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 (H_2O_2) 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 H_2O_2 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. The only exception is eosinophil peroxidase that produces hypochlorous acid at pH below 5. The chlorination activity of MPO has a neutral pH optimum, therefore the assay conditions can be set so that only MPO activity is specifically measured.

About This Assay

Myeloperoxidase Chlorination Assay provides a convenient fluorescence-based method for detecting the MPO chlorination activity in both crude cell lysates and purified enzyme preparations. The 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 (See Figure 1).[7] Fluorescein fluorescence is analyzed with an excitation wavelength of 480-490 nm and an emission wavelength of 515-520 nm. The kit includes a MPO-specific inhibitor for distinguishing MPO activity from MPO-independent fluorescence.

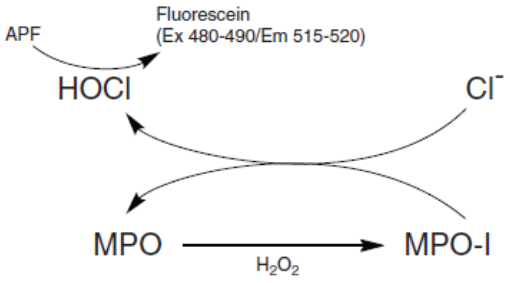

Figure 1. The ferric, or native, MPO reacts with hydrogen peroxide to form the active redox and enzyme ntermediate compound MPO-I, which oxidizes chloride (Cl-) to HOCl.

Material Supplied

Kit will arrive packaged as a -20℃ kit. For best results, remove components and store as stated below.

Item	Quantity	Storage
MPO Assay Buffer	1 vial	4℃
MPO Chlorination Substrate	2 vials	4℃
Myeloperoxidase Control	1 vial	-20℃
MPO Inhibitor	1 vial	4℃
MPO Hydrogen Peroxide	1 vial	4℃
Fluorescein Standard	1 vial	4℃
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 an excitation wavelength of 480-490 nm and an emission wavelength of 515-520 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℃. The rest of the components should be stored at 4℃. This kit will perform as specified if 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 - Each 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 Working Solution.
- ✓ 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 assaying, dilute 10 µl of MPO with 3.99 ml of assay buffer for a final MPO concentration of 250 ng/ml. 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] Prior to

assaying, 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 assaying, dilute 10 µl with 90 µl of assay buffer to yield a 3% solution. Then 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 Solution. The diluted solutions are stable for two hours.
- ✓ Fluorescein Standard - The vial contains 100 µl of a 1 mM solution of fluorescein. The reagent is ready to use to prepare the fluorescein standard curve.

Sample Preparation

The kit is designed for detection of MPO activity in cell lysates and in purified solutions. This assay is not applicable for use with serum samples. Many reagents interfere with the chlorination assay. Before collecting cells, check the interference section for reagents that will not interfere with the assay.

Cell Lysate

1. Collect cells (~7 x 10^6) by centrifugation (*i.e.*, 1,000-2,000 x g for 10 minutes at 4°C). For adherent cells, do not harvest using proteolytic enzymes; rather use a rubber policeman.
2. Sonicate cell pellet in 0.5-1 ml of cold 1X PBS, pH 7.4, on ice.
3. Centrifuge at 10,000 x g for 10 minutes at 4°C.
4. Remove the supernatant and store on ice.
5. We recommend assaying for MPO activity on the same day of collection. If this is not possible, freeze the sample at -80°C. The sample will be stable for at least one week.

Plate Set Up

There is no specific pattern for using the wells on the plate. However, a fluorescein standard curve in duplicate has to be assayed with two wells for the sample and the MPO positive control. We suggest that each sample be assayed at least in duplicate. We also recommend assaying each sample in the presence and absence of the MPO inhibitor to allow for the correction of non-MPO-independent fluorescence and to record the contents of each well on. A typical layout of samples to be measured in duplicate is shown in Figure 2 below.

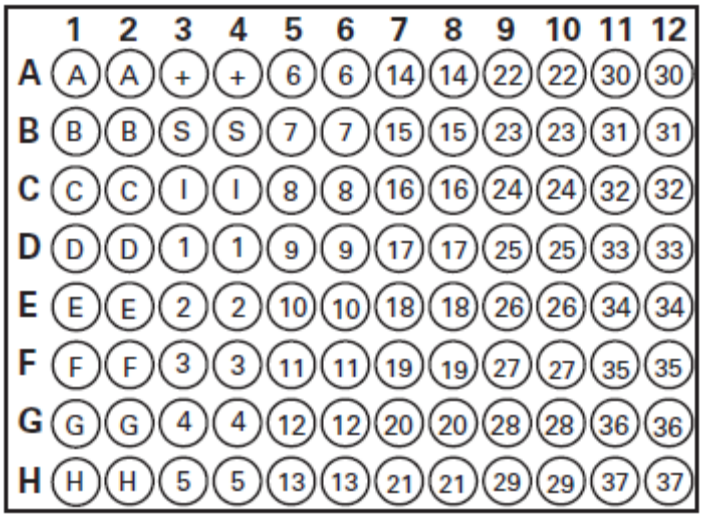

Figure 2. Sample plate format

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 MPO Positive Control and samples 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.
- ✓ The assay is performed at room temperature.
- ✓ Monitor the fluorescence with an excitation wavelength of 480-490 nm and an emission wavelength of 515-520 nm.

Standard Preparation

Dilute 10 µl of the fluorescein standard with 990 µl of assay buffer to yield a concentration of 10 µM. Dilute 100 µl of this 10 µM standard with 900 µl of assay buffer to yield a stock concentration of 1 µM. Take eight clean glass test tubes and mark them A-H. Add the amount of fluorescein standard (1 µM) and assay buffer to each tube as described in Table 1. The diluted standards are stable for four hours at room temperature.

Tube	1 µM Fluorescein Standard (µl)	Assay Buffer (µl)	Final Concentration (nM)
A	0	1,000	0
B	5	995	5
C	10	990	10
D	25	975	25
E	50	950	50
F	100	900	100
G	150	850	150
H	200	800	200

Performing the Assay

1. **Standard Wells -** add 60 µl of assay buffer and 50 µl of standard (tubes A-H) per well in the designated wells on the plate (see Sample plate format, Figure 2).
2. Read the plate after five minutes in a fluorometer using an excitation wavelength of 480-490 nm and an emission wavelength of 515-520 nm. This will allow you to establish an appropriate GAIN for detecting the entire range of the standards. This GAIN will then be used when assaying the samples.
3. In a suitable tube, prepare the Working Solution according to the table below. The solution will turn yellow.

Reagents	50 wells	100 wells	150 wells	200 wells
Assay Buffer	2.44 ml	4.88 ml	7.32 ml	9.76 ml
Chlorination Substrate	40 µl	80 µl	120 µl	160 µl
Hydrogen Peroxide (5 mM)	20 µl	40 µl	60 µl	80 µl

Table 2. Working Solution preparation

4. **MPO Positive Control Wells -** add 10 µl of assay buffer and 50 µl of 25 ng/ml MPO to two wells.
5. **Sample Wells -** add 10 µl of assay buffer and 50 µl of sample to two wells. To obtain reproducible results, the amount of myeloperoxidase added to the wells should fall within the range of the assay. When necessary, samples should be diluted with assay buffer or concentrated with an Amicon centrifuge concentrator with a molecular weight cut-off of 30,000 to bring the enzymatic activity to this level.
6. **Inhibitor Wells -** add 10 µl of diluted MPO inhibitor and 50 µl of sample to two wells.
7. Initiate the reactions by quickly adding 50 µl of the Working Solution to the positive control, sample, and inhibitor wells only.
8. Read the plate in a fluorometer every minute for 30 minutes using an excitation wavelength of 480-490 nm and an emission wavelength of 515-520 nm.

Well Type	Assay Buffer	MPO (25 ng/ml)	Sample	MPO Inhibitor	Working Solution
Positive Control	10 µl	50 µl	-	-	50 µl
Sample	10 µl	-	50 µl	-	50 µl
Inhibitor	-	-	50 µl	10 µl	50 µl

Table 3. Pipetting summary

Calculations

Plot the Standard Curve

1. Determine the average fluorescence of the standards. Subtract the fluorescence value of the standard A from itself and all other standards. This is the corrected fluorescence.
2. Plot the corrected fluorescence values (from step 1 above) of each standard as a function of the final concentration of fluorescein from Table 1. See Figure 3, for a typical standard curve.

Determine MPO Activity

1. Determine the average fluorescence of each sample and sample plus inhibitor.
2. Determine the change in fluorescence (RFU) per minute for the sample and sample plus inhibitor by:
   a. Plotting the fluorescence values as a function of time to obtain the slope (rate) of the linear portion of the curve. An example of human myeloid leukemia HL60 10,000 x g supernatant assayed with and without MPO inhibitor over time is shown in Figure 4,

   OR

   b. Select two points on the linear portion of the curve and determine the change in fluorescence during that time using the following equation:

$$\text{RFU/min.} = \frac{\text{RFU(Time 2)} - \text{RFU(Time 1)}}{\text{Time 2(min.)} - \text{Time 1(min.)}}$$

3. Calculate the MPO activity using the equation. One unit is defined as the amount of enzyme that will cause the formation of 1 pmol of fluorophore per minute at 25℃.

$$\text{Myeloperoxidase Activity (pmol/min/ml)} = \frac{\text{RFU/min.(Sample)} - \text{RFU/min.(Sample + Inhibitor)}}{\text{Slope from Fluorescein standard curve (RFU/nM)}} \times \text{Sample Dilution}$$

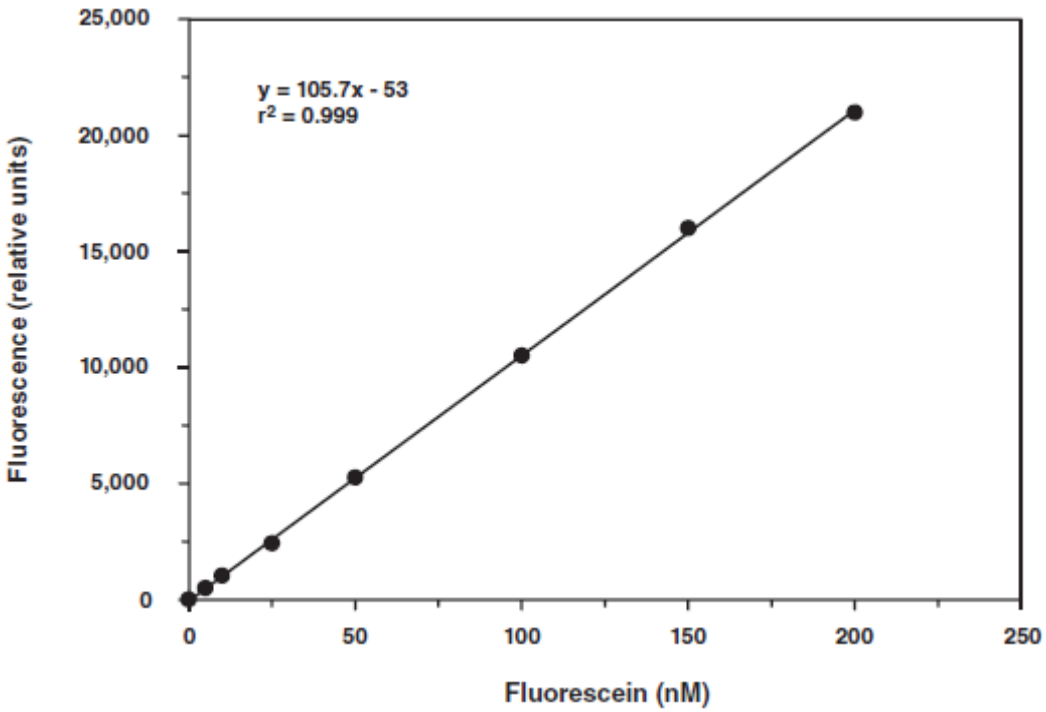

Figure 3. Fluorescein standard curve

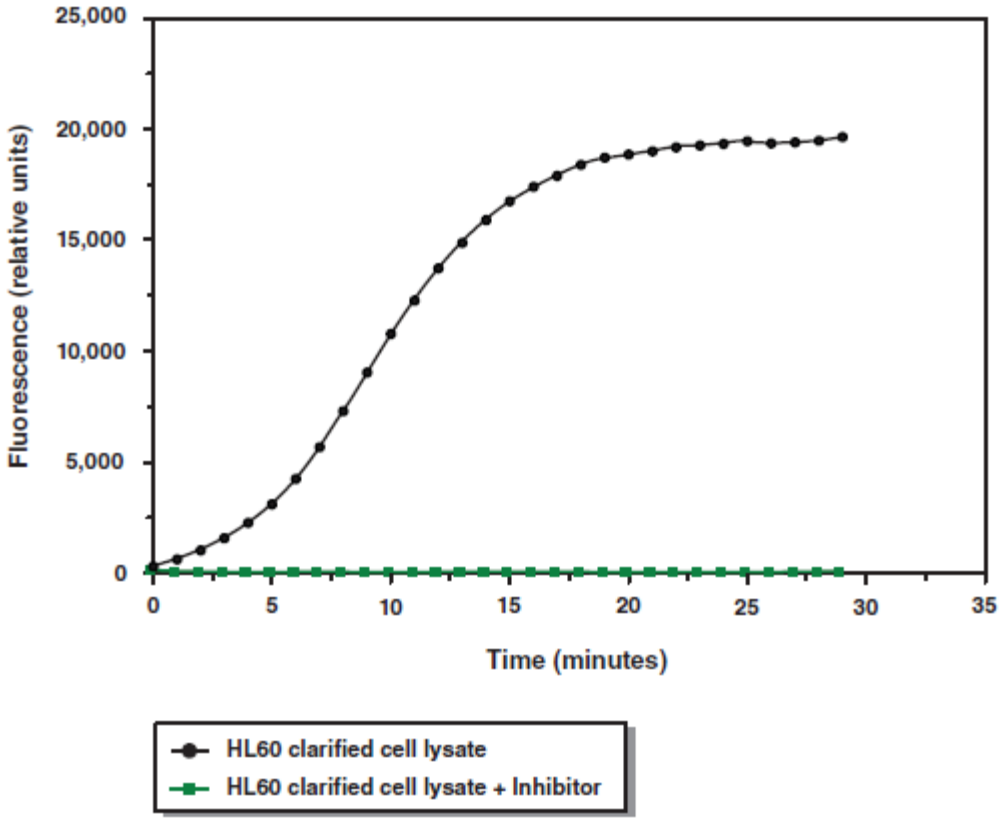

Figure 4. Human myeloid leukemia HL60 10,000 x g supernatant assayed with and without MPO inhibitor.

Precision

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%.

Assay Range

Under the standardized conditions of the assay described in this booklet, the dynamic range of the kit is 0-200 nM of fluorescein.

Interferences

	Reagent	Will Interfere (Yes or No)
Buffers	Tris	Yes
	HEPES	Yes
	Phosphate	No
Detergents	Tween 20 (0.1%)	Yes
	Tween 20 (1%)	Yes
	Triton X-100 (0.1%)	Yes
	Triton X-100 (1%)	Yes
Protease Inhibitors/ Chelators/ Enzymes	EDTA (1 mM)	Yes
	EGTA (1 mM)	Yes
	Trypsin (10 µg/ml)	Yes
	PMSF (1 mM)	Yes
	Leupeptin (10 µg/ml)	No
	Antipain (10 µg/ml)	No
	Chymostatin (10 µg/ml)	No
DSolvents	Ethanol (10 µl)	Yes
	Methanol (10 µl)	Yes
	Dimethylsulfoxide (10 µl)	Yes
Others	BSA (0.1%)	Yes
	Glutathione (1 mM)	Yes
	Glycerol (5%)	Yes

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 detected in the sample wells	Sample was too dilute	A. Re-assay the sample using a lower dilution B. Concentrate the sample with an Amicon concentrator with a 30,000 MW cut-off
The fluorometer exhibited 'MAX' values for the wells	The GAIN setting is too high	A. Reduce the GAIN and re-read B. Make sure to establish the GAIN using the fluorescein standards before assaying your samples
No inhibition was seen with the MPO inhibitor	A. MPO activity is too low to detect B. The sample does not contain MPO C. Sample contains something that is interfering with the assay	A. Re-assay the sample using a lower dilution B. Check the interference section for possible interfering reagents

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