MMP Inhibitor Profiling Kit, Fluorometric RED

Designed to examine the specificity of inhibitors against a panel of ten matrix metalloproteinase enzymes, using a quenched fluorogenic peptide.

Instruction Manual

BML-AK308

For research use only

MMP Inhibitor Profiling Kit, Fluorometric RED – BML-AK308

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[1-3]. 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[1-3]. 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[4].

The *MMP Inhibitor Profiling Kit, Fluorometric RED* is a complete assay system designed to examine the specificity of inhibitors against a panel of ten matrix metalloproteinase enzymes, using a quenched fluorogenic substrate OmniMMP™ RED: TQ3-GABA-Pro-Cha-Abu-Smc-His-Ala-Dab(6-TAMRA)-Ala-Lys-NH_2 [TQ3=quencher; GABA=4-aminobutyric acid; Cha=L-cyclohexylalanine; Abu=2-aminobutyric acid; Smc=S-methyl-L-cysteine; Dab=2,4-diaminobutyric acid; 6-TAMRA=6-tetramethylrhodamine]. TAMRA fluorescence is thoroughly quenched by the TQ3 group until cleavage by MMPs separates the two moieties.

The OmniMMP™ RED substrate offers key advantages over other MMP substrates. 1) Emission at the red end of the spectrum (576nm after excitation at 545nm) avoids the interference at lower wavelengths often exhibited by screening compounds, and by substances commonly found in biological samples and tissue culture medium. 2) MMP substrate peptides display poor aqueous solubility, often with K_ms near their limits of solubility, making enzyme and inhibitor kinetics difficult. MMP K_ms for OmniMMP™ RED substrate are below its solubility limit. 3) In addition to the efficient binding as exhibited by low K_ms, OmniMMP™ RED is avidly cleaved by MMPs, with k_{cat}/K_ms in the range of 10^4-10^6 $M^{-1}sec^{-1}$. 4) The ultra-strong fluorescence of OmniMMP™ RED allows for substrate concentrations much lower than the K_m, a condition generally desirable in inhibitor screening assays.

The assays are performed in a convenient 96-well microplate format. The kit is useful to screen inhibitors of MMP-19, a potential therapeutic target. The compound NNGH[5] 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. J.F. Woessner and H. Nagase Metalloproteinases and TIMPs. 2000 Oxford University Press
5. *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. **DO NOT PERFORM THIS ASSAY IN WHITE PLATES DUE TO PLATE AUTOFLUORESCENCE AT THE WAVELENGTHS USED.**

COMPONENTS OF BML-AK308 KIT

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

FORM: Recombinant human MMP-1 catalytic domain (19.9kDa). One U=100 pmoL/min@ 37°C, 100μM thiopeptide (BML-P125). Purity >95% by SDS-PAGE.

STORAGE: -80°C; Avoid freeze/thaw cycles

QUANTITY: 2000 U

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

FORM: Recombinant human MMP-2 catalytic domain (40kDa). One U=100 pmoL/min@ 37°C, 100μM thiopeptide (BML-P125). Purity >95% by SDS-PAGE.

STORAGE: -80°C; Avoid freeze/thaw cycles

QUANTITY: 150 U

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

FORM: Recombinant human MMP-3 catalytic domain (19.5kDa). One U=100 pmoL/min@ 37°C, 100μM thiopeptide (BML-P125). Purity >95% by SDS-PAGE.

STORAGE: -80°C; Avoid freeze/thaw cycles

QUANTITY: 300 U

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

FORM: Recombinant human MMP-7 catalytic domain (20.4kDa). One U=100 pmoL/min@ 37°C, 100μM thiopeptide (BML-P125). Purity >95% by SDS-PAGE.

STORAGE: -80°C; Avoid freeze/thaw cycles

QUANTITY: 150 U

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

FORM: Recombinant human MMP-8 catalytic domain (20.3kDa). One U=100 pmoL/min@ 37°C, 100μM thiopeptide (BML-P125). Purity >95% by SDS-PAGE.

STORAGE: -80°C; Avoid freeze/thaw cycles

QUANTITY: 200 U

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

FORM: Recombinant human MMP-9 catalytic domain (39kDa). One U=100 pmoL/min@ 37°C, 100µM thiopeptide (BML-P125). Purity >95% by SDS-PAGE.

STORAGE: -80°C; Avoid freeze/thaw cycles

QUANTITY: 130 U

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

FORM: Recombinant human MMP-12 catalytic domain (20.3kDa). One U=100 pmoL/min@ 37°C, 100µM thiopeptide (BML-P125). Purity >95% by SDS-PAGE.

STORAGE: -80°C; Avoid freeze/thaw cycles

QUANTITY: 140 U

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

FORM: Recombinant human MMP-13 catalytic domain (20.4kDa). One U=100 pmoL/min@ 37°C, 100µM thiopeptide (BML-P125). Purity >95% by SDS-PAGE.

STORAGE: -80°C; Avoid freeze/thaw cycles

QUANTITY: 180 U

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

FORM: Recombinant human MMP-14 catalytic domain (22.5kDa). One U=100 pmoL/min@ 37°C, 100µM thiopeptide (BML-P125). Purity >95% by SDS-PAGE.

STORAGE: -80°C; Avoid freeze/thaw cycles

QUANTITY: 300 U

BML-SE561-9090 MMP-19 ENZYME (HUMAN, RECOMBINANT)

FORM: Recombinant human MMP-19 catalytic domain (19.2kDa). One U=100 pmoL/min@ 37°C, 100µM thiopeptide (BML-P125). Purity >95% by SDS-PAGE.

STORAGE: -80°C; Avoid freeze/thaw cycles

QUANTITY: 14 U

BML-P277-9090 SUBSTRATE (OmniMMP™ RED fluorogenic substrate peptide; MW=1910.7)

FORM: 250µM in DMSO

STORAGE: -80°C

QUANTITY: 40µL

BML-KI582-0010 6-TAMRA CALIBRATION STANDARD

FORM: 10µM in DMSO; MW=430.4

STORAGE: -80°C

QUANTITY: 20µL

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

FORM: 1.3mM in DMSO

STORAGE: -20°C

QUANTITY: 50µL

BML-KI175-0020 ASSAY BUFFER

50mM HEPES, 10mM $CaCl_2$, 0.05% Brij-35, pH 7.5

STORAGE: Ambient (or -20°C)

QUANTITY: 20mL

80-2409 96-WELL BLACK NBS MICROPLATE

STORAGE: Ambient

OTHER MATERIALS REQUIRED

- Fluorescent microplate reader capable of excitation at 545nm and emission at 576nm. The following Ex/Em has also been used: 540/590.
- Pipetmen or multi-channel pipetmen capable of pipetting 1-100µL accurately.
- 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 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 BML-P277-9090, BML-KI582-0010, and BML-PI115-9090 to RT to thaw DMSO.
2. Dilute control inhibitor (NNGH, BML-PI115-9090) 1/200 in assay buffer BML-KI175 as follows: add 2µl inhibitor to 398µL assay buffer, in a separate tube (for a 6.5µM stock). Higher concentrations can be used (see Figure 1).
3. Thaw the DMSO stock vial of substrate BML-P277-9090 and dilute sufficient volume to 7.5µM in assay buffer (10µL needed per well). This is a recommended 10x substrate concentration only. Higher and lower concentrations of this substrate have been used successfully with these MMPs. See Table 2 for the isoform specific substrate K_m data.
4. Shortly before assay, dilute MMP enzymes in assay buffer to required total volume (20µL of the 5x are needed per well), at the following concentrations:

MMP-1: 640 mU/µL	MMP-9: 17 mU/µL
MMP-2: 55 mU/µL	MMP-12: 42 mU/µL
MMP-3: 63mU/µL	MMP-13: 17 mU/µL
MMP-7: 64 mU/µL	MMP-14: 300 mU/µL
MMP-8: 115 mU/µL	MMP-19: 6.4 mU/µL

Notes:

a) MMP-3 is unique in that its pH optimum is 6.0, Activity of this enzyme in the pH 7.5 buffer is somewhat reduced; the dilution has been adjusted accordingly to achieve a similar reaction rate as other provided MMP isoforms.

b) The above enzyme & substrate concentrations are provided as a general guideline for our customers. They should be used to find optimal screening conditions.

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)

Example of plate:

well#	sample
A1	Calibration
B1	Calibration
C1	Calibration
D1	Control/MMP-1
E1	Control/MMP-1
F1	Inhibitor NNGH/MMP-1
G1	Inhibitor NNGHMMP-1
H1	Test inhibitor/MMP-1
A2	Test inhibitor/MMP-1
B2	Control/MMP-2
C2	Control/MMP-2
D2	Test inhibitor/MMP-2
E2	Test inhibitor/MMP-2…

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 (below) for example of inhibition of MMPs by NNGH.
9. Add desired volume of test inhibitor to appropriate wells. See Table 1.
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=545/576nm, with cutoff set at 570nm: Pre-warm 80µl assay buffer to reaction temperature in 3 wells in the microplate, then to each add 10µl BML-P277-9090 substrate peptide to give the concentration to be used in the assay (e.g., for 0.75µM final add 10µl 7.5µM) 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 (diluted in assay buffer) calibration standard BML-KI582 to give 3 different final molar concentrations ranging between 2 and 10% of the substrate peptide molar concentration (e.g., 15, 45, and 75nM) and measure their fluorescence. Use these values to build a standard curve

relating micromolar BML-KI582 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 should 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-P277-9090 substrate (diluted and equilibrated to reaction temperature in step 3). Final substrate concentration = 0.75µM.
13. Continuously read plates in the fluorescent microplate reader, using Ex/Em=545/576nm, with cutoff set at 570nm. For example, record data at 1 minute time intervals for 10 minutes at set reaction temperature (e.g., 37°C). An endpoint read could also be done at a fixed timepoint.
14. 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 (7.5µM)	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, each in duplicate. Average the slopes of duplicate samples.

Data Reduction & Sample Data

To determine inhibitor % remaining activity:

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

See figure for example of results.

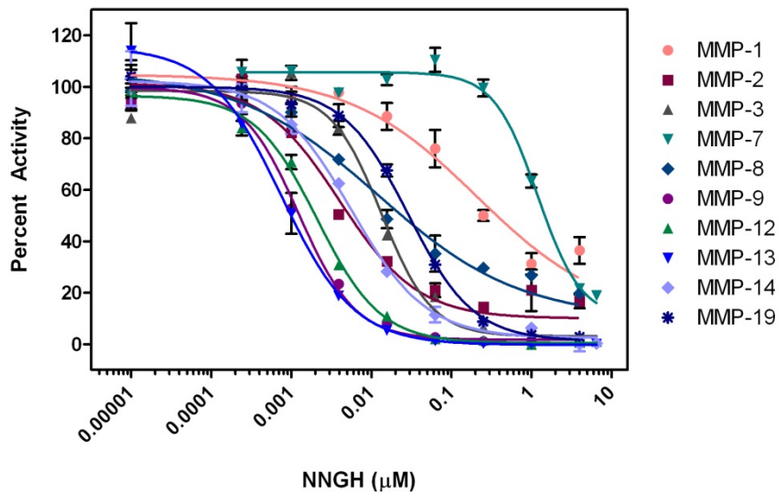

Figure 1. Inhibitor dose response curve: NNGH was pre-incubated with MMP enzyme before reactions were started by the addition of substrate. The dose-response curve was derived from a fit to a four parameter Hill-Slope model, Y=Bottom + (Top-Bottom) / (1+10^((LogIC_{50}-X)*HillSlope)) using Graphpad Prism software where the bottom was constrained to 0. Data are presented as mean of duplicate wells ± SEM. Data were collected on a Biotek Synergy2 plate reader.

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).

TABLE 2. Kinetic parameters.

Isoform	K_m (μM)
MMP-1	7
MMP-2	3
MMP-3	9
MMP-7	5
MMP-8	5
MMP-9	2
MMP-12	4
MMP-13	3
MMP-14	6
MMP-19	14

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