



MPO Inhibitor Screening Assay Kit

Catalog Number KA1337

96 assays

Version: 02

Intended for research use only

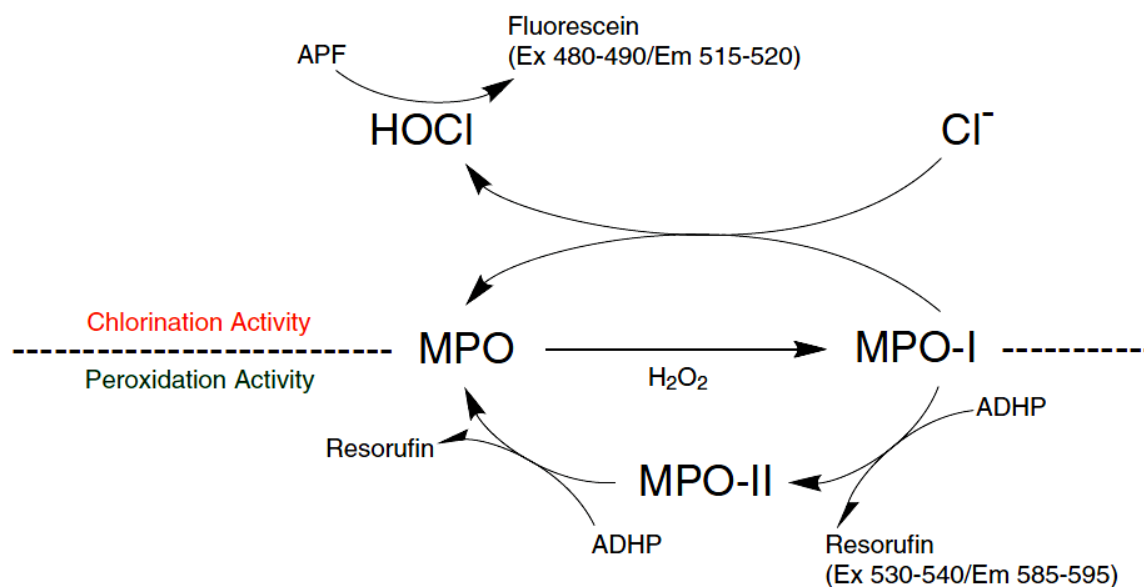
Background

Myeloperoxidase (MPO) is a member of the heme peroxidase superfamily and is stored within the azurophilic granules of leukocytes.¹ MPO is found within circulating neutrophils, monocytes, and some tissue macrophages.² 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.³ Recently, evidence has emerged that MPO-derived oxidants contribute to tissue damage and the initiation and propagation of acute and chronic vascular inflammatory diseases.^{4,5} 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.⁶ 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.

About This Assay

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 480-490 nm and an emission wavelength of 515-520 nm. The peroxidation assay utilizes the peroxidase component of MPO. 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.



Material Supplied

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
MPO Peroxidation Substrate	2 vials	4 °C
Myeloperoxidase Control	1 vial	-20 °C
MPO Inhibitor	1 vial	4 °C
MPO Hydrogen Peroxide	1 vial	4 °C
MPO DMSO	1 vial	4 °C
96-Well Plate (black)	2 plates	Room temperature
96-Well Cover Sheets	2 covers	Room temperature

WARNING: This product is for laboratory research use only; not for administration to humans. Not for human or veterinary diagnostic or therapeutic use.

Materials Needed But Not Supplied

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

Storage and Stability

Remove the Myeloperoxidase Control from the kit and store at -20°C. The rest of the components should be stored at 4°C. This kit will perform as specified if stored at -20°C and used before the expiration date indicated on the outside of the box.

Reagent Preparation

- ✓ MPO Assay Buffer - The vial contains 50 ml of 1X phosphate-buffered saline (PBS), pH 7.4. It is ready to use in the assay.
- ✓ 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 Working Solution.
- ✓ MPO Peroxidation Substrate - Each vial contains a clear lyophilized powder of ADHP (10-acetyl-3,7-dihydroxyphenoxazine). Immediately prior to preparing the Peroxidation Working Solution, dissolve the contents of one vial with 100 µl of Dimethylsulfoxide and then add 400 µl of assay buffer for a final concentration of 1 mM. This is enough substrate to assay 100 wells. Prepare additional vials as needed. The reconstituted substrate is stable for 30 minutes. After 30 minutes, increased background fluorescence will occur.
- ✓ 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, dilute 10 µl of MPO with 3.99 ml of assay buffer for a final MPO concentration of 250 ng/ml. This is enough enzyme to assay 80 wells. The diluted enzyme is stable for one hour on ice.
- ✓ MPO Inhibitor - The vial contains 300 µl of 50 mM 4-aminobenzhydrazide, a MPO inhibitor.^{8,9} The inhibitor's use is optional but may be used to standardize the assay. Prior to use, dilute 10 µl of inhibitor with 490 µl of assay buffer. This is enough inhibitor to assay 50 wells. The diluted inhibitor is stable for four hours.
- ✓ 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 Working Solutions. The diluted solutions are stable for two hours.
- ✓ MPO DMSO - The vial contains 1 ml of dimethylsulfoxide (DMSO). The reagent is ready to use as supplied.

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. We suggest that each inhibitor sample be assayed in triplicate and that you record the contents of each well. A typical layout of samples to be measured in duplicate is shown in Figure 2.

	1	2	3	4	5	6	7	8	9	10	11	12
A	BW	BW	BW	7	7	7	15	15	15	23	23	23
B	A	A	A	8	8	8	16	16	16	24	24	24
C	1	1	1	9	9	9	17	17	17	25	25	25
D	2	2	2	10	10	10	18	18	18	26	26	26
E	3	3	3	11	11	11	19	19	19	27	27	27
F	4	4	4	12	12	12	20	20	20	28	28	28
G	5	5	5	13	13	13	21	21	21	29	29	29
H	6	6	6	14	14	14	22	22	22	30	30	30

BW - Background Wells

A - 100% Initial Activity Wells

1-30 - Inhibitor/Activator 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 μ l 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 480-490 nm and an emission wavelength of 515-520 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 Working Solution according to the table below. The solution will turn yellow.

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

Table 1. Chlorination Working Solution preparation

2. **100% Initial Activity Wells** - add 10 μ l of assay buffer and 50 μ l of 250 ng/ml MPO to three wells.
3. **Background Wells** - add 60 μ l of assay buffer to three wells.
4. **Inhibitor Wells** - add 10 μ l of inhibitor* and 50 μ l of 250 ng/ml MPO to three wells.
5. Initiate the reactions by quickly adding 50 μ l of the Chlorination Working 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 480-490 nm and an emission wavelength of 515-520 nm.

*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 μ l. 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.

Performing the Peroxidation Assay

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

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

Table 2. Peroxidation Working Solution preparation

2. **100% Initial Activity Wells** - add 10 μ l of assay buffer and 50 μ l of 250 ng/ml MPO to three wells.
3. **Background Wells** - add 60 μ l of assay buffer to three wells.
4. **Inhibitor Wells** - add 10 μ l of inhibitor* and 50 μ l of 250 ng/ml MPO to three wells.
5. Initiate the reactions by quickly adding 50 μ l of the Peroxidation Working 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.

*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 μ l. 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.

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.

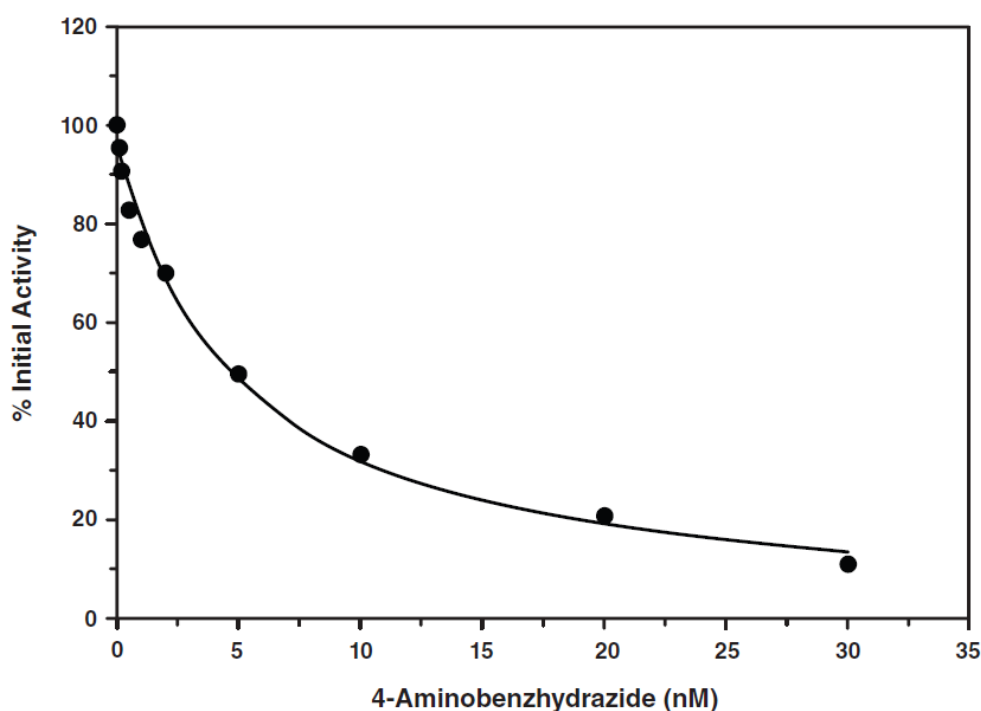


Figure 3. Inhibition of MPO chlorination activity by 4-aminobenzhydrazide ($IC_{50} = 5$ nM).

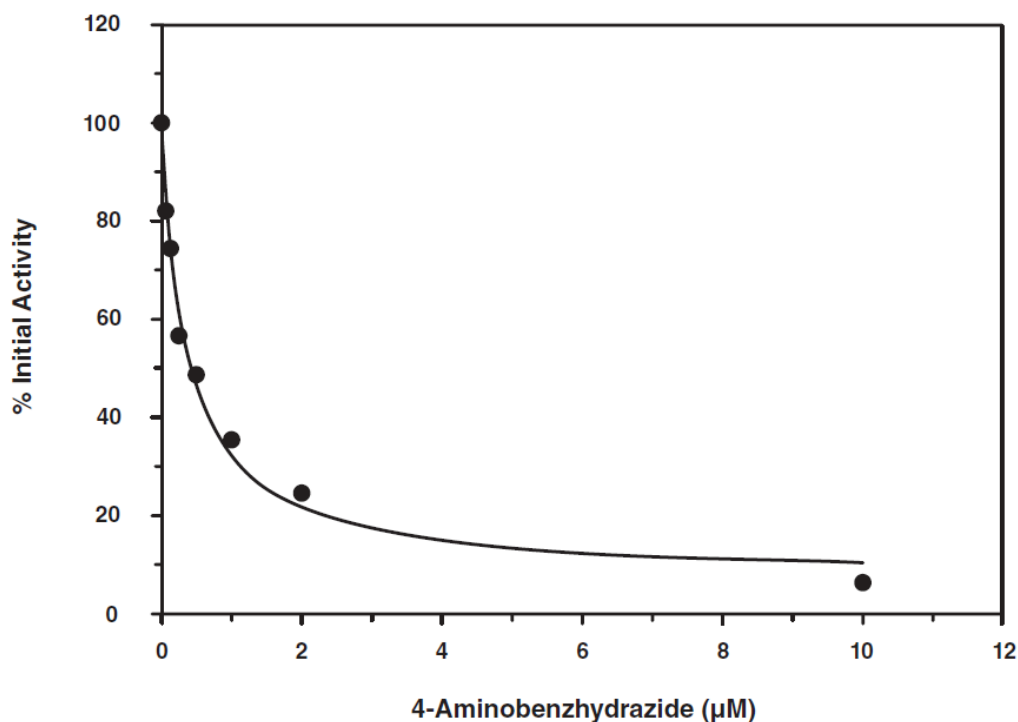


Figure 4. Inhibition of MPO peroxidation activity by 4-aminobenzhydrazide ($IC_{50} = 0.4 \mu M$).

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%.

Troubleshooting

Problem	Possible Causes	Recommended Solutions
Erratic values; dispersion of duplicates/triplicates	A. Poor pipetting/technique B. Bubble in the well(s)	A. Carefully tap the side of the plate with your finger to remove bubbles B. Be careful not to splash the contents of the wells
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

References

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