Enabling Discovery in Life Science®

MMP-12 Fluorometric Drug Discovery Kit, GREEN

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

Instruction Manual
BML-AK312

For research use only

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[1-3]. Targets of MMP-12 include elastin, fibronectin, laminin, plasminogen, u-PAR, and tissue factor pathway inhibitor[1-6]. MMP-12 is secreted as a 53 kDa proenzyme (as measured by SDS-PAGE), and activated by cleavage to forms of 45-22 kDa[7]. MMP-12 is an important target for inhibitor screening due to its involvement in diseases such as cancer, allergy and emphysema[1-3, 8].

The *MMP-12 Fluorescent Drug Discovery Kit, GREEN* is a complete assay system designed to screen MMP-12 inhibitors using a quenched MMP-3 Fluorogenic Substrate: 5-FAM-Arg-Pro-Lys-Pro-Val-Glu-Nva-Trp-Arg-Lys(TQ2W)-NH_2 [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 green Fluorogenic MMP substrate provided in the kit, which is cleaved by both MMP-3 and MMP-12, offers key advantages over other MMP substrates. 1) Emission in the green region 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 allows for substrate concentrations much lower than the K_m, 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-12, a potential therapeutic target. The compound NNGH[7] 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. *Proteolysis of the urokinase-type plasminogen activator receptor by metalloproteinase-12: implication for angiogenesis in fibrin matrices.* P. Koolwijk *et al.* Blood **97,** 3123 (2001)
6. *Matrix metalloproteinases cleave tissue factor pathway inhibitor. Effects on coagulation* A. Belaaouaj *et al.* J. Biol. Chem*.* **275,** 27123 (2000)
7. *Hydrolysis of a broad spectrum of extracellular matrix proteins by human macrophage elastase*. T.J. Gronski, Jr. *et al.* J. Biol. Chem*.* 1997 **272,** 12189 (1997)
8. *Matrix metalloproteinase-12 is a therapeutic target for asthma in children and young adults***.** Mukhopadhyay S. *et a*l.; J Allergy Clin Immunol **126**, 70 (2010).

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-AK312 KIT

BML-SE138-9090 MMP-12 ENZYME (HUMAN, RECOMBINANT)
FORM: *E. coli* recombinant human MMP-12 catalytic domain (calculated MW 20.3 kDa). 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: 140 U
PRESENTATION: Frozen liquid in screw-cap microfuge vial.

BML-P278-9090 SUBSTRATE (MMP-3 Fluorogenic Substrate; peptide MW=2156.9)
FORM: 250μM in DMSO (dimethylsulfoxide)
STORAGE: -70°C
PRESENTATION: 40μl in amber screw-cap microfuge vial.

BML-KI583-0010 5-FAM CALIBRATION STANDARD
FORM: 10μM in DMSO; MW=376.3
STORAGE: -70°C
PRESENTATION: 20μl 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-KI175-0020 ASSAY BUFFER
50mM HEPES, 10mM $CaCl_2$, 0.05% Brij-35, pH 7.5
FORM: Liquid in screw-cap plastic bottle
STORAGE: Room temperature
QUANTITY: 20ml

80-2409 96-WELL BLACK NBS MICROPLATE
STORAGE: Room temperature.

OTHER MATERIALS REQUIRED

1. Fluorescent microplate reader capable of excitation at 494 nm and emission at 521 nm.
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

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 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-P278-9090, BML-KI583-0010, 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 1µl inhibitor into 200µl assay buffer, in a separate tube.
3. Thaw the DMSO stock vial of substrate BML-P278-9090 and dilute sufficient volume to 5µM in assay buffer (10µl needed per well).
4. Dilute MMP-12 enzyme to 50 mU/µl in assay buffer to required total volume (20µl 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) = 70µl

   Inhibitor NNGH = 50µl

   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 20µl MMP-12 (diluted in step 4) to the control, inhibitor NNGH, and test inhibitor wells. Final amount of MMP-12 will be 1 U per well (10 mU/µl).
8. Add 20µl NNGH inhibitor (diluted in step 2) to the inhibitor NNGH wells only. Final inhibitor concentration = 1.3µM.
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 80µl 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, for example, using Ex/Em=494/521 nm, with cutoff ~510 nm. 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-12 (50 mU/µl)	Inhibitor (6.5µM)	Substrate (5µM)	Total Volume
Control	70µl	20µl	0	10µl	100µl
Inhibitor NNGH	50µl	20µl	20µl	10µl	100µl
Test inhibitor*	Xµl	20µl	Yµl	10µl	100µl

*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 100µl.

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.

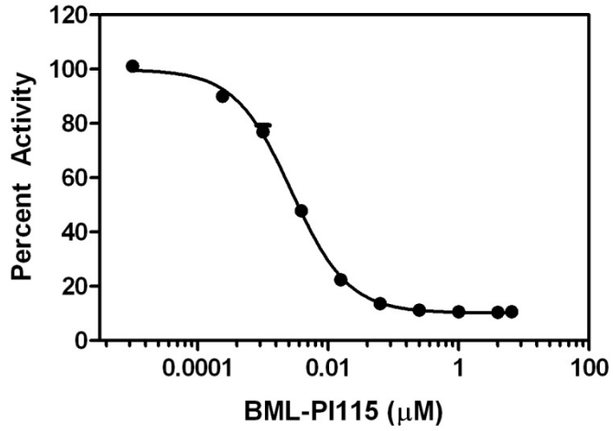

Figure 1. Inhibitor dose response curve: NNGH was pre-incubated with MMP-12 enzyme before reactions were started by the addition of substrate. Final concentrations of reagents were 10 mU/µl MMP-12, 750 nM MMP-3 Fluorogenic Substrate, 0.5 % DMSO. IC_{50} = 3 nM. Data were collected on a Biotek Synergy2 plate reader. 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.

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)

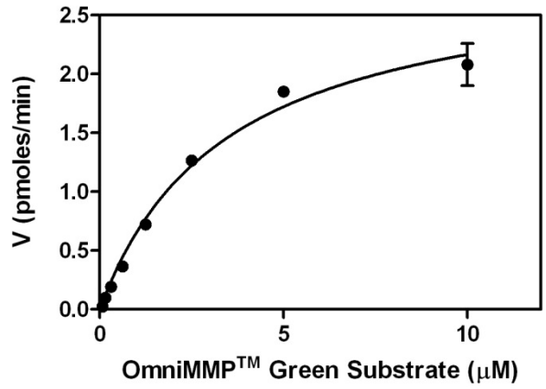

Figure 2. Example graph for k_{cat}/K_m determination for MMP-12. K_m = 3.5μM, k_{cat}/K_m = 1.1 x 10^5 $M^{-1}sec^{-1}$. Data shown are the mean of duplicate wells ± SEM.

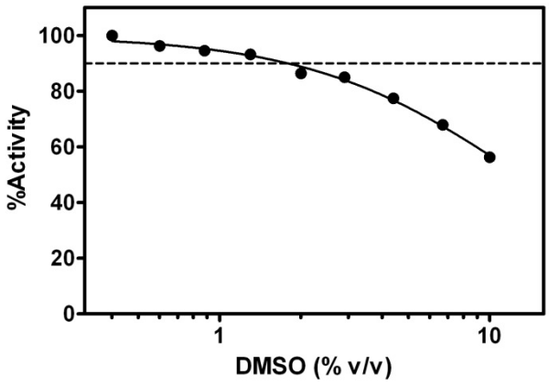

Figure 3. DMSO Sensitivity: Final concentrations of reagents were 10 mU/ul MMP-12 enzyme, 1μM MMP-3 Fluorogenic Substrate, variable DMSO concentration. Data are presented as mean of duplicate wells.

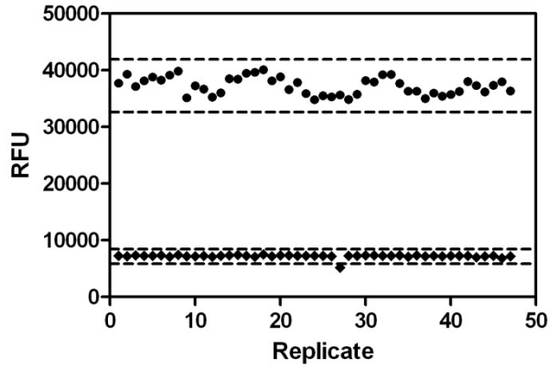

Figure 4. Z analysis: MMP-12 enzyme (●) or BML-KI175 buffer (◆) was prewarmed to 37°C before the addition of MMP-3 Fluorogenic Substrate. After the addition of substrate, the reaction proceeded for 45 minutes before the fluorescence was measured at 485 nm/528 nm (510 nm cutoff) on a BioTek Synergy2 plate reader. Final concentrations were 500 nM substrate, 10 mU/μl MMP-12, 0.5 % DMSO. The Z factor for this assay was 0.80, (Z factor = 1-((3SD^{enzyme} + 3SD^{buffer})/($mean^{enzyme}$ – $mean^{buffer}$)). Dashed lines indicate 3 * standard deviation.

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