

ab139450

MMP13 Inhibitor

Screening Assay Kit

(Colorimetric)

Instructions for Use

For the screening of MMP13 Inhibitors

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

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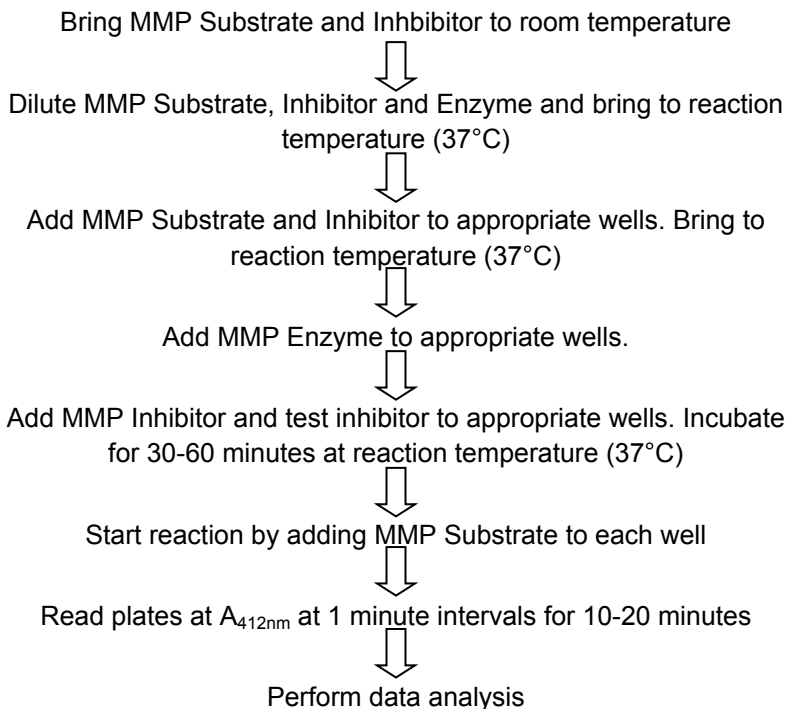
1. Background

MMP13 (Matrix metalloproteinase-13, collagenase-3) 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 MMP13 include collagen, gelatin, aggrecan, plasminogen, and CXCL12. MMP13 is secreted as a 60 kDa proenzyme (as measured by SDS-PAGE), and activated by cleavage to 48 kDa and below. MMP13 is an important target for inhibitor screening due to its involvement in diseases such as cancer and arthritis.

2. Principle of the Assay

Abcam MMP13 Inhibitor Screening Assay Kit (Colorimetric) (ab139450) is a complete assay system designed to screen MMP13 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 MMP13, 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
MMP13 Enzyme (Human, Recombinant) 3.45 U/ μ L	1 x 53 μ L	-80°C
MMP Substrate (25 mM (16.4 mg/ml) n DMSO).	1 x 50 μ L	-20°C
MMP Inhibitor (1.3 mM NNGH n DMSO).	1 x 50 μ L	-20°C
Colorimetric Assay Buffer	1 x 20 mL	-20°C

5. Storage and Stability

- Store components as stated in table for the highest stability.
- The MMP13 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, MMP13 enzyme is stable for at least 5 freeze/thaw cycles. To minimize the number of freeze/thaw cycles, aliquot the MMP13 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 MMP3 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 reading $A_{412\text{nm}}$ to ≥ 3 -decimal accuracy
- Pipettes or multi-channel pipettes capable of pipetting 10-100 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 (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. Dilute MMP 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 MMP13 enzyme 1/50 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 MMP13)=90 μL Colorimetric Assay Buffer
 - Control (no inhibitor)=70 μL Colorimetric Assay Buffer
 - MMP Inhibitor =50 μL Colorimetric Assay Buffer
 - Test inhibitor=varies (see Table 1)
6. Allow microplate to equilibrate to assay temperature (e.g. 37°C).

7. Add 20 μL MMP13 (diluted in step 4) to the Control, MMP Inhibitor, and Test inhibitor wells. Final amount of MMP13 will be 1.38 U per well (13.8 mU/ μL). Remember to not add MMP13 to blanks!
8. Add 20 μL MMP Inhibitor (diluted in step 2) to the inhibitor wells only! Final inhibitor concentration=1.3 μM . See Figure 2 for inhibition of MMP13 by MMP Inhibitor.
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 MMP 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	MMP13 (69 mU//μL)	MMP Inhibitor (6.5 μM)	MMP 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 Colorimetric 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).

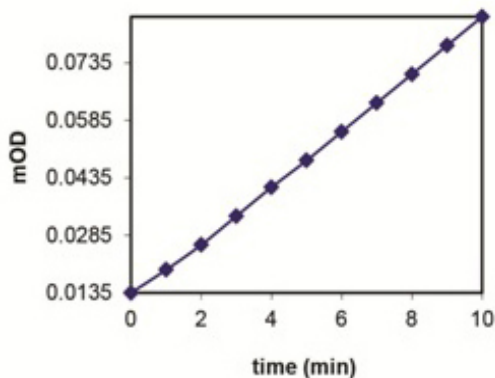


Figure 1. Plot of OD vs. time. Slope= $V=7.00\text{E-}03$ OD/min

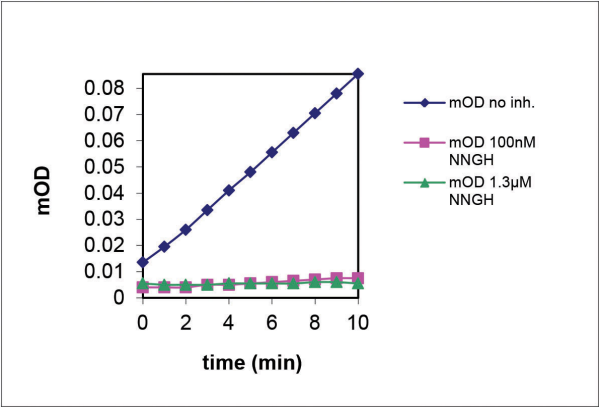
2. Determine the range of time points during which the reaction is linear. Typically, points from 1 to 10 min are sufficient.
3. Obtain the reaction velocity (V) in OD/min: determine the slope of a line fit to the linear portion of the data plot using an appropriate routine.
4. Average the slopes of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

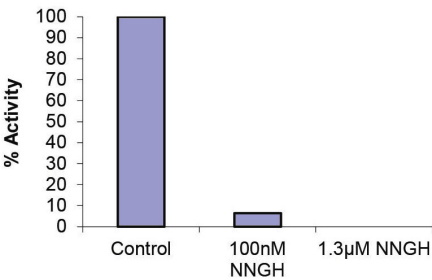
Figure 2. Inhibition of MMP13 by NNGH. Example of inhibitor data.



control slope = 7.00E-03 OD/min

inhibitor (100nM) slope = 4.50E-04 OD/min

inhibitor % activity remaining = $(4.50\text{E-}04 / 7.00\text{E-}03) \times 100 = 6.4\%$



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

Employ the following equation:

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

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 100 μL 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.

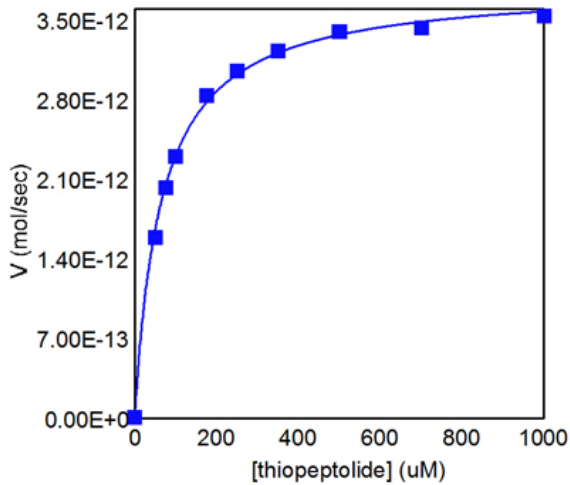


Figure 3. Example graph for K_m and V_{max} determination:

$K_m=65.3 \mu\text{M}$

$V_{max}=3.81 \text{ pmol/sec}$

Example calculation for activity:

Activity of a control sample =

$$(9.40\text{E-}03\text{OD/min} \times 1\text{E-}04\text{L}) / (13,600\text{M}^{-1}\text{cm}^{-1} \times 0.5\text{cm}) =$$

1.38E-10 mol/min at 37°C, 100 μM thiopeptide

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