Myeloperoxidase Inhibitor Screening Assay Kit

Item No. 700170

www.caymanchem.com
Customer Service 800.364.9897
Technical Support 888.526.5351
1180 E. Ellsworth Rd · Ann Arbor, MI · USA

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GENERAL INFORMATION

Materials Supplied

This kit will arrive packaged as a 4°C kit. After opening the kit, store individual components as stated below.

Item Number	Item	Quantity	Storage
700164	MPO Assay Buffer	1 vial	4°C
700165	MPO Chlorination Substrate	1 vial	4°C
700002	ADHP Assay Reagent	2 vials	-20°C
700166	Myeloperoxidase Assay Reagent	1 vial	-20°C
700167	MPO Inhibitor	1 vial	4°C
700168	MPO Hydrogen Peroxide	1 vial	4°C
700001	DMSO Assay Reagent	1 ml	RT
400017	96-Well Solid Plate (black)	2 plates	RT
400012	96-Well Cover Sheets	2 covers	RT

If any of the items listed above are damaged or missing, please contact our Customer Service department at (800) 364-9897 or (734) 971-3335. We cannot accept any returns without prior authorization.

WARNING: THIS PRODUCT IS FOR RESEARCH ONLY - NOT FOR HUMAN OR VETERINARY DIAGNOSTIC OR THERAPEUTIC USE.

Safety Data

This material should be considered hazardous until further information becomes available. Do not ingest, inhale, get in eyes, on skin, or on clothing. Wash thoroughly after handling. Before use, the user must review the complete Safety Data Sheet, which has been sent *via* email to your institution.

Precautions

Please read these instructions carefully before beginning this assay.

If You Have Problems

Technical Service Contact Information

Phone: 888-526-5351 (USA and Canada only) or 734-975-3888

Email: techserv@caymanchem.com

In order for our staff to assist you quickly and efficiently, please be ready to supply the lot number of the kit (found on the outside of the box).

Storage and Stability

This kit will perform as specified if stored as directed in the **Materials Supplied** section on page 3 and used before the expiration date indicated on the outside of the box.

Materials Needed But Not Supplied

1. A plate reader with the ability to measure fluorescence using excitation wavelengths of 480-495 and 530-540 nm and emission wavelengths of 515-525 and 585-595 nm
2. Adjustable pipettes and a multichannel pipette
3. A source of pure water; glass distilled water or HPLC-grade water is acceptable

INTRODUCTION

Background

Myeloperoxidase (MPO) is a heme-containing enzyme and the most abundant protein in polymorphonuclear leukocytes (PMNs).[1] It is composed of two subunits linked by a disulfide bridge with each subunit containing a light and heavy polypeptide chain. MPO is stored in azurophilic granules of PMNs and is released from activated or necrotic PMNs, after which it can bind to and modify acidic serum proteins, as well as recruit additional PMNs.[1] It can oxidize a variety of substrates and catalyzes the formation of highly reactive (pseudo)hypohalous acids and radicals, including hypochlorous acid (HOCl), using hydrogen peroxide (H_2O_2) for chlorination or peroxidation.[1] The use of H_2O_2 by MPO for either its chlorination or peroxidation activities depends on the relative concentrations of chloride and the reducing substrate.[2] MPO also has roles in PMN apoptosis and antimicrobial defense systems, including neutrophil extracellular trap (NET) formation and NETosis.[1,3,4] It enhances neutrophil elastase-induced chromatin decondensation and produces reactive oxygen species (ROS), which trigger NET formation.[5] MPO-derived oxidants and chlorinated products are enriched in LDL and human atherosclerotic lesions.[6-8] In addition, MPO levels in leukocytes and the blood are elevated in patients with coronary artery disease (CAD), and elevated serum levels of MPO in patients with acute coronary syndromes are considered a risk factor for subsequent cardiovascular events.[7-9] MPO inhibitors improve endothelial function in mouse models of vascular inflammation and atherosclerosis and reduce the severity of cigarette smoke-induced lung damage in a guinea pig model of chronic obstructive pulmonary disease (COPD).[10,11] Development of novel MPO inhibitors has the potential to improve the treatment of conditions caused by oxidative stress.

About This Assay

Cayman's MPO Inhibitor Screening Assay provides convenient fluorescence-based methods for screening inhibitors to both the chlorination and peroxidation activities of MPO. The chlorination assay utilizes the non-fluorescent 2-[6-(4-aminophenoxy)-3-oxo-3H-xanthen-9-yl]-benzoic acid (APF), which is selectively cleaved by hypochlorite (-OCl) to yield the highly fluorescent compound fluorescein.[7] Fluorescein fluorescence is analyzed with an excitation wavelength of 480-495 nm and an emission wavelength of 515-525 nm. The peroxidation assay utilizes the peroxidase component of MPO, where a single two electron oxidation of native enzyme (MPO) to compound I (MPO-I) is followed by two successive one electron reductions back to native enzyme by compound II (MPO-II).[8] The reaction between hydrogen peroxide and ADHP (10-acetyl-3,7-dihydroxyphenoxazine) produces the highly fluorescent compound resorufin. Resorufin fluorescence is analyzed with an excitation wavelength of 530-540 nm and an emission wavelength of 585-595 nm. The assay schemes are outlined in Figure 1.

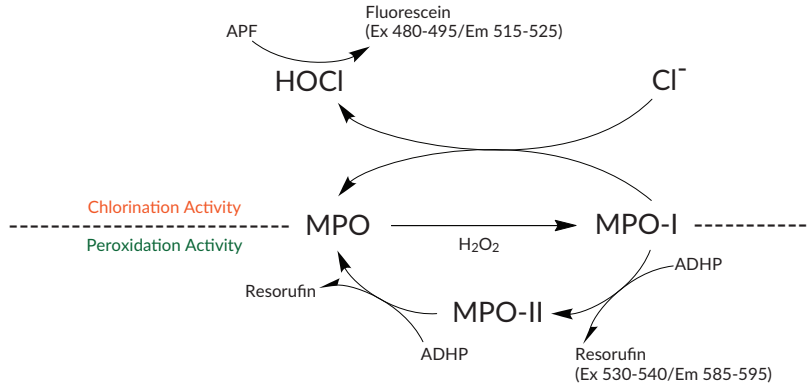


Figure 1. Assay scheme

PRE-ASSAY PREPARATION

Reagent Preparation

1. **MPO Assay Buffer - (Item No. 700164)**

   This bottle contains 50 ml of 1X Assay Buffer. It is ready to use in the assay.

2. **MPO Inhibitor - (Item No. 700167)**

   This vial contains 300 µl of 50 mM 4-aminobenzhydrazide, an MPO inhibitor.[9,10] Using this inhibitor is optional, but it can be used as a positive control. To use this supplied solution as a positive control inhibitor, dilute in buffer to 110 µM. This will give a final concentration of 10 µM when diluted into the well, and result in total inhibition of the enzyme. The diluted inhibitor is stable for four hours.

3. **MPO Hydrogen Peroxide - (Item No. 700168)**

   This vial contains 100 µl of a 30% solution of hydrogen peroxide. Prior to use, mix 10 µl with 90 µl of MPO Assay Buffer to yield a 3% solution. Prepare a 5 mM solution by diluting 10 µl of the 3% solution with 1.74 ml of MPO Assay Buffer. The 5 mM solution will be used to prepare the Initiator Solutions (see Tables 1 and 2 on pages 13 and 14, respectively). The diluted solutions are stable for two hours.

4. **DMSO Assay Reagent - (Item No. 700001)**

   This vial contains 1 ml of dimethylsulfoxide (DMSO). The reagent is ready to use as supplied.

5. **Myeloperoxidase Assay Reagent - (Item No. 700166)**

   This vial contains 50 µl of a 100 µg/ml suspension of human polymorphonuclear leukocyte MPO. Thaw and store the enzyme on ice while preparing the reagents for the assay. Prior to use, pipette up and down to mix thoroughly because this enzyme settles over time. Dilute 25 µl of MPO with 1,975 µl of MPO Assay Buffer for a final MPO concentration of 1.25 µg/ml. The diluted enzyme is stable for one hour on ice.

6. **MPO Chlorination Substrate - (Item No. 700165)**

   This vial contains 100 µl of 2.5 mM 2-[6-(4-aminophenoxy)-3-oxo-3H-xanthen-9-yl]-benzoic acid (APF) in DMSO. It is ready to use to prepare the Chlorination Initiator Solution.

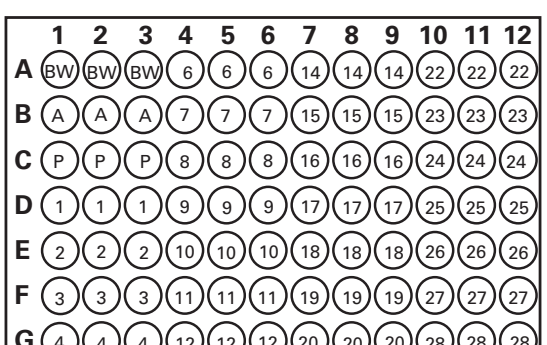
7. **MPO Peroxidation Substrate**

   Immediately prior to preparing the Initiator Solution, add 120 µl of DMSO Assay Reagent (Item No. 700001) to one vial of ADHP Assay Reagent (Item No. 700002) and vortex until dissolved. Then, add 470 µl of MPO Assay Buffer for a final MPO Peroxidation Substrate concentration of 1 mM. This is enough MPO Peroxidation Substrate to assay 100 wells. Prepare additional vials as needed. The reconstituted MPO Peroxidation Substrate is stable for 15 minutes. After 15 minutes, increased background fluorescence will occur.

ASSAY PROTOCOL

Plate Set Up

Chlorination and peroxidation activities cannot be measured simultaneously. There is no specific pattern for using the wells on the plate. However, it is necessary to have three wells designated as 100% Initial Activity and three wells designated as Background Wells. It is recommended to assay each inhibitor sample in triplicate, and record the contents of each well on the template sheet provided on page 18. A typical layout of samples to be measured in triplicate is shown in Figure 2.



BW - Background Wells
A - 100% Initial Activity Wells
P - Positive Control Wells
1-29 - Test Compound Wells

Figure 2. Sample plate format

Pipetting Hints

- It is recommended that a multichannel pipette be used to deliver reagents to the wells. This saves time and helps to maintain more precise incubation times.
- Before pipetting each reagent, equilibrate the pipette tip in that reagent (*i.e.*, slowly fill the tip and gently expel the contents, repeat several times).
- Do not expose the pipette tip to the reagent(s) already in the well.

General Information

- The final volume of the assay is 110 µl in all the wells.
- All reagents except the enzyme must be equilibrated to room temperature before beginning the assay.
- It is not necessary to use all the wells on the plate at one time.
- It is recommended to assay the samples in triplicate, but it is at the user's discretion to do so.
- Both assays are performed at room temperature.
- Chlorination and peroxidation activities cannot be measured simultaneously.
- Monitor the Chlorination fluorescence using an excitation wavelength of 480-495 nm and an emission wavelength of 515-525 nm.
- Monitor the Peroxidation fluorescence using an excitation wavelength of 530-540 nm and an emission wavelength of 585-595 nm.

Performing the Chlorination Assay

1. In a suitable tube, prepare the Chlorination Initiator Solution according to the table below. The solution will turn yellow.

Reagents	50 wells	100 wells
MPO Assay Buffer	2.44 ml	4.88 ml
Chlorination Substrate (2.5 mM)	40 µl	80 µl
Hydrogen Peroxide (5 mM)	20 µl	40 µl

Table 1. Chlorination Initiator Solution preparation

2. **100% Initial Activity Wells -** add 50 µl of MPO Assay Buffer and 10 µl of 1.25 µg/ml MPO to three wells.
3. **Background Wells -** add 60 µl of MPO Assay Buffer to three wells.
4. **Inhibitor Wells -** add 40 µl of MPO Assay Buffer, 10 µl of Inhibitor*, and 10 µl of 1.25 µg/ml MPO to three wells.
5. Begin the reactions by quickly adding 50 µl of the Chlorination Initiator Solution to all of the wells being used.
6. Cover the plate with the plate cover and incubate on a shaker for 10 minutes at room temperature.
7. Remove the plate cover. Read the plate using an excitation wavelength of 480-495 nm and an emission wavelength of 515-525 nm.

*Inhibitors can be dissolved in ethanol, methanol, or DMSO but need to be further diluted into MPO Assay Buffer before being added to the assay in a final volume of 10 µl. Solvents dramatically interfere with the assay. In the event that the appropriate concentration of Inhibitor needed for MPO inhibition is completely unknown, it is recommended that several concentrations of the compound be assayed.

Performing the Peroxidation Assay

1. In a suitable tube, prepare the Peroxidation Initiator Solution according to the table below:

Reagents	50 wells	100 wells
MPO Assay Buffer	2.24 ml	4.48 ml
Peroxidation Substrate (1 mM)	250 µl	500 µl
Hydrogen Peroxide (5 mM)	10 µl	20 µl

Table 2. Peroxidation Initiator Solution preparation

2. **100% Initial Activity Wells -** add 50 µl of MPO Assay Buffer and 10 µl of 1.25 µg/ml MPO to three wells.
3. **Background Wells -** add 60 µl of MPO Assay Buffer to three wells.
4. **Inhibitor Wells -** add 40 µl MPO Assay Buffer, 10 µl of Inhibitor*, and 10 µl of 1.25 µg/ml MPO to three wells.
5. Begin the reactions by quickly adding 50 µl of the Peroxidation Initiator Solution to to all of the wells being used.
6. Cover the plate with the plate cover and incubate on a shaker for five minutes at room temperature.
7. Remove the plate cover. Read the plate using an excitation wavelength of 530-540 nm and an emission wavelength of 585-595 nm.

*Inhibitors can be dissolved in ethanol, methanol, or DMSO but need to be further diluted into MPO Assay Buffer before being added to the assay in a final volume of 10 µl. Solvents dramatically interfere with the assay. In the event that the appropriate concentration of Inhibitor needed for MPO inhibition is completely unknown, it is recommended that several concentrations of the compound be assayed.

ANALYSIS

Calculations

1. Determine the average fluorescence of the 100% Initial Activity, Background, and Inhibitor Wells.
2. Subtract the fluorescence of the Background Wells from the fluorescence of the 100% Initial Activity and Inhibitor Wells.
3. Determine the percent inhibition for each inhibitor. To do this, subtract each inhibitor sample value from the 100% Initial Activity sample value. Divide the result by the 100% Initial Activity value and then multiply by 100 to give the percent inhibition.

$$\% \text{ Inhibition} = \left[\frac{\text{Initial Activity - Inhibitor}}{\text{Initial Activity}}\right] \times 100$$

4. If multiple concentrations of inhibitor are tested, graph either the Percent Inhibition or Percent Initial Activity as a function of the inhibitor concentration to determine the IC_{50} value (concentration at which there was 50% inhibition). Examples of MPO chlorination and peroxidation inhibition by the MPO inhibitor, 4-aminobenzhydrazide are shown in Figures 3 and 4, on pages 16-17.

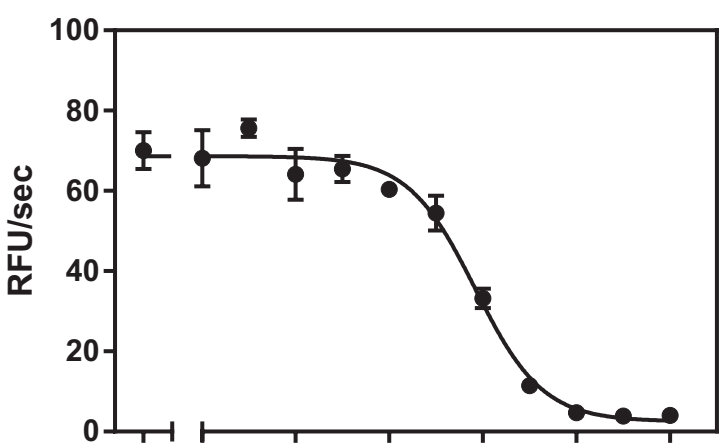


Figure 3. The IC_{50} range for a typical 4-aminobenzahydrazide inhibition curve using the MPO chlorination assay should fall between 10 and 46.2 nM. "Veh" represents compound vehicle control.

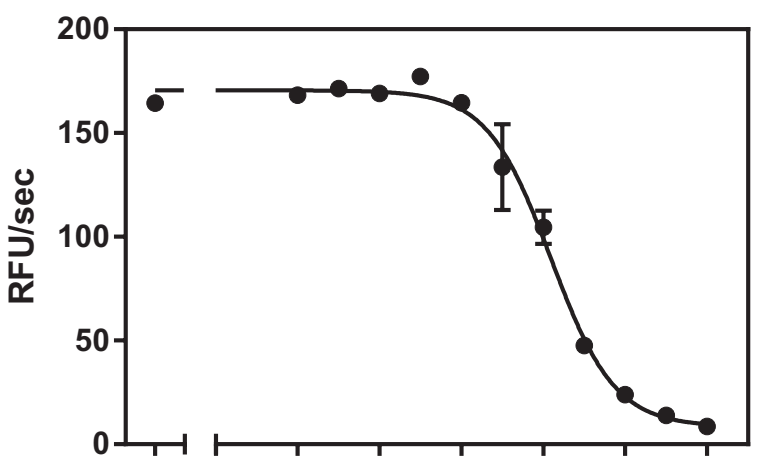


Figure 4. The IC_{50} range for a typical 4-aminobenzahydrazide inhibition curve using the MPO peroxidation assay should fall between 26.6 and 76.4 nM. "Veh" represents compound vehicle control.

Performance Characteristics

Precision:

Chlorination Assay: When a series of sixteen MPO measurements were performed on the same day, the intra-assay coefficient of variation was 3.7%. When a series of sixteen MPO measurements were performed on five different days under the same experimental conditions, the inter-assay coefficient of variation was 3.6%.

Peroxidation Assay: When a series of sixteen MPO measurements were performed on the same day, the intra-assay coefficient of variation was 2.8%. When a series of sixteen MPO measurements were performed on five different days under the same experimental conditions, the inter-assay coefficient of variation was 3.1%.

RESOURCES

Troubleshooting

Problem	Possible Causes	Recommended Solutions
Erratic values; dispersion of duplicates/triplicates	A. Poor pipetting/technique B. Bubble in the well(s)	A. Be careful not to splash the contents of the wells B. Carefully tap the side of the plate with your finger to remove bubbles
No fluorescence above background is seen in the inhibitor wells	Inhibitor concentration is too high and inhibited all of the enzyme activity	Reduce the concentration of the inhibitor and re-assay
The plate reader exhibited 'MAX' values for the wells	The *gain* setting is too high	Reduce the *gain* and re-read
No inhibition was seen with the MPO inhibitor	A. The inhibitor concentration is not high enough B. The compound is not an inhibitor of the enzyme	Increase the inhibitor concentration and re-assay

References

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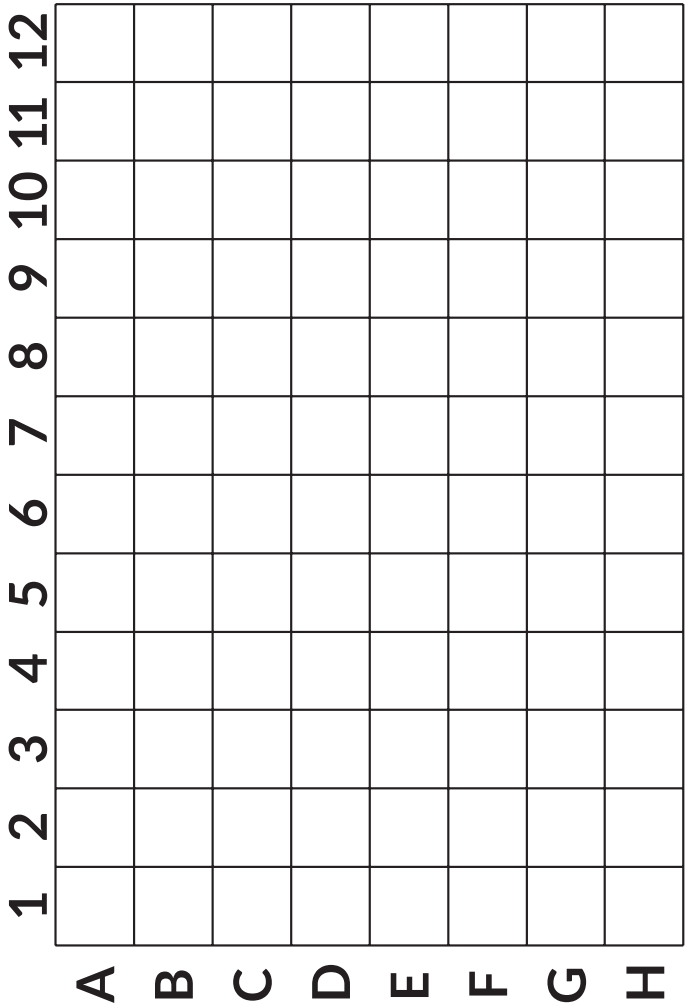

NOTES

Warranty and Limitation of Remedy

Buyer agrees to purchase the material subject to Cayman's Terms and Conditions. Complete Terms and Conditions including Warranty and Limitation of Liability information can be found on our website.