Product Manual

# MMP-1 Colorimetric Drug Discovery Kit

Catalog #: BML-AK404

Designed to screen MMP-1 inhibitors using a thiopeptide as a chromogenic substrate.

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Product Manual

Please read entire booklet before proceeding with the assay.

Carefully note the handling and storage conditions of each kit component.

Please contact Enzo Life Sciences Technical Support if necessary.

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## BACKGROUND

Matrix metalloproteinase-1 (MMP-1, interstitial collagenase, fibroblast 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 specificities1. Targets of MMP-1 include collagen, gelatin, entactin, pro-TNF-$\alpha$, and the chemokine SDF-11-4. MMP-1 is secreted as a 52-56kDa glycosylated proenzyme (as measured by SDS-PAGE), and activated by cleavage to forms of 22-46kDa5. MMP-1 is an important target for inhibitor screening due to its involvement in diseases such as cancer3.

The *MMP-1 Colorimetric Drug Discovery Kit* is a complete assay system designed to screen MMP-1 inhibitors using a thiopeptide as a chromogenic substrate (Ac-PLG-[2-mercapto-4-methyl-pentanoyl]-LG-OC2H5)6,7. 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 ($\varepsilon$=13,600 M-1cm-1 at pH 6.0 and above8). The assays are performed in a convenient 96-well microplate format. The kit is useful to screen inhibitors of MMP-1, a potential therapeutic target. An inhibitor, NNGH9, is also included as a prototypic control inhibitor. Thiol inhibitors should not be used with this kit, as they may interfere with the colorimetric assay.

**REFERENCES:**


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Avoid freeze / thaw cycles

Reagents require separate storage conditions.

## MATERIALS SUPPLIED

1. **MMP-1 ENZYME (HUMAN, RECOMBINANT)**
   **Catalog No. BML-SE180-9090:**

   **FORM**: E. coli recombinant human MMP-1 catalytic domain (calculated MW 19.9kDa), 30.6 U/µl. One U=100 pmol/min@ 37°C, 100µM thiopeptolide P125.

   **STORAGE**: -70°C; Avoid freeze/thaw cycles

   **QUANTITY**: 2000 U

2. **MMP SUBSTRATE (chromogenic; MW=655.9)**
   **Catalog No. BML-P125-9090:**

   **FORM**: 25mM (16.4mg/ml) in DMSO

   **STORAGE**: -20°C

   **QUANTITY**: 50µl

3. **INHIBITOR (NNGH; MW=316.4)**
   **Catalog No. BML-PI115-9090:**

   **FORM**: 1.3mM in DMSO

   **STORAGE**: -20°C

   **QUANTITY**: 50µl

4. **ASSAY BUFFER, Catalog No. BML-KI173-0020:**

   50mM HEPES, 10mM $CaCl_2$, 0.05% Brij-35, 1mM DTNB, pH7.5

   **STORAGE**: -20°C

   **QUANTITY**: 20 ml

5. **96-WELL MICROPLATE, Catalog No. 80-2404:**

   **STORAGE**: Room temperature.

## OTHER MATERIALS NEEDED

1. Microplate reader capable of measuring A412 to ≥3-decimal accuracy.
2. Pipetman or multi-channel pipetman capable of pipetting 10-100µl accurately (note: reagents can be diluted to increase the minimal pipetting volume to >10µl).
3. Ice bucket to keep reagents cold until use.
4. Water bath or incubator for component temperature equilibration.

## EXPERIMENTAL METHODS

**Note on storage**: *Store all components except the microplate (room temperature) at -70°C for the highest stability. The MMP-1 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, MMP-1 enzyme is stable for at least 4 freeze/thaw cycles. To minimize the number of freeze/thaw cycles, aliquot the MMP-1 into separate tubes and store at -70°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*.

**To start assay:**

1. Briefly warm kit components BML-P125-9090 and BML-PI115-9090 to RT to thaw DMSO.
2. Dilute inhibitor (NNGH, BML-PI115-9090) 1/200 in assay buffer BML-KI173 as follows. Add 1µl inhibitor into 200µl assay buffer, in a separate tube. Warm to reaction temperature (e.g., 37°C).
3. Dilute substrate BML-P125-9090 1/25 in assay buffer to required total volume (10µl are needed per well). For example, for 15 wells dilute 6.4µl BML-P125-9090 into 153.6µl assay buffer, in a separate tube. Warm to reaction temperature (e.g., 37°C).
4. Dilute MMP-1 enzyme 1/40 in assay buffer to required total volume (20µl 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 MMP-1)=90µl Assay Buffer

   **Control** (no inhibitor)=70µl Assay Buffer

   **Inhibitor** NNGH=50µl Assay Buffer

   **Test inhibitor**=varies (see Table 1, below)
6. Allow microplate to equilibrate to assay temperature (e.g., 37°C).
7. Add 20µl MMP-1 (diluted in step 4) to the control, inhibitor NNGH, and test inhibitor wells. Final amount of MMP-1 will be 15.3 U per well (153 mU/µl). Remember to not add MMP-1 to the blanks!
8. Add 20µl NNGH inhibitor (diluted in step 2) to the inhibitor NNGH wells only! Final inhibitor concentration=1.3µM. **Note**: 1µM NNGH will not completely inhibit MMP-1 under these conditions (see **Figure 2**).
9. Add desired volume of test inhibitor to appropriate wells. See **Table 1**, following.
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 10µl BML-P125-9090 substrate (diluted and equilibrated to reaction temperature in step 3). Final substrate concentration=100µM.
12. Continuously read plates at A412nm in a microplate reader. Record data at 1 min. time intervals for 10 to 20 min.
13. Perform data analysis (see below).

**NOTE**: Retain microplate for future use of unused wells.

**Table 1. Example of Samples.**

| Sample | Assay buffer | MMP-1 (765mU/µl) | Inhibitor (6.5µM) | Substrate (1mM) | Total Volume |
|---|---|---|---|---|---|
| **Blank** | 90µl | 0 | 0 | 10µl | 100µl |
| **Control** | 70µl | 20µl | 0 | 10µl | 100µl |
| **Inhibitor NNGH** | 50µl | 20µl | 20µl | 10µl | 100µl |
| **Test inhibitor*** | Xµl | 20µl | Yµl | 10µl | 100µl |

*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 100µl.

**Example of plate:**

| well# | Sample |
|---|---|
| A1 | Blank |
| B1 | Blank |
| C1 | Control |
| D1 | Control |
| E1 | Inhibitor NNGH |
| F1 | Inhibitor NNGH |
| G1 | Test inhibitor |
| H1 | Test inhibitor |

## DATA ANALYSIS

### Plotting

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

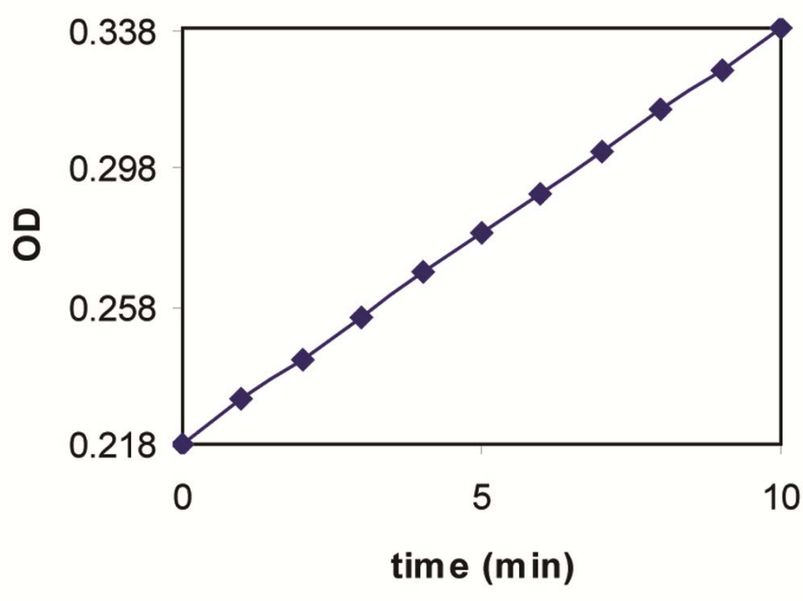
### Data Reduction

5. If the blank has a significant slope, subtract this number from all samples.
6. To determine inhibitor % remaining activity:

   Inhibitor % activity remaining = (**V** inhibitor / **V** control) x 100

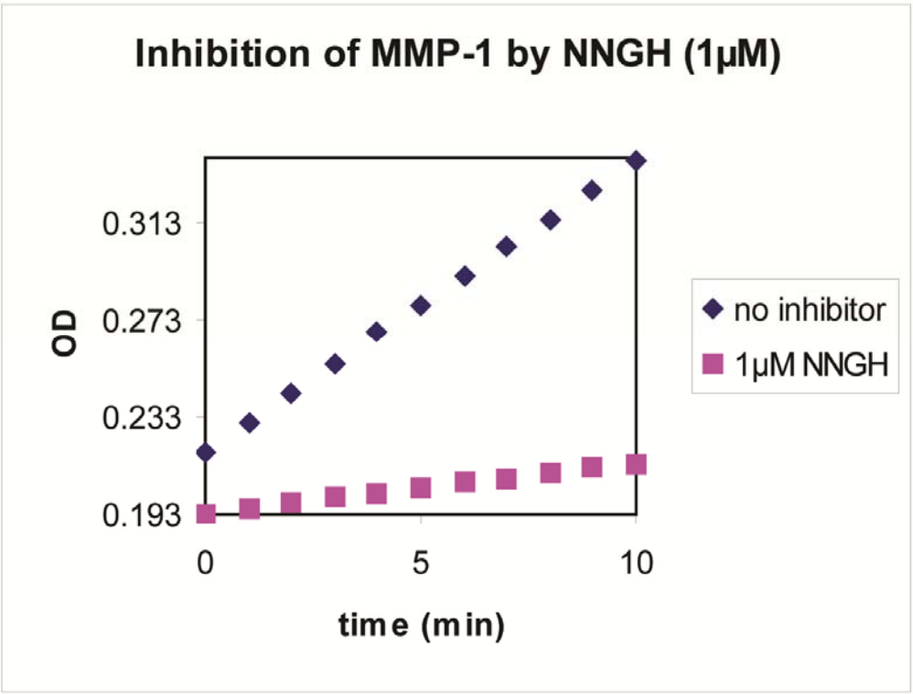
   See **Figure 2** for example.

**Figure 1. Plot of OD vs. time**




Slope=V=1.20E-02 OD/min

**Figure 2**

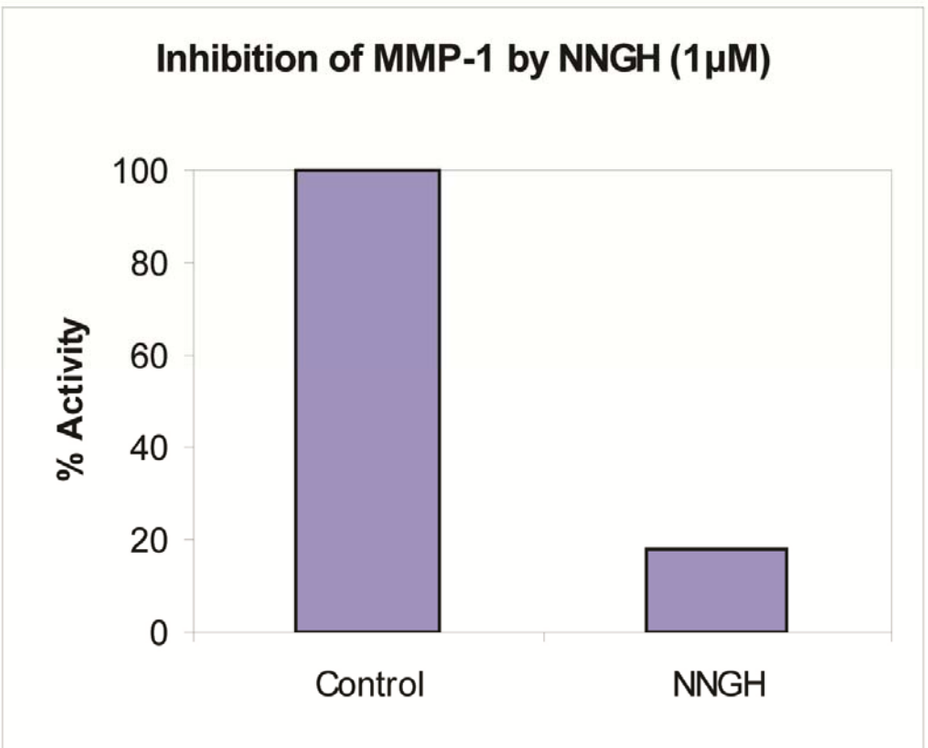



control slope = 1.20E-02 OD/min

inhibitor slope = 2.13E-03 OD/min

inhibitor % activity remaining = (2.13E-03/1.20E-02) x 100 = 17.7%

**Figure 3**



7. 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 $\ell$)

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), and $\ell$ is the path length of light through the sample in cm (for 100µl in the supplied microplate, $\ell$ is 0.5 cm).

**Note**: The above equation determines enzyme activity in terms of moles of thiopeptolide substrate P125 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 calculation for activity:**

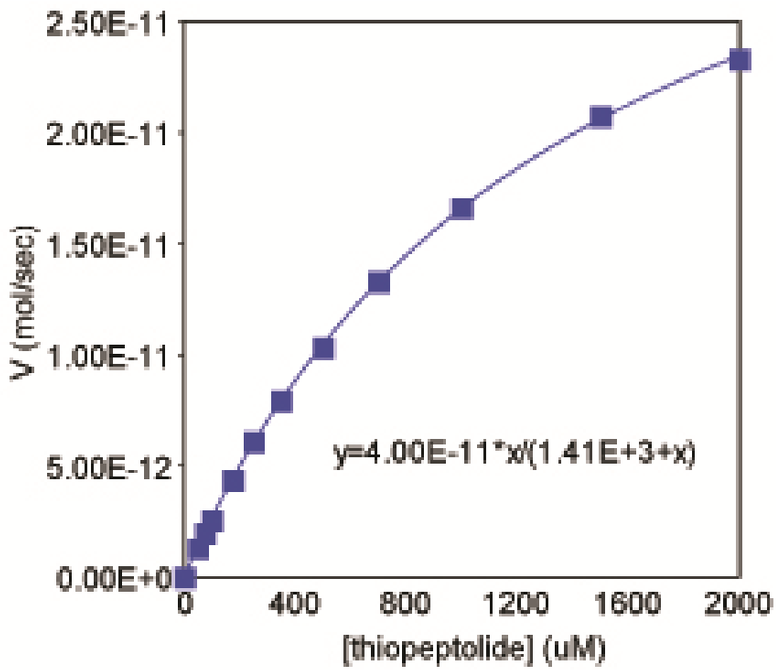
Activity of a control sample =

(1.04E-02OD/min x 1E-04L)/(13,600$M^{-1}cm^{-1}$ x 0.5cm)=

1.53E-10 mol/min at 37°C, 100µM thiopeptolide P125

**Example graph for $K_m$ and $V_{max}$ determination:**

**Figure 4**




Km=1410µM

Vmax=40.0 pmol/sec

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