ab139453

MMP8 Inhibitor Screening Assay Kit (Fluorometric)

Instructions for Use

For the screening of MMP8 inhibitors

This product is for research use only and is not intended for diagnostic use.

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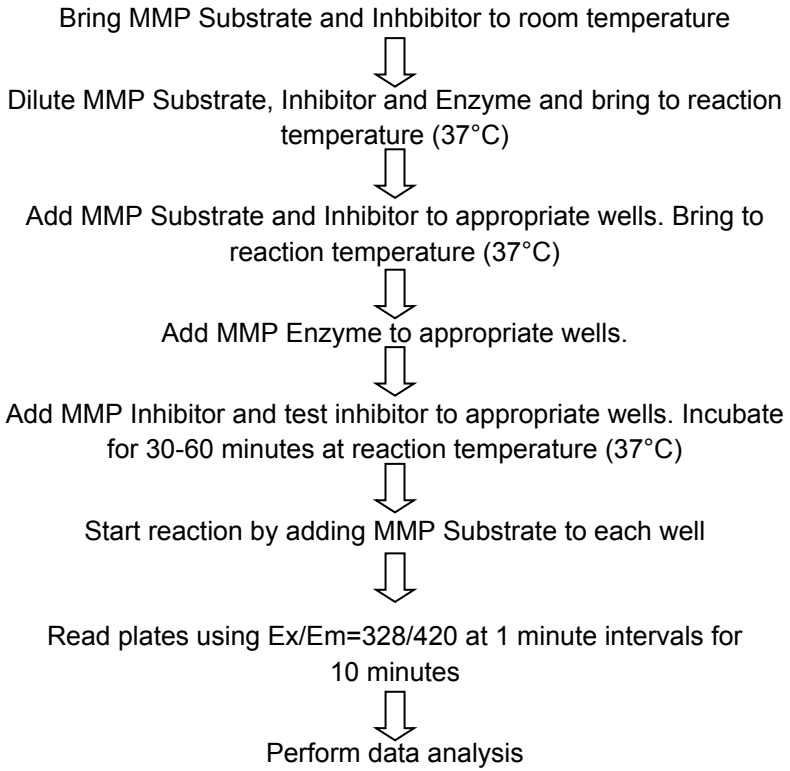
1. Background

Matrix metalloproteinase-8 (MMP8, 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. Targets of MMP8 include collagen, gelatin, aggrecan, entactin, and α1-proteinase inhibitor. MMP8 is secreted as a 55-80 kDa glycosylated proenzyme (as measured by SDS-PAGE), and activated by cleavage to 46 kDa and below. MMP8 is an important target for inhibitor screening due to its involvement in diseases such as cancer, arthritis, and asthma.

2. Principle of the Assay

Abcam MMP8 Inhibitor Screening Assay Kit (Fluorometric) (ab139453) is a complete assay system designed to screen MMP8 inhibitors using a quenched fluorogenic peptide: MMP 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]. Mca fluorescence is quenched by the Dpa group until cleavage by MMPs at the Gly-Leu bond separates the two moieties. The assays are performed in a convenient 96-well microplate format. The kit is useful to screen inhibitors of MMP8, a potential therapeutic target. The compound NNGH is also included as a prototypic control inhibitor.

3. Protocol Summary



4. Materials Supplied

Item	Quantity	Storage
96-well White Microplate (½ Volume)	1	RT
MMP8 Enzyme (Human, Recombinant) (9.2 U/ µL)	1 x 22 µL	-80ºC
MMP Fluorogenic Substrate (400 µM (437 µg/ml) in DMSO)	1 x 200 µL	-80ºC
MMP Inhibitor (1.3mM NNGH in DMSO)	1 x 50 µL	-80ºC
MMP Calibration Standard (40 µM (17.8 µg/ml) in DMSO)	1 x 50 µL	-80ºC
Fluorometric Assay Buffer	1 x 20 mL	RT

5. Storage and Stability

- Store components as stated in table for the highest stability.
- The MMP8 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, MMP8 enzyme is stable for 5 freeze/thaw cycles. To minimize the number of freeze/thaw cycles, aliquot the MMP8 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.
- One U MMP8 Enzyme is defined as the amount of enzyme that will hydrolyze 100mM thiopeptide Ac-PLG-[2-mercapto-4-methyl-pentanoyl]-LG-OC_2H_5 at 100 pmol/min@ 37°C.

6. Materials Required, Not Supplied

- Fluorescent microplate reader capable of excitation at 328 nm and emission at 420nm. The following Ex/Em have also been used: 320,340/393,400,405.
- Pipettes or multi-channel pipettes capable of pipetting 1-100 µL accurately.
- Ice bucket to keep reagents cold until use.
- Water bath or incubator for component temperature equilibration.

7. Assay Protocol

1. Briefly warm kit components MMP Fluorogenic Substrate, MMP Calibration Standard and MMP Inhibitor to RT to thaw DMSO.
2. Dilute MMP inhibitor 1/200 in Fluorometric Assay Buffer as follows. Add 1 µL inhibitor into 200 µL Fluorometric Assay Buffer, in a separate tube. Warm to reaction temperature (e.g. 37°C).
3. Thaw the DMSO stock vial of MMP Fluorogenic Substrate and dilute sufficient volume to 40 µM in Fluorometric Assay Buffer (10 µL needed per well). Warm to reaction temperature (e.g. 37°C).
4. Dilute MMP8 enzyme 1/100 in Fluorometric Assay Buffer to required total volume (20 µL are needed per well). Warm to reaction temperature (e.g. 37°C) shortly before assay.
5. Pipette Fluorometric 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) = 70 µL
   MMP Inhibitor = 50 µL
   Test inhibitor = varies (see Table 1, below)
6. Allow microplate to equilibrate to assay temperature (e.g. 37°C).

7. Add 20 µL MMP8 (diluted in step 4) to the control, MMP Inhibitor, and test inhibitor wells. Final amount of MMP8 will be 1.84 U per well (18.4 mU/ µL). Remember to not add MMP8 to the calibration wells!
8. Add 20 µL MMP Inhibitor (diluted in step 2) to the MMP Inhibitor wells only. Final inhibitor concentration = 1.3 µM
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=328/420: Prewarm Fluorometric Assay Buffer to reaction temperature in 3 wells in the microplate, then to each add 10 µL MMP8 substrate peptide to give the concentration to be used in the assay (e.g., for 4 µM final add 10 µL 40 µM) 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 10 µL calibration standard peptide to give 3 different final molar concentrations ranging between 2 and 10% of the substrate peptide molar concentration (e.g., 80, 200, and 400 nM) and measure their fluorescence. Use these values to build a standard curve relating micromolar Calibration Standard 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 10 µL MMP Fluorogenic Substrate (diluted and equilibrated to reaction temperature in step 3). Final substrate concentration = 4 µM.
13. Continuously read plates 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 next section).

NOTE: Retain microplate for future use of unused wells.

Table 1. Example of Samples

Sample	Assay Buffer	MMP8 (92 mU/ µL)	Inhibitor (6.5 µM)	Substrate (40 µM)	Total Volume
Control	70 µL	20 µL	0 µL	10 µL	100 µL
MMP Inhibitor	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	Calibration
B1	Calibration
C1	Calibration
D1	Control
E1	Control
F1	MMP Inhibitor
G1	MMP Inhibitor
H1	Test inhibitor
A2…	Test inhibitor...

8. Data Analysis

1. Plot data as RFUs (minus Blank RFU value determined during calibration, Section 7, step 11) versus time for each sample.

2. Determine the range of initial time points during which the reaction is linear.

3. 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.

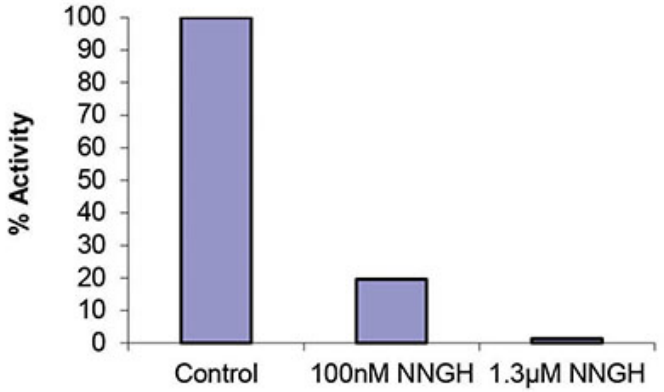
4. It is best to use a range of inhibitor concentrations, each in duplicate. Average the slopes of duplicate samples.

A. To determine inhibitor % remaining activity:

Inhibitor % activity remaining = $(V_{inhibitor}/V_{control})$ x 100

See Figure 1 for example.

Figure 1. Inhibition of MMP8 by NNGH. Example of inhibitor data.



B. To determine the activity of the samples expressed as picomoles substrate hydrolyzed per minute

Employ the following equation:

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 8.3),
vol. is the reaction volume in microliters (100).

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