MMP-8 Fluorometric Drug Discovery Kit

Designed to screen MMP-8 inhibitors using a quenched fluorogenic peptide.

Instruction Manual

BML-AK415

For research use only

✦*MMP-8 Fluorometric Drug Discovery Kit – BML-AK415*✦

BACKGROUND

Matrix metalloproteinase-8 (MMP-8, neutrophil collagenase, collagenase-2) 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-8 include collagen, gelatin, aggrecan, entactin, and α1-proteinase inhibitor[1,2]. MMP-8 is secreted as a 55-80 kDa glycosylated proenzyme (as measured by SDS-PAGE), and activated by cleavage to 46 kDa and below[3-5]. MMP-8 is an important target for inhibitor screening due to its involvement in diseases such as cancer, arthritis, and asthma[3,6-8].

The *MMP-8 Fluorometric Drug Discovery Kit* is a complete assay system designed to screen MMP-8 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][9]. Mca fluorescence is quenched by the Dpa group until cleavage by MMPs at the Gly-Leu bond separates the two moieties[9,10]. The assays are performed in a convenient 96-well microplate format. The kit is useful to screen inhibitors of MMP-8, a potential therapeutic target. The compound NNGH[11] is also included as a prototypic control inhibitor.

REFERENCES:

1. L.J. McCawley and L.M. Matrisian *Curr. Opin. Cell Biol.* 2001 **13** 534
2. E.C. Arner *et al. J. Biol. Chem.* 1997 **272** 9294
3. R. Hanemaaijer *et al. J. Biol. Chem.* 1997 **272** 31504
4. S.K. Mallya *et al. Biochem.* 1990 **29** 10628
5. A.A. Cole *et al. J. Biol. Chem.* 1996 **271** 11023
6. W.G. Stetler-Stevenson and A.E. Yu *Semin. Cancer Biol.* 2001 **11** 143
7. F. Grams *et al. Biol. Chem.* 2001 **382** 1277
8. K. Prikk *et al. Lab. Invest.* 2002 **82** 1535
9. C.G. Knight *et al. FEBS Lett.* 1992 **296** 263
10. M.M. Smith *et al. J. Biol. Chem.* 1995 **270** 6440
11. 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.

COMPONENTS OF BML-AK415 KIT

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

FORM: *E. coli* recombinant human MMP-8 catalytic domain (calculated MW 20.3 kDa), 9.2 U/µl.

UNIT DEFINITION: One unit is defined as the amount of enzyme that will hydrolyze 100µM thiopeptide 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: 200 U

PRESENTATION: 22µl 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-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: 20 ml

80-2406 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 1-100µl 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 and assay buffer (room temperature) at -70°C for the highest stability. The MMP-8 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-8 enzyme is stable for at least 5 freeze/thaw cycles. To minimize the number of freeze/thaw cycles, aliquot the MMP-8 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 KI-175 as follows. Add 1µl inhibitor into 200µl 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-8 enzyme 1/100 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 = 80 µl 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
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-8 (diluted in step 4) to the control, inhibitor NNGH, and test inhibitor wells. Final amount of MMP-8 will be 1.84 U per well (18.4 mU/µl). Remember to **not** add MMP-8 to the calibration wells!
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.
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 4µM 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 400nm) 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 10µl BML-P126-9090 substrate (diluted and equilibrated to reaction temperature in step 3). Final substrate concentration = 4µM.
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-8 (92mU/µl)	Inhibitor (6.5µM)	Substrate (40µM)	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

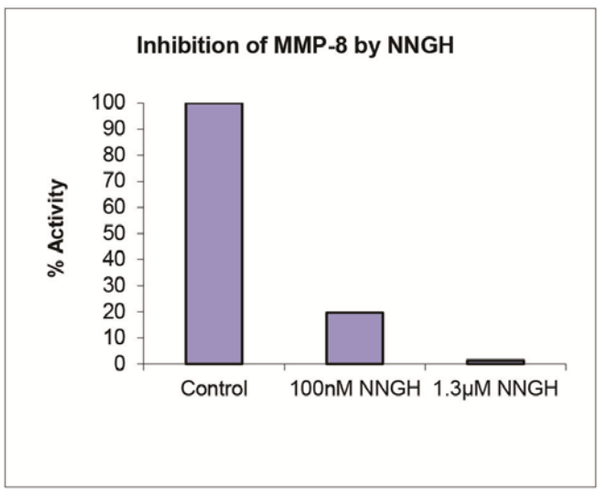
15. Plot data as RFUs (minus Blank RFU value determined during calibration, step 11) versus time for each sample.
16. Determine the range of initial time points during which the reaction is linear.
17. Obtain the initial reaction velocity (**V**) in RFUs/min: determine the slope of a line fit to the 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

To determine inhibitor % remaining activity:

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

See figure 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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