

ab139449

MMP9 Inhibitor

**Screening Assay Kit
(Fluorometric)**

Instructions for Use

For the screening of MMP9 inhibitors

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

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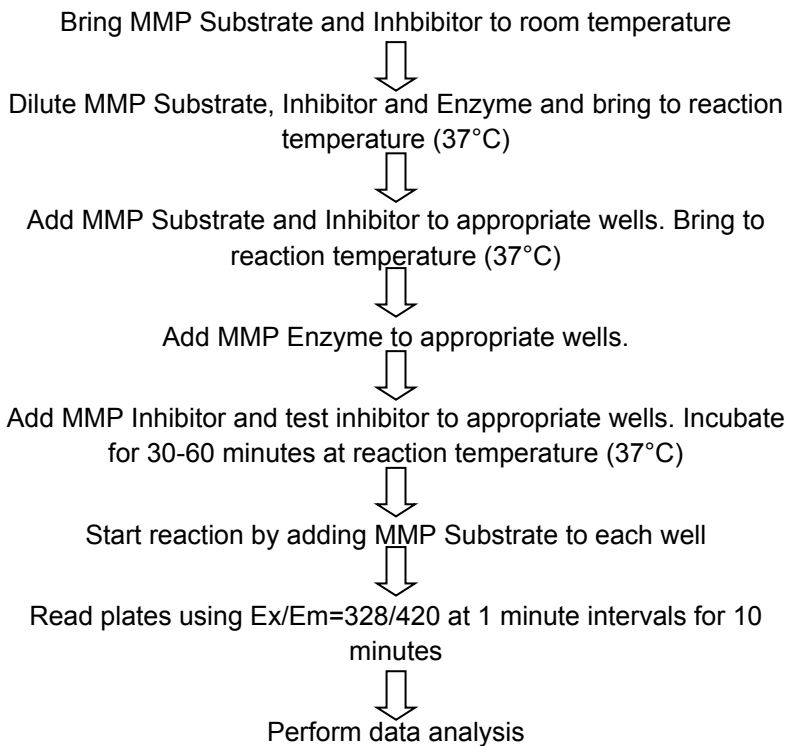
1. Background

Matrix metalloproteinase-9 (MMP9, gelatinase B, 92 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. Targets of MMP9 include native and denatured collagens, fibronectin, elastin, laminin, pro-TNF- α , and interleukins and their receptors. MMP9 is secreted as a 92 kDa proenzyme (as measured by SDS-PAGE), and activated by cleavage to forms 82 kDa and smaller. MMP9 is an important target for inhibitor screening due to its involvement in diseases such as alopecia, cancer, angiogenesis, and metastasis.

2. Principle of the Assay

Abcam MMP9 Inhibitor Screening Assay Kit (Fluorometric) (ab139449) is a complete assay system designed to screen MMP9 inhibitors using a quenched fluorogenic peptide: MMP Fluorogenic Substrate Mca-Pro-Leu-Gly-Leu-Dpa-Ala-Arg-NH₂ [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 MMP9, 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 Clear Microplate (1/2 Volume)	1	RT
MMP9 Enzyme (Human, Recombinant) (2.68 U/ μ L)	1 x 48.5 μ L	-80°C
MMP Calibration Standard (40 μ M (17.8 μ g/mL) in DMSO)	1 x 50 μ L	-80°C
MMP Inhibitor (1.3 mM NNGH in DMSO)	1 x 50 μ L	-80°C
MMP Fluorogenic Substrate (400 μ M (437 μ g/mL) in DMSO)	1 x 200 μ L	-80°C
Fluorometric Assay Buffer	1 x 20 mL	RT

5. Storage and Stability

- Store components as stated in table for the highest stability.
- The MMP9 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, MMP9 enzyme is stable for at least 5 freeze/thaw cycles. To minimize the number of freeze/thaw cycles, aliquot the MMP9 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.
- For the MMP9 Enzyme, 1 unit is defined as the amount of enzyme that will hydrolyze 100 μ M thiopeptide Ac-PLG-[2-mercapto-4-methyl-pentanoyl]-LG-OC₂H₅ at 100 pmol/min at 37°C.

6. Materials Required, Not Supplied

- Fluorescent microplate reader capable of excitation at 328 nm and emission at 420 nm. 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.
(Note: reagents can be diluted to increase the minimal pipetting volume to $>10 \mu\text{L}$)
- 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 Substrate, MMP Calibration Standard and MMP Inhibitor) to RT to thaw DMSO.
2. Dilute MMP inhibitor (NNGH) 1/200 in Assay Buffer as follows. Add 1 μL inhibitor into 200 μL Assay Buffer, in a separate tube. Warm to reaction temperature (e.g. 37°C).
3. Thaw the DMSO stock vial of MMP substrate and dilute sufficient volume to 40 μM in assay buffer (10 μL needed per well). Warm to reaction temperature (e.g. 37°C).
4. Dilute MMP9 enzyme 1/60 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. Pipette 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)
6. Allow microplate to equilibrate to assay temperature (e.g. 37°C).

7. Add 20 μL MMP9 (diluted in step 4) to the control, MMP Inhibitor, and test inhibitor wells. Final amount of MMP9 will be 0.9 U per well (9 mU/ μL). Remember to not add MMP9 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 . Note: 1.3 μM NNGH will inhibit MMP9 by 96% under these conditions (see Figure 1).
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 assay buffer to reaction temperature in 3 wells in the microplate, then to each add 10 μL MMP Substrate 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 MMP 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 MMP 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 μ L MMP substrate (diluted and equilibrated to reaction temperature in step 3).
Final substrate concentration = 4 μ M.
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	MMP9 (45 U/μL)	Inhibitor (6.5 μM)	Substrate (40 μM)	Total Volume
Control	70 μ L	20 μ L	0	10 μ L	100 μ L
MMP Inhibitor	50 μ L	20 μ L	20 μ L	10 μ L	100 μ L
Test inhibitor*	X μ L	20 μ L	Y μ L	10 μ L	100 μ L

* 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 μ L.

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

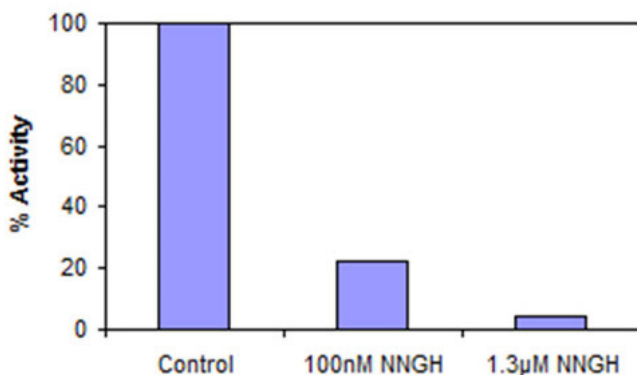
1. Plot data as RFUs (minus Blank RFU value determined during calibration, step 11) versus time for each sample.
2. Determine the range of initial time points during which the reaction is linear.
3. Obtain the initial reaction velocity (V) in RFUs/min: determine the slope of a line fit to the initial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

$$\text{Inhibitor \% activity remaining} = (V_{\text{inhibitor}}/V_{\text{control}}) \times 100$$

See Figure 1 for example.

Figure 1. Inhibition of MMP9 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:

$$\mathbf{X} \text{ pmoles substrate/min} = 1/\mathbf{CF} \times \mathbf{V} \times \mathbf{vol}$$

Where

CF is the conversion factor (micromolar concentration/RFUs

V is initial reaction velocity (RFUs/min)

vol is the reaction volume in microliters (100)

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