MMP-2 Colorimetric Drug Discovery Kit

Designed to screen MMP-2 inhibitors using a thiopeptide as a chromogenic substrate.

Instruction Manual

BML-AK408

For research use only

BACKGROUND

Matrix metalloproteinase-2 (MMP-2, gelatinase A, 72 kDa type IV 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 specificities[1]. Targets of MMP-2 include native and denatured collagens, fibronectin, elastin, laminin-5, pro-TNF-α, and neurocan[1-5]. MMP-2 is secreted as a 72 kDa proenzyme (as measured by SDS-PAGE), and activated by cleavage to 62 and 59 kDa[6]. MMP-2 is an important target for inhibitor screening due to its involvement in diseases such as atherosclerosis[7], and cancer growth, angiogenesis, and metastasis[2,8].

The *MMP-2 Colorimetric Drug Discovery Kit* is a complete assay system designed to screen MMP-2 inhibitors using a thiopeptide as a chromogenic substrate (Ac-PLG-[2-mercapto-4-methyl-pentanoyl]-LG-OC_2H_5)[9,10]. The MMP cleavage site peptide bond is replaced by a thioester bond in the thiopeptide. Hydrolysis of this bond by an MMP produces a sulfhydryl group, which reacts with DTNB [5,5'-dithiobis(2-nitrobenzoic acid), Ellman's reagent] to form 2-nitro-5-thiobenzoic acid, which can be detected by its absorbance at 412 nm (ε=13,600 $M^{-1}cm^{-1}$ at pH 6.0 and above[11]). The assays are performed in a convenient 96-well microplate format. The kit is useful to screen inhibitors of MMP-2, a potential therapeutic target. An inhibitor, NNGH[12], is also included as a prototypic control inhibitor. Thiol inhibitors should not be used with this kit, as they may interfere with the colorimetric assay.

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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 KIT BML-AK408

BML-SE237-9090 MMP-2 ENZYME (HUMAN, RECOMBINANT)

FORM: *P. pastoris* recombinant human MMP-2 catalytic domain (calculated MW 40 kDa), One U=100 pmol/min@ 37°C, 100 µM thiopeptide P125.

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

QUANTITY: 150 U

BML-P125-9090 MMP SUBSTRATE (chromogenic; MW=655.9)

FORM: 25 mM (16.4 mg/ml) in DMSO

STORAGE: -20°C

QUANTITY: 50 µl

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

FORM: 1.3 mM in DMSO

STORAGE: -70°C

QUANTITY: 50 µl

BML-KI173 ASSAY BUFFER

50mM HEPES, 10mM $CaCl_2$, 0.05% Brij-35, 1mM DTNB, pH7.5

STORAGE: -20°C

QUANTITY: 20 ml

80-2404 CLEAR ½ AREA MICROTITER PLATE

STORAGE: Room temperature.

OTHER MATERIALS REQUIRED

- Microplate reader capable of measuring A_{412} to ≥3-decimal accuracy.
- Pipetman or multi-channel pipetman capable of pipetting 10-100 µl accurately (note: reagents can be diluted to increase the minimal pipetting volume to >10 µl).
- 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 (room temperature) at -70°C for the highest stability. The MMP-2 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-2 enzyme is stable for at least 5 freeze/thaw cycles. To minimize the number of freeze/thaw cycles, aliquot the MMP-2 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-P125-9090 and BML-PI115-9090 to RT to thaw DMSO.
2. Dilute inhibitor (NNGH, BML-PI115-9090) 1/200 in assay buffer KI-173 as follows. Add 1 µl inhibitor into 200 µl assay buffer, in a separate tube. Warm to reaction temperature (e.g. 37°C).
3. Dilute substrate BML-P125-9090 1/25 in assay buffer to required total volume (10 µl are needed per well). For example, for 15 wells dilute 6.4 µl BML-P125-9090 into 153.6 µl assay buffer, in a separate tube. Warm to reaction temperature (e.g. 37°C).
4. Dilute MMP-2 enzyme 1/56 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:
   - Blank (no MMP-2)=90 µl Assay Buffer
   - Control (no inhibitor)=70 µl Assay Buffer
   - Inhibitor NNGH=50 µl Assay Buffer
   - Test inhibitor=varies (see Table 1, below)
6. Allow microplate to equilibrate to assay temperature (e.g. 37°C).
7. Add 20 µl MMP-2 (diluted in step 4) to the control, inhibitor NNGH, and test inhibitor wells. Final amount of MMP-2 will be 1.16 U per well (11.6 mU/µl). Remember to **not** add MMP-2 to the blanks!
8. Add 20 µl NNGH inhibitor (diluted in step 2) to the inhibitor NNGH wells only! Final inhibitor concentration=1.3 µM. Note: 1.3 µM NNGH will inhibit MMP-2 by approximately 94% under these conditions (see Figure 2).
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. Start reaction by the addition of 10 µl BML-P125-9090 substrate (diluted and equilibrated to reaction temperature in step 3). Final substrate concentration=100 µM.
12. Continuously read plates at A_{412nm} in a microplate reader. Record data at 1 min. time intervals for 10 to 20 min.
13. Perform data analysis (see below).

NOTE: Retain microplate for future use of unused wells.

TABLE 1. Example of Samples.

Sample	Assay buffer	MMP-2 (58 mU/µl)	Inhibitor (6.5 µM)	Substrate (1 mM)	Total Volume
Blank	90 µl	0	0	10 µl	100 µl
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.

Example of plate:

well#	sample
A1	Blank
B1	Blank
C1	Control
D1	Control
E1	Inhibitor NNGH
F1	Inhibitor NNGH
G1	Test inhibitor
H1	Test inhibitor

DATA ANALYSIS

Plotting

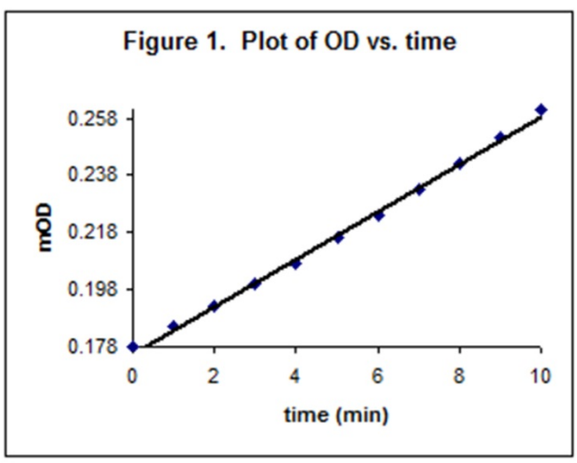
1. Plot data as OD versus time for each sample (see Fig. 1).
2. Determine the range of time points during which the reaction is linear. Typically, points from 1 to 10 min are sufficient.
3. Obtain the reaction velocity (**V**) in OD/min: determine the slope of a line fit to the linear portion of the data plot using an appropriate routine.
4. Average the slopes of duplicate samples.

Data Reduction

5. *If the blank has a significant slope, subtract this number from all samples.*
6. To determine inhibitor % remaining activity:

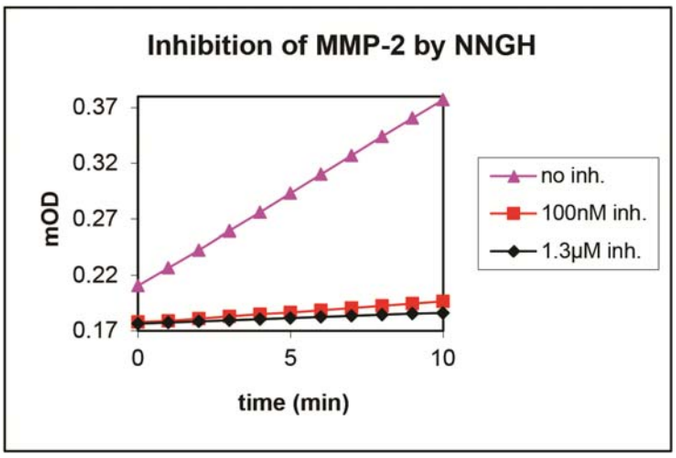
Inhibitor % activity remaining = (**V** inhibitor / **V** control) x 100

See Figure 2 for example.



Slope=**V**=8.60E-03 OD/min

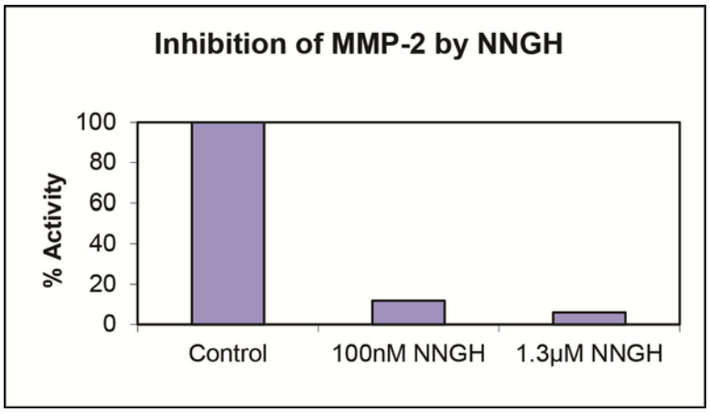
Figure 2. Example of inhibitor data:



control slope = 1.65E-02 OD/min

inhibitor slope = 9.80E-04 OD/min

inhibitor % activity remaining = (9.80E-04/1.65E-02) x 100 = 5.94%



7. To find the activity of the samples expressed as mol substrate/min, employ the following equation:

X mol substrate/min=(**V** x vol.)/(ε x ℓ)

Where **V** is reaction velocity in OD/min, vol. is the reaction volume in liters, ε is the extinction coefficient of the reaction product (2-nitro-5-thiobenzoic acid)(13,600 $M^{-1}cm^{-1}$), and ℓ is the path length of light through the sample in cm (for 100µl in the supplied microplate, ℓ is 0.5 cm).

Note: The above equation determines enzyme activity in terms of moles of thiopeptolide substrate P125 converted per minute. Under these conditions, the secondary substrate DTNB is saturating, and the velocity of DTNB conversion to 2-nitro-5-thiobenzoic acid is not rate-limiting.

See Figure 3 for activity and kinetic calculations.

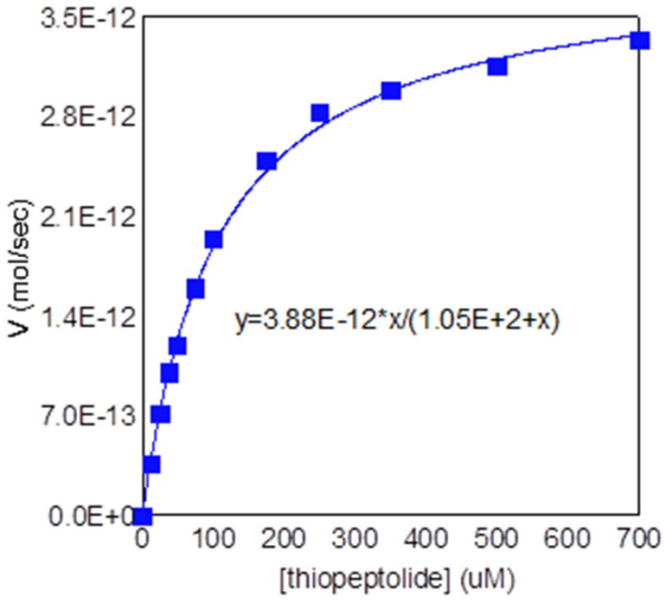
Figure 3. Example calculation for activity:

Activity of a control sample =

(7.88E-03OD/min x 1E-04L)/(13,600$M^{-1}cm^{-1}$ x 0.5cm)=

1.16E-10 mol/min at 37°C, 100µM thiopeptolide P125

Example graph for K_m and V_{max} determination:



K_m=105 µM

V_{max}=3.9 pmol/sec

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