

ab139448

MMP9 Inhibitor

**Screening Assay Kit
(Colorimetric)**

Instructions for Use

For the screening of MMP9 inhibitors

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

Table of Contents

1.	Background	3
2.	Principle of the Assay	4
3.	Protocol Summary	5
4.	Materials Supplied	6
5.	Storage and Stability	7
6.	Materials Required, Not Supplied	8
7.	Assay Protocol	9
8.	Data Analysis	12

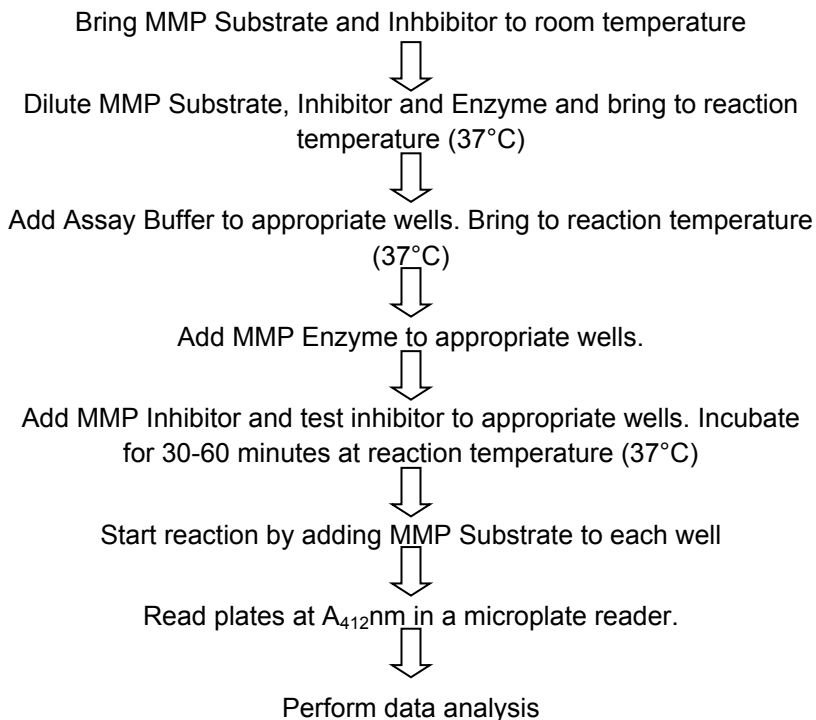
1. Background

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 (Colorimetric) (ab139448) is a complete assay system designed to screen MMP9 inhibitors using a thiopeptide as a chromogenic substrate (Ac-PLG-[2-mercapto-4-methyl-pentanoyl]-LG-OC₂H₅). 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 ($\epsilon=13,600 \text{ M}^{-1}\text{cm}^{-1}$ at pH 6.0 and above). The assays are performed in a convenient 96-well microplate format. The kit is useful to screen inhibitors of MMP9, a potential therapeutic target. An inhibitor, 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 Inhibitor (1.3 mM NNGH in DMSO)	1 x 50 μ L	-80°C
MMP Substrate (25 mM (16.4 mg/mL) in DMSO)	1 x 50 μ L	-80°C
Colorimetric 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.
- One U MMP9 Enzyme = 100 pmol/min@ 37°C, 100 μ M thiopeptide.
- Thiol inhibitors should not be used with this kit, as they may interfere with the colorimetric assay.

6. Materials Required, Not Supplied

- Microplate reader capable of measuring A_{412} to ≥ 3 -decimal accuracy.
- 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 and MMP Inhibitor to RT to thaw DMSO.
2. Dilute MMP inhibitor 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. Dilute MMP9 substrate 1/25 in assay buffer to required total volume (10 μL are needed per well). For example, for 15 wells dilute 6.4 μL MMP substrate into 153.6 μL Assay Buffer, in a separate tube. 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:
Blank (no MMP9)=90 μL Assay Buffer
Control (no inhibitor)=70 μL Assay Buffer
MMP Inhibitor =50 μL Assay Buffer
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 blanks!
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 approximately 96% under these conditions (see Figure 2).
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. Start reaction by the addition of 10 μL MMP9 substrate (diluted and equilibrated to reaction temperature in step 3). Final substrate concentration=100 μM .
12. Continuously read plates at $A_{412\text{nm}}$ in a microplate reader. Record data at 1 min. time intervals for 10 to 20 min.
13. Perform data analysis (see next section).

NOTE: Retain microplate for future use of unused wells.

Table 1. Example of Samples

Sample	Assay Buffer	MMP9 (45 mU/μL)	Inhibitor (6.5 μM)	Substrate (1 mM)	Total Volume
Blank	90 μ L	0 μ L	0 μ L	10 μ L	100 μ L
Control	70 μ L	20 μ L	0 μ L	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	Blank
B1	Blank
C1	Control
D1	Control
E1	MMP Inhibitor
F1	MMP Inhibitor
G1	Test inhibitor
H1...	Test inhibitor...

8. Data Analysis

1. Plot data as OD versus time for each sample (see Fig. 1).

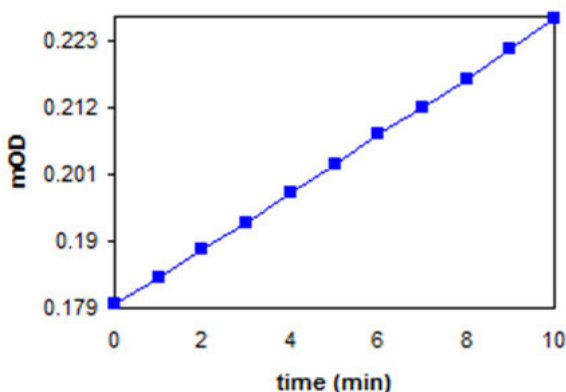


Figure 1. Plot of OD vs time. Slope= $V=4.69\text{E-}03$ OD/min

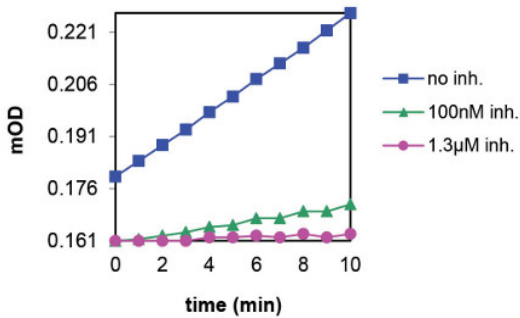
2. Determine the range of time points during which the reaction is linear. Typically, points from 1 to 10 min are sufficient.
3. Obtain the reaction velocity (V) in OD/min: determine the slope of a line fit to the linear portion of the data plot using an appropriate routine.
4. Average the slopes of duplicate samples.
5. If the blank has a significant slope, subtract this number from all samples.

A. To determine inhibitor % remaining activity:

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

See Figure 2 for example.

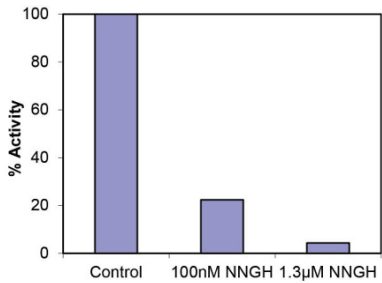
Figure 2. Inhibition of MMP9 by NNGH. Example of inhibitor data.



Control slope = 4.69E-03 OD/min

Inhibitor slope = 2.04E-04 OD/min

Inhibitor % activity remaining = $(2.04\text{E-}04/4.69\text{E-}03) \times 100 = 4.34\%$



B. To find the activity of the samples expressed as mol substrate/min:

Employ the following equation:

$$\mathbf{X} \text{ mol substrate/min} = (V \times \text{vol.})/(\epsilon \times l)$$

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⁻¹cm⁻¹)

l is the path length of light through the sample in cm (for 100μL in the supplied microplate, l is 0.5 cm).

Note: The above equation determines enzyme activity in terms of moles of thiopeptide substrate 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 for activity and kinetic calculations.

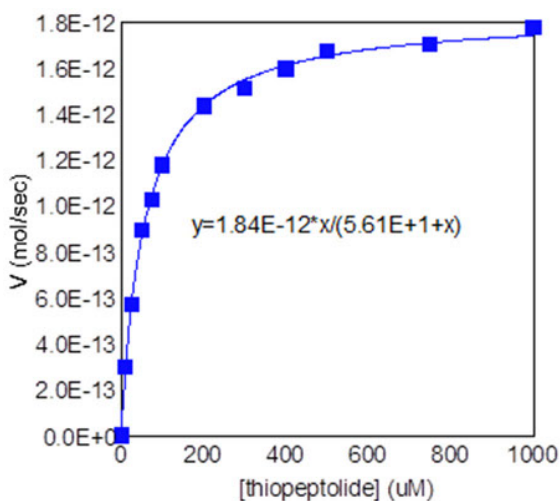


Figure 3. Example graph for Km and Vmax determination

$K_m = 56.1 \mu\text{M}$

$V_{\text{max}} = 1.8 \text{ pmol/sec}$

Example calculation for activity:

Activity of a control sample =

$$(4.81\text{E-}03\text{OD/min} \times 1\text{E-}04\text{L}) / (13,600\text{M}^{-1}\text{cm}^{-1} \times 0.5\text{cm}) =$$

$$7.07\text{E-}11 \text{ mol/min at } 37^\circ\text{C, } 100\mu\text{M thiopeptide}$$

UK, EU and ROW

Email: technical@abcam.com | Tel: +44-(0)1223-696000

Austria

Email: wissenschaftlicherdienst@abcam.com | Tel: 019-288-259

France

Email: supportscientifique@abcam.com | Tel: 01-46-94-62-96

Germany

Email: wissenschaftlicherdienst@abcam.com | Tel: 030-896-779-154

Spain

Email: soportecientifico@abcam.com | Tel: 911-146-554

Switzerland

Email: technical@abcam.com

Tel (Deutsch): 0435-016-424 | Tel (Français): 0615-000-530

US and Latin America

Email: us.technical@abcam.com | Tel: 888-77-ABCAM (22226)

Canada

Email: ca.technical@abcam.com | Tel: 877-749-8807

China and Asia Pacific

Email: hk.technical@abcam.com | Tel: 400 921 0189 / +86 21 2070 0500

Japan

Email: technical@abcam.co.jp | Tel: +81-(0)3-6231-0940

www.abcam.com | www.abcam.cn | www.abcam.co.jp