ab133080 – Myeloperoxidase (MPO) Inhibitor Screening Assay Kit

Instructions for Use

The Myeloperoxidase (MPO) Inhibitor Screening Assay provides convenient fluorescence-based methods for screening inhibitors to both the chlorination and peroxidation activities of MPO.

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

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1. Overview

Abcam'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. Fluorescein fluorescence is analyzed with an excitation wavelength of 480-490 nm and an emission wavelength of 515-520 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). 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.

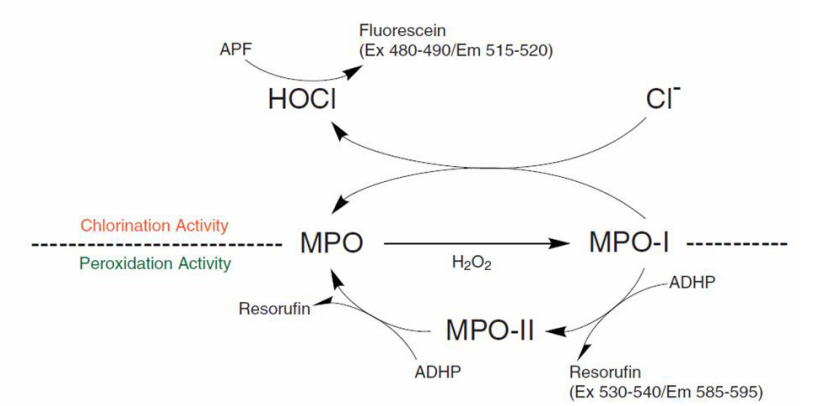


Figure 1.

2. Background

Myeloperoxidase (MPO) is a member of the heme peroxidase superfamily and is stored within the azurophilic granules of leukocytes. MPO is found within circulating neutrophils, monocytes, and some tissue macrophages. A unique activity of MPO is its ability to use chloride as a cosubstrate with hydrogen peroxide to generate chlorinating oxidants such as hypochlorous acid, a potent antimicrobial agent. Recently, evidence has emerged that MPO-derived oxidants contribute to tissue damage and the initiation and propagation of acute and chronic vascular inflammatory diseases. The fact that circulating levels of MPO have been shown to predict risks for major adverse cardiac events and that levels of MPO-derived chlorinated compounds are specific biomarkers for disease progression, has attracted considerable interest in the development of therapeutically useful MPO inhibitors. MPO also oxidizes a variety of substrates, including phenols and anilines, via the classic peroxidation cycle. The relative concentrations of chloride and the reducing substrate determine whether MPO uses hydrogen peroxide for chlorination or peroxidation. Assays based on measurement of chlorination activity are more specific for MPO than those based on peroxidase substrates because peroxidases generally do not produce hypochlorous acid. However, it is important that when screening for MPO inhibition that both the chlorination and peroxidation activities be tested. This determines whether the inhibitor specifically interferes with the chlorination and/or peroxidation cycle or whether the inhibitor simply acts as a scavenger for hypochlorous acid. Also, many reversible inhibitors act by diverting MPO from the chlorinating cycle to the peroxidase cycle.

3. Components and Storage

For best results, remove components and store as stated below.

Item	Quantity	Storage
MPO Assay Buffer	1 vial	4°C
MPO Chlorination Substrate	1 vial	4°C
ADHP Assay Reagent	2 vials	-20°C
Myeloperoxidase Assay Reagent	1 vial	-20°C
MPO Inhibitor	1 vial	4°C
MPO Hydrogen Peroxide	1 vial	4°C
MPO DMSO	1 vial	RT
96-Well Solid Plate (black)	2 plates	RT
96-Well Cover Sheets	2 covers	RT

Materials Needed but Not Supplied

- A fluorometer with the capacity to measure fluorescence using excitation wavelengths of 480-490 nm and 530-540 nm and emission wavelengths of 515-520 nm and 585-595 nm.
- Adjustable pipettes and a repeat pipettor.
- A source of pure water; glass distilled water or HPLC-grade water is acceptable.

4. Pre-Assay Preparation

1. MPO Assay Buffer

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

2. MPO Chlorination Substrate

The 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.

3. MPO Peroxidation Substrate

Immediately prior to preparing the Peroxidation Initiator Solution, add 120 µl of MPO DMSO to one vial of ADHP Assay Reagent 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 Substrate to assay 100 wells. Prepare additional vials as needed. The reconstituted Substrate is stable for 15 minutes. After 15 minutes, increased background fluorescence will occur.

4. Myeloperoxidase Control

The vial contains 50 µl of a 100 µg/ml solution 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 1975 µl of Assay Buffer for a final MPO concentration of 1.25 µg/ml. The diluted enzyme is stable for one hour on ice.

5. MPO Inhibitor

The vial contains 300 µl of 50 mM 4-aminobenzhydrazide, a MPO inhibitor. The Inhibitor's use is optional but may be used to standardize the assay. 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. For practicality, an intermediate dilution can be done. For instance, dilute 10 µl of 50 mM Inhibitor with 490 µl of Assay Buffer to prepare a working solution at 1 mM. Then dilute it further down to 110 µM in Assay Buffer. The diluted Inhibitor solutions are stable for four hours.

6. MPO Hydrogen Peroxide

The vial contains 100 µl of a 30% solution of hydrogen peroxide. Prior to use, dilute 10 µl with 90 µl of 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 Assay Buffer. The 5 mM solution will be used to prepare the Initiator Solutions. The diluted solutions are stable for two hours.

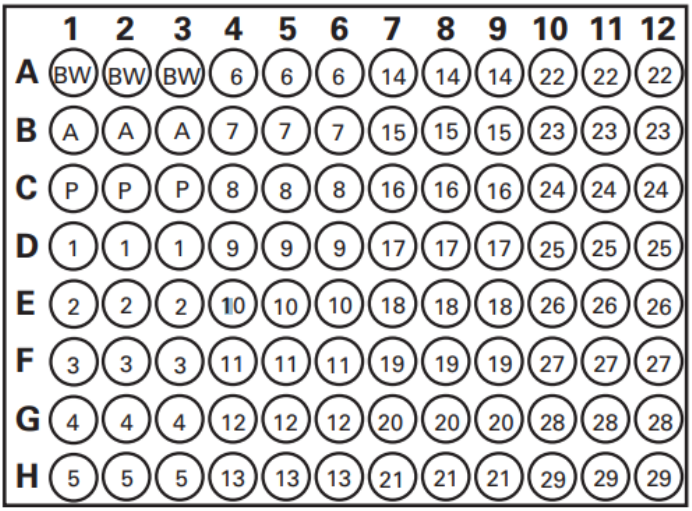
7. MPO DMSO

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

5. Assay Protocol

A. Plate Setup

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. We suggest that each inhibitor sample be assayed in triplicate. A typical layout of samples to be measured in duplicate 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 repeating pipettor 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.*
- *We recommend assaying samples in triplicate, but it is 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.*

B. Performing the Chlorination Assay

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

Component	50 wells	100 wells
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 Assay Buffer and 10 µl of 1.25 µg/ml MPO to three wells.
3. **Background Wells** - add 60 µl of Assay Buffer to three wells.
4. **Sample (inhibitor) Wells** - add 40 µl of Assay Buffer, 10 µl of sample (inhibitor) and 10 µl of 1.25 µg/ml MPO to three wells.
5. Initiate 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.

Sample (inhibitors) can be dissolved in ethanol, methanol, or DMSO but need to be further diluted into 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, we

recommend that several concentrations of the compound be assayed.

C. Performing the Peroxidation Assay

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

Component	50 wells	100 wells
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 Assay Buffer and 10 µl of 1.25 µg/ml MPO to three wells.
3. **Background Wells** - add 60 µl of Assay Buffer to three wells.
4. **Sample (inhibitor) Wells** - add 40 µl of Assay Buffer, 10 µl of sample (inhibitor) and 10 µl of 1.25 µg/ml MPO to three wells.
5. Initiate the reactions by quickly adding 50 µl of the Peroxidation Initiator Solution to all of the wells being used.
6. Cover the plate with the plate cover and incubate on a shaker for 5 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.

Sample (inhibitors) can be dissolved in ethanol, methanol, or DMSO but need to be further diluted into 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, we

recommend that several concentrations of the compound be assayed.

6. Data Analysis

A. 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.

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

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.

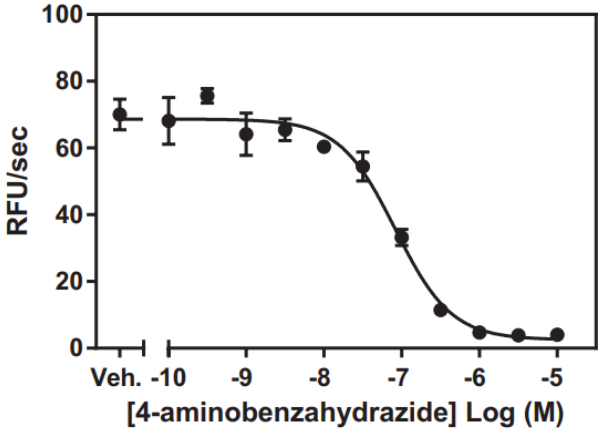


Figure 3. The IC50 range for a typical 4-aminobenzahydrazide inhibition curve using the MPO chlorination assay should fall between 10 and 46.2 nM. “Veh” represents compound vehicle control.

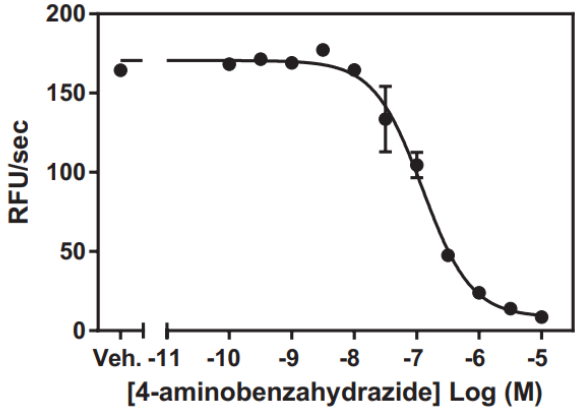


Figure 4. The IC50 range for a typical 4-aminobenzahydrazide inhibition curve using the MPO peroxidation assay should fall between 26.6 and 76.4 nM. “Veh” represents compound vehicle control.

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%.

7. 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 fluorometer 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

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