Product Manual

MMP Inhibitor Profiling Kit, Colorimetric

Designed to examine the specificity of inhibitors against a panel of ten matrix metalloproteinase enzymes.

Instruction Manual

BML-AK015

For research use only

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BACKGROUND

The matrix metalloproteinases, or MMPs, are extracellular proteases that function at a neutral pH to cleave a wide variety of substrates. These include basement membrane and extracellular matrix components, growth and death factors, cytokines, and cell and matrix adhesion moLecules[1,2,3]. The broad range of substrate specificities and expression patterns of MMPs results in their involvement in many different processes, both normal and pathological. Aberrant expression has been noted in cancer, angiogenesis, arthritis, inflammation, periodontal disease, emphysema, multiple sclerosis, pre-eclampsia, and chronic wounds, among others[1,2,4]. The general structure of an MMP protein consists of a pre domain to direct secretion from the cell, a pro domain, a catalytic domain, and a C-terminal hemopexin domain. The catalytic site involves a coordinately-bound zinc ion. The inactive, or zymogen, form of the enzyme is activated by disruption of one of the coordinate bonds, usually via proteolytic removal of the pro domain[5].

The *MMP Inhibitor Profiling Kit* is a complete assay system designed to examine the specificity of inhibitors against a panel of ten matrix metalloproteinase enzymes. A thiopeptide is used as a chromogenic substrate (Ac-PLG-[2-mercapto-4-methyl-pentanoyl]-LG-OC_2H_5)[6,7]. 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[8]). The assays are performed in a convenient 96-well microplate format. An inhibitor, NNGH[9], 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.

Please contact Enzo Life Sciences for kit components in bulk.

REFERENCES:

1. G. Bergers and L.M. Coussens *Curr. Opin. Genet. Dev.* 2000 **10** 120
2. S.D. Shapiro *Curr. Opin. Cell Biol.* 1998 **10** 602
3. V. Noe *et al. J. Cell Sci.* 2001 **114** 111
4. A.R. Nelson *et al. J. Clin. Oncol.* 2000 **18** 1135
5. J.F. Woessner and H. Nagase Metalloproteinases and TIMPs. 2000 Oxford University Press
6. H. Weingarten and J. Feder *Anal. Biochem.* 1985 **147** 437
7. H. Weingarten *et al. Biochemistry* 1985 **24** 6730
8. L. Yu and E.A. Dennis *Methods EnzymoL.* 1991 **197** 65
9. L.J. MacPherson *et al. J. Med. Chem.* 1997 **40** 2525

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.

MATERIALS SUPPLIED

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

FORM: Recombinant human MMP-1 catalytic domain (19.9kDa); 30.6U/µL. One U=100 pmol/min@ 37°C, 100µM thiopeptide P125.
STORAGE: -80°C; Avoid freeze/thaw cycles
QUANTITY: 2000U

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

FORM: Recombinant human MMP-2 catalytic domain (40kDa); 3.28U/µL. One U=100 pmol/min@ 37°C, 100µM thiopeptide.
STORAGE: -80°C; Avoid freeze/thaw cycles
QUANTITY: 150U

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

FORM: Recombinant human MMP-3 catalytic domain (19.5kDa); 10U/µL. One U=100 pmol/min@ 37°C, 100µM thiopeptide.
STORAGE: -80°C; Avoid freeze/thaw cycles
QUANTITY: 300U

BML-SE181-9090 MMP-7 ENZYME (HUMAN, RECOMBINANT)

FORM: Recombinant human MMP-7 catalytic domain (20.4kDa); 4.5U/µL. One U=100 pmol/min@ 37°C, 100µM thiopeptide.
STORAGE: -80°C; Avoid freeze/thaw cycles
QUANTITY: 150U

BML-SE255-9090 MMP-8 ENZYME (HUMAN, RECOMBINANT)

FORM: Recombinant human MMP-8 catalytic domain (20.3kDa); 9.2U/µL. One U=100 pmol/min@ 37°C, 100µM thiopeptide.
STORAGE: -80°C; Avoid freeze/thaw cycles
QUANTITY: 200U

BML-SE360-9090 MMP-9 ENZYME (HUMAN, RECOMBINANT)

FORM: Recombinant human MMP-9 catalytic domain (39kDa); 2.68U/µL.One U=100 pmol/min@ 37°C, 100µM thiopeptide.
STORAGE: -80°C; Avoid freeze/thaw cycles
QUANTITY: 130U

BML-SE329-9090 MMP-10 ENZYME (HUMAN, RECOMBINANT)

FORM: Recombinant human MMP-10 catalytic domain (19.4kDa); 5U/µL. One U=100 pmol/min@ 37°C, 100µM thiopeptide.
STORAGE: -80°C; Avoid freeze/thaw cycles
QUANTITY: 150U

BML-SE138-9090 MMP-12 ENZYME (HUMAN, RECOMBINANT)

FORM: Recombinant human MMP-12 catalytic domain (20.3kDa); 10U/µL. One U=100 pmol/min@ 37°C, 100µM thiopeptide.
STORAGE: -80°C; Avoid freeze/thaw cycles
QUANTITY: 140U

BML-SE246-9090 MMP-13 ENZYME (HUMAN, RECOMBINANT)

FORM: Recombinant human MMP-13 catalytic domain (20.4kDa); 3.45U/µL. One U=100 pmol/min@ 37°C, 100µM thiopeptide.
STORAGE: -80°C; Avoid freeze/thaw cycles
QUANTITY: 180U

BML-SE259-9090 MMP-14 ENZYME (HUMAN, RECOMBINANT)

FORM: Recombinant human MMP-14 catalytic domain (22.5kDa); 12U/µL. One U=100 pmol/min@ 37°C, 100µM thiopeptide.
STORAGE: -80°C; Avoid freeze/thaw cycles
QUANTITY: 300U

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

FORM: 25mM (16.4mg/ml) in DMSO
STORAGE: -20°C
QUANTITY: 50µL

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

FORM: 1.3mM in DMSO
STORAGE: -20°C
QUANTITY: 50µL

BML-KI173-0020 ASSAY BUFFER

FORM: 50mM HEPES, 10mM $CaCl_2$, 0.05% Brij-35, 1mM DTNB, pH7.5
STORAGE: -20°C
QUANTITY: 20 ml

80-2404 ½ Volume clear 96-Well Microplate

STORAGE: Ambient

OTHER MATERIALS REQUIRED

1. Microplate reader capable of measuring A_{412} to ≥3-decimal accuracy.
2. Pipetman or multi-channel pipetman capable of pipetting 10-100µL accurately (note: reagents can be diluted to increase the minimal pipetting volume to >10µL).
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 at -80°C for the highest stability. Components with storage temperatures other than -80°C can be stored at the temperature listed OR at -80°C. The MMP enzymes should be handled carefully in order to retain maximal enzymatic activity. They are stable, in diluted or concentrated form, for several hours on ice. As supplied, the MMP enzymes are stable for at least 5 freeze/thaw cycles. If necessary, to minimize the number of freeze/thaw cycles, aliquot the MMPs into separate tubes and store at -80°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 P125-9090 and PI115-9090 to RT to thaw DMSO.
2. Dilute control inhibitor (NNGH, PI115-9090) 1/200 in assay buffer KI173 as follows. Add 1µL inhibitor into 200µL assay buffer, in a separate tube. Warm to reaction temperature (e.g., 37°C).
3. Dilute substrate P125-9090 1/25 in assay buffer to required total volume (10µL are needed per well). For example, for 15 wells dilute 6.4µL P125-9090 into 153.6µL assay buffer, in a separate tube. Warm to reaction temperature (e.g., 37°C).

4. Shortly before assay, dilute MMP enzymes in assay buffer to required total volume (20µL are needed per well), using the following ratios for dilution:

MMP-1: 1/80	MMP-9: 1/60
MMP-2: 1/70	MMP-10: 1/100
MMP-3: 1/70	MMP-12: 1/285
MMP-7: 1/70	MMP-13: 1/50
MMP-8: 1/100	MMP-14: 1/100

Note: MMP-3 is unique in that its pH optimum is 6.0. Activity of this enzyme in pH 7.5 buffer is somewhat reduced; thus the dilution for MMP-3 has been adjusted accordingly.

Warm to reaction temperature (e.g., 37°C).

5. Pipet assay buffer into each desired well of the 1/2 volume microplate as follows:

Blank (no MMP)=90µL Assay Buffer

Control (no inhibitor)=70µL Assay Buffer

Inhibitor NNGH=50µL Assay Buffer

Test inhibitor=varies (see Table 1, below)

6. Allow microplate to equilibrate to assay temperature (e.g., 37°C).

7. Add 20µL MMP (diluted in step 4) to the control, inhibitor NNGH, and test inhibitor wells.

8. Add 20µL NNGH inhibitor (diluted in step 2) to the inhibitor NNGH wells only! Final inhibitor concentration=1.3µM. See Figure 2 for example of inhibition of MMPs by NNGH.

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 10µL P125-9090 substrate (diluted and equilibrated to reaction temperature in step 3). Final substrate concentration=100µM.

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	Inhibitor (6.5µM)	Substrate (1mM)	Total Volume
Blank	90µL	0	0	10µL	100µL
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.

Example of plate:	well#	sample
	A1	Blank
	B1	Blank
	C1	Control/MMP-1
	D1	Control/MMP-1
	E1	Inhibitor NNGH/MMP-1
	F1	Inhibitor NNGH/MMP-1
	G1	Test inhibitor/MMP-1
	H1	Test inhibitor/MMP-1
	A2	Control/MMP-2
	B2	Control/MMP-2
	C2	Test inhibitor/MMP-2
	D2	Test inhibitor/MMP-2

DATA ANALYSIS

Plotting

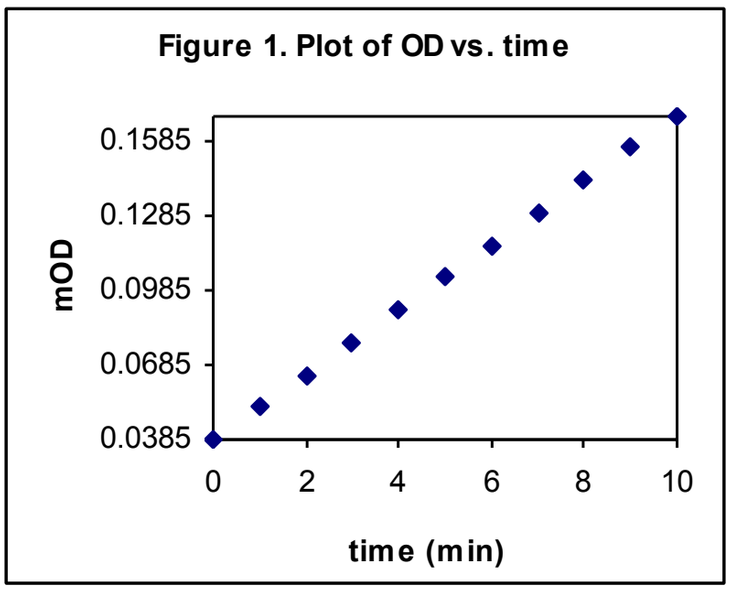
1. Plot data as OD versus time for each sample (see Fig. 1).
2. Determine the range of time points during which the reaction is linear. Typically, points from 1 to 10 min are sufficient.
3. Obtain the reaction velocity (**V**) in OD/min: determine the slope of a line fit to the linear portion of the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

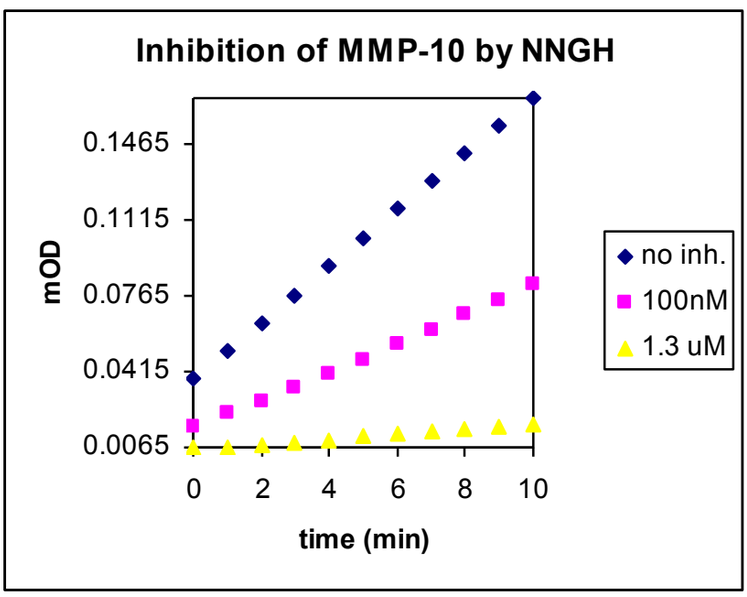
6. To determine inhibitor % remaining activity:
   Inhibitor % activity remaining = (**V** inhibitor / **V** control) x 100

See **Figure 2** for example.



Slope=**V**=1.30E-02 OD/min

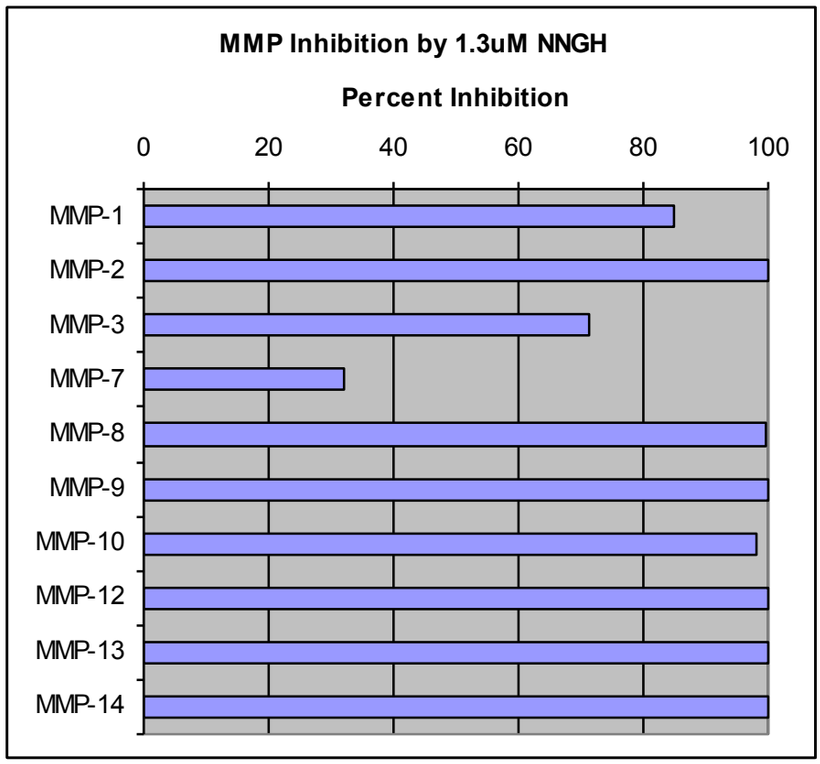
Figure 2. Example of inhibitor data:



control slope = 1.30E-02 OD/min

inhibitor (100nM) slope = 6.64E-03 OD/min

inhibitor % activity remaining = (6.63E-03/1.30E-02) x 100 = 51.0%



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 100ml 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** (following page) for activity and kinetic calculations.

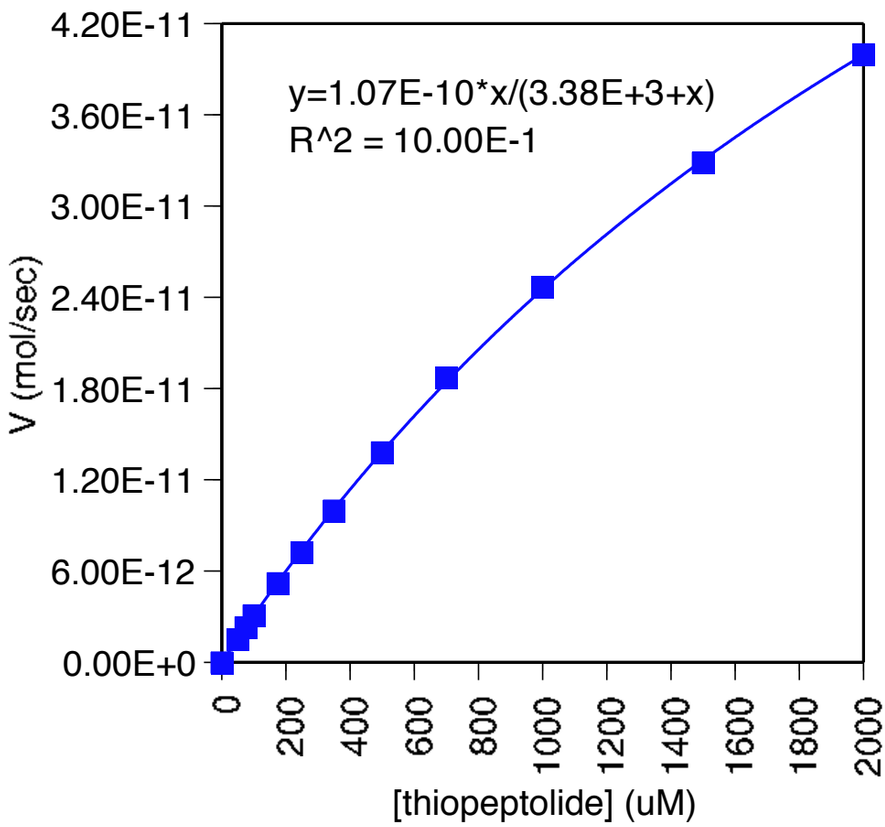
Figure 3. Example calculation for activity:

Activity of a control sample =

(1.25E-02OD/min x 1E-04L)/(13,600$M^{-1}cm^{-1}$ x 0.5cm)=

1.84E-10 moL/min at 37°C, 100µM thiopeptolide P125

Example graph for K_m and V_{max} determination for MMP-10 with substrate P-125:



Km=3380μM

Vmax=107 pmol/sec

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