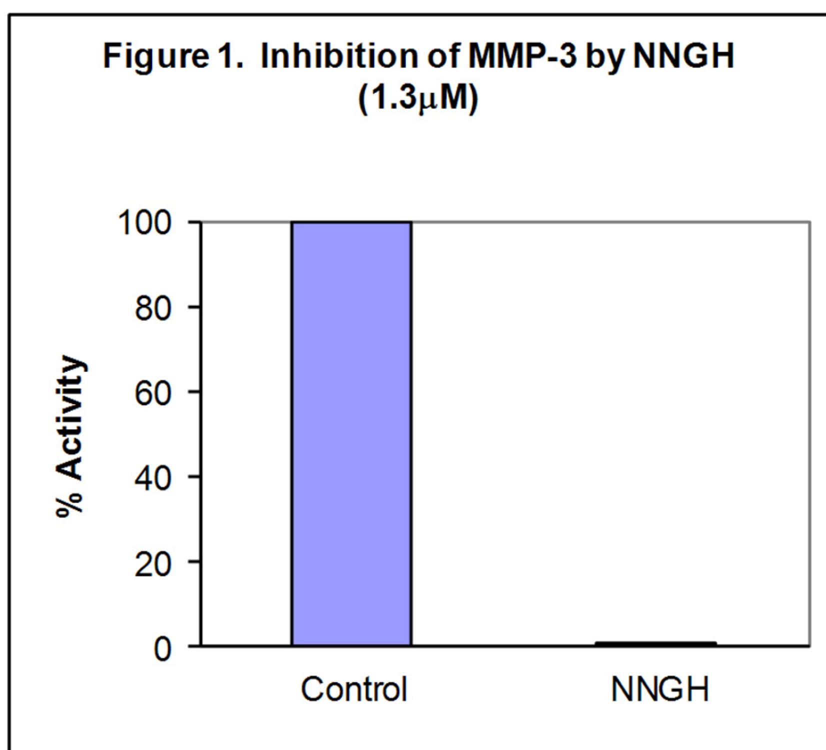
Plotting

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, 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 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).

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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.

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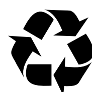

www.enzolifesciences.com

Enabling Discovery in Life

Global Headquarters
Enzo Life Sciences Inc.
10 Executive Blvd
Farmingdale, NY 11735
(p) 1-800-942-0430
(f) 1-631-694-7501
(e) info-usa@enzolifesciences.com

Enzo Life Sciences (ELS) AG
Industriestrasse 17, Postfach
CH-4415 Lause / Switzerland
(p) +41/0 61 926 89 89
(f) +41/0 61 926 89 79
(e) info-ch@enzolifesciences.com

Please visit our website at www.enzolifesciences.com for additional contact information.