ab139441

MMP12 Inhibitor Screening Assay Kit (Colorimetric)

Instructions for Use

For the screening of MMP12 inhibitors

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

Version 1 Last Updated 13 May 2013

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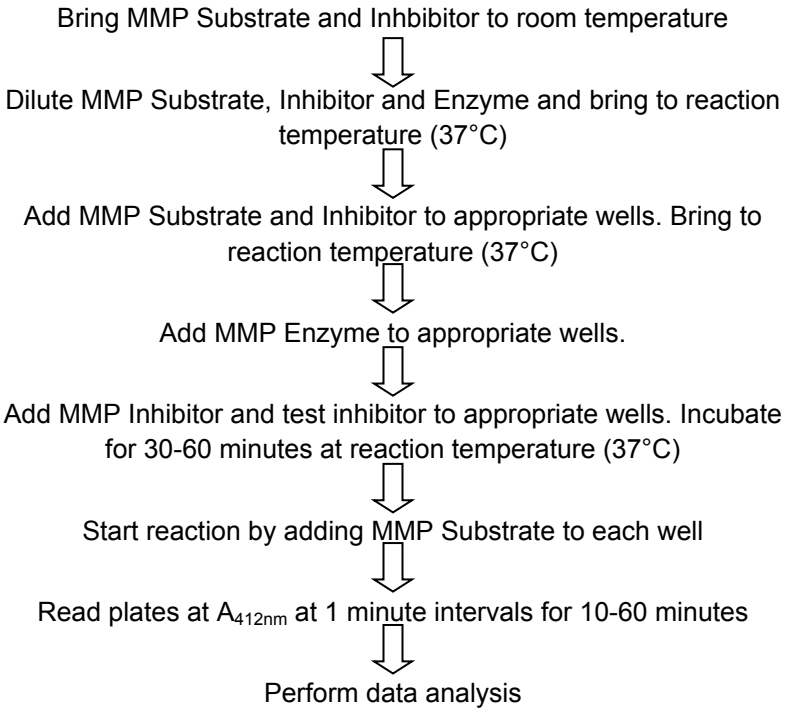
1. Background

Matrix metalloproteinase-12 (MMP12, metalloelastase, macrophage elastase, commonly confused with neutrophil elastase) 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 MMP12 include elastin, fibronectin, laminin, plasminogen, u-PAR, and tissue factor pathway inhibitor. MMP12 is secreted as a 53 kDa proenzyme (as measured by SDS-PAGE), and activated by cleavage to forms of 22-45 kDa. MMP12 is an important target for inhibitor screening due to its involvement in diseases such as cancer and emphysema.

2. Principle of the Assay

Abcam's MMP12 Inhibitor Screening Assay Kit (Colorimetric) (ab139441) is a complete assay system designed to screen MMP12 inhibitors using a thiopeptide as a chromogenic substrate (Ac-PLG-[2-mercapto-4-methyl-pentanoyl]-LG-OC_2H_5). 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 (e=13,600 $M^{-1}cm^{-1}$ 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 MMP12, 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 unit	4°C
MMP12 Enzyme (Human, Recombinant) (10 U/µL)	1 x 14µL	-80°C
MMP Inhibitor (1.3mM NNGH in DMSO)	1 x 50 µL	-80°C
MMP Substrate (25 mM (16.4 mg/ml) in DMSO)	1 x 50 µL	-80°C
Colorimetric Assay Buffer	1 x 20 mL	-80°C

5. Storage and Stability

- Store all components except the microplate at -80°C for the highest stability.

- The MMP12 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, MMP12 enzyme is stable for at least 4 freeze/thaw cycles. To minimize the number of freeze/thaw cycles, aliquot the MMP12 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 MMP12 Enzyme = 100 pmol/min@ 37°C, 100 µM 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_{412nm} 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 µL).

- 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 MMP12 enzyme 1/285 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. Pipet assay buffer into each desired well of the 1/2 volume microplate as follows:

   Blank (no MMP12)=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 MMP12 (diluted in step 4) to the control, MMP Inhibitor, and test inhibitor wells. Final amount of MMP12 will be 0.7 U per well (7.0 mU/ µL). Remember to not add MMP12 to blanks!
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.
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_{412nm} in a microplate reader. Record data at 1 min. time intervals for 10 to 60 min.
13. Perform data analysis (see below).

NOTE: Retain microplate for future use of unused wells.

Table 1. Example of Samples

Sample	Assay Buffer	MMP12 (35 mU/µL)	Inhibitor (6.5 µM)	Substrate (1 mM)	Total Volume
Blank	90 µL	0 µL	0 µL	10 µL	100 µL
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	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).

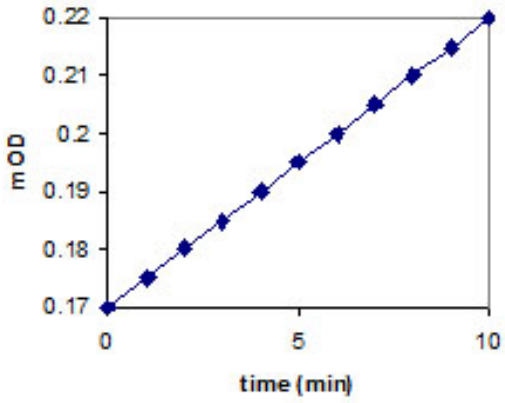


Figure 1. Plot of OD vs. time. Slope=V=4.85E-03 OD/min

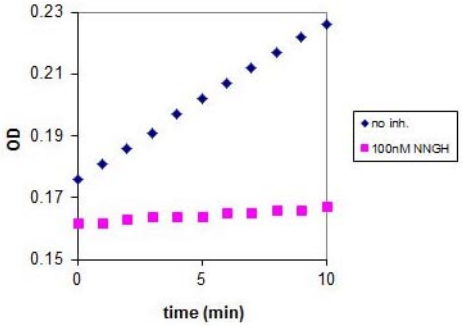
2. Determine the range of time points during which the reaction is linear. Typically, points from 1 to 10 min are sufficient.
3. Obtain the reaction velocity (V) in OD/min: determine the slope of a line fit to the linear portion of the data plot using an appropriate routine.
4. Average the slopes of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

A. To determine inhibitor % remaining activity:

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

See Figure 2 for example.

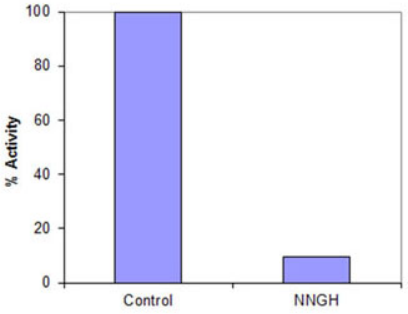
Figure 2. Inhibition of MMP12 by NNGH (100 nM). Example of inhibitor data.



control slope = 5.08E-03 OD/min

inhibitor slope (100nM) = 4.82E-04 OD/min

inhibitor % activity remaining = (4.82E-04/5.08E-03) x 100 = 9.49%



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

Employ the following equation:

X mol substrate/min=(V x vol.)/(ε x *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 $M^{-1}cm^{-1}$)
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.

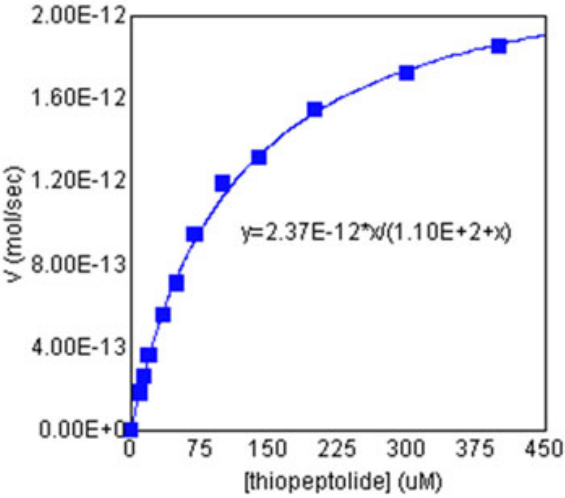


Figure 3. Example graph for Km and Vmax determination:

Km=110 μM

Vmax=2.37 pmol/sec

Example calculation for activity:

Activity of a control sample =

(4.85-03OD/min x 1E-04L)/(13,600$M^{-1}cm^{-1}$ x 0.5cm) =

7.13E-11 mol/min at 37°C, 100 μM thiopeptide

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