



Myeloperoxidase Chlorination Fluorometric Assay Kit

Item No. 10006438

www.caymanchem.com

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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 bottle | 4°C |
| 700169 | Fluorescein Standard | 1 vial | 4°C |
| 700167 | MPO Inhibitor | 1 vial | 4°C |
| 700168 | MPO Hydrogen Peroxide | 1 vial | 4°C |
| 700166 | Myeloperoxidase Assay Reagent | 1 vial | -20°C |
| 700165 | MPO Chlorination Substrate | 2 vials | 4°C |
| 400017 | 96-Well Solid Plate (black) | 2 plates | RT |
| 400012 | 96-Well Cover Sheet | 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

Fax: 734-971-3640

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 an excitation wavelength of 480-495 nm and an emission wavelength of 515-525 nm
2. Adjustable pipettes and a multichannel or repeating 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 hydrogen peroxide (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

About This Assay

Cayman's Myeloperoxidase Chlorination Fluorometric 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 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 480-495 nm and an emission wavelength of 515-525 nm. The kit includes a MPO-specific inhibitor for distinguishing MPO activity from MPO-independent fluorescence.

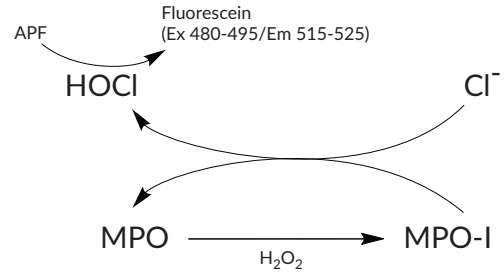


Figure 1. The ferric, or native, MPO reacts with hydrogen peroxide to form the active redox and enzyme intermediate compound MPO-I, which oxidizes chloride (Cl^-) to HOCl.

PRE-ASSAY PREPARATION

Reagent Preparation

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

This bottle contains 50 ml of 1X Assay Buffer, pH 7.4. It is ready to use in the assay.

2. Fluorescein Standard - (Item No. 700169)

This vial contains 100 μ l of a 1 mM solution of fluorescein. The reagent is ready to use to prepare the fluorescein standard curve.

3. MPO Inhibitor - (Item No. 700167)

This vial contains 300 μ l of 50 mM 4-aminobenzhydrazide, a MPO inhibitor.^{8,9} Prior to assaying, mix 10 μ l of Inhibitor with 490 μ l of MPO Assay Buffer. This is enough Inhibitor to assay 50 wells. The diluted Inhibitor is stable for four hours.

4. MPO Hydrogen Peroxide - (Item No. 700168)

This vial contains 100 μ l of a 30% solution of hydrogen peroxide. Prior to assaying, mix 10 μ l with 90 μ l of MPO Assay Buffer to yield a 3% solution. Then mix 10 μ l of the 3% solution with 1.74 ml of MPO Assay Buffer to prepare a 5 mM solution. The 5 mM solution will be used to prepare the Initiator Solution. The diluted solutions are stable for two hours.

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

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

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

Each vial contains 100 μ l 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 Initiator Solution.

Sample Preparation

The kit is designed for detection of MPO activity in cell lysates and in purified solutions. This assay is not compatible for use with serum samples. Some reagents have been shown to interfere with this assay, see page 19 for a list of compatible reagents.

Cell Lysate

1. Collect cells ($\sim 7 \times 10^6$) 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.

ASSAY PROTOCOL

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. It is also recommended to assay 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 the template sheet provided on page 22. A typical layout of samples to be measured in duplicate is shown in Figure 2 below.

| | 1 | 2 | 3 | 4 | 5 | 6 | 7 | 8 | 9 | 10 | 11 | 12 |
|---|-----|-----|-----|-----|------|------|------|------|------|------|------|------|
| A | (A) | (A) | (+) | (+) | (6) | (6) | (14) | (14) | (22) | (22) | (30) | (30) |
| B | (B) | (B) | (S) | (S) | (7) | (7) | (15) | (15) | (23) | (23) | (31) | (31) |
| C | (C) | (C) | (I) | (I) | (8) | (8) | (16) | (16) | (24) | (24) | (32) | (32) |
| D | (D) | (D) | (1) | (1) | (9) | (9) | (17) | (17) | (25) | (25) | (33) | (33) |
| E | (E) | (E) | (2) | (2) | (10) | (10) | (18) | (18) | (26) | (26) | (34) | (34) |
| F | (F) | (F) | (3) | (3) | (11) | (11) | (19) | (19) | (27) | (27) | (35) | (35) |
| G | (G) | (G) | (4) | (4) | (12) | (12) | (20) | (20) | (28) | (28) | (36) | (36) |
| H | (H) | (H) | (5) | (5) | (13) | (13) | (21) | (21) | (29) | (29) | (37) | (37) |

A-H = Fluorescein Standards
+ = MPO Positive Control
S = Sample Wells
I = Sample + Inhibitor Wells
1-37 = Other Samples

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 achieve 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 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 480-495 nm and an emission wavelength of 515-525 nm.

Standard Preparation

Mix 20 μl of the Fluorescein Standard with 980 μl of MPO Assay Buffer to yield a concentration of 20 μM . Mix 10 μl of this 20 μM Standard with 990 μl of MPO Assay Buffer to yield a stock concentration of 200 nM. Add the amount of Fluorescein Standard (200 nM) and MPO Assay Buffer to each well of a dilution plate (or test tube) as described in Table 1. The diluted Standards are stable for four hours at room temperature.

| Well (or tube) | 200 nM Fluorescein Standard (μl) | MPO Assay Buffer (μl) | Final Concentration (nM) |
|----------------|---|------------------------------------|--------------------------|
| A | 0 | 200 | 0 |
| B | 5 | 195 | 5 |
| C | 10 | 190 | 10 |
| D | 25 | 175 | 25 |
| E | 50 | 150 | 50 |
| F | 100 | 100 | 100 |
| G | 150 | 50 | 150 |
| H | 200 | 0 | 200 |

Table 1. Preparation of the Fluorescein Standards

Performing the Assay

- Standard Wells** - add 60 μl of MPO Assay Buffer and 50 μl of Standard (wells or tubes, A-H) per well in the designated wells on the plate (see Sample plate format, Figure 2, page 10).
- Read the plate in a plate reader using an excitation wavelength of 480-495 nm and an emission wavelength of 515-525 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.
- In a suitable tube, prepare the Initiator Solution according to the table below. The solution will turn yellow.

| Reagents | 50 wells | 100 wells | 150 wells | 200 wells |
|--------------------------|------------------|------------------|-------------------|-------------------|
| MPO 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. Initiator Solution Preparation

- MPO Positive Control Wells** - add 50 μl of MPO Assay Buffer and 10 μl of 1.25 $\mu\text{g}/\text{ml}$ Myeloperoxidase Assay Reagent to two wells.
- Sample Wells** - add 50 μl of MPO Assay Buffer and 10 μl of experimental 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 MPO Assay Buffer or concentrated with a centrifugal concentrator with a molecular weight cut-off of 30 kDa to bring the enzymatic activity to this level.
- Inhibitor Wells** - add 40 μl of MPO Assay Buffer, 10 μl of diluted MPO Inhibitor, and 10 μl of sample to two wells.

| Well Type | MPO Assay Buffer | Myeloperoxidase Assay Reagent (1.25 µg/ml) | Sample | MPO Inhibitor |
|------------------|------------------|--|--------|---------------|
| Positive Control | 50 µl | 10 µl | - | - |
| Sample | 50 µl | - | 10 µl | - |
| Inhibitor | 40 µl | - | 10 µl | 10 µl |

Table 3. Pipetting summary

7. Begin the reactions by quickly adding 50 µl of the Initiator Solution to the positive control, sample, and inhibitor wells.
8. Read the plate in a plate reader every 30 seconds for 30 minutes using an excitation wavelength of 480-495 nm and an emission wavelength of 515-525 nm.

ANALYSIS

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, on page 16, 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 polymorphonuclear leukocyte MPO assayed with and without MPO Inhibitor over time is shown in Figure 4, on page 17.

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 below. One unit is defined as the amount of enzyme that will cause the formation of 1 pmol of fluorophore per minute at 25°C.

Myeloperoxidase activity (pmol/min/ml) =

$$\frac{\text{Sample slope (RFU/min)} - \text{Inhibitor slope (RFU/min)}}{\text{Fluorescein standard curve slope}} \times \text{Sample dilution}$$

Sample Data:

The data shown here is an example of the data typically produced with this kit. Your results may vary, and therefore should not be directly compared to these samples.

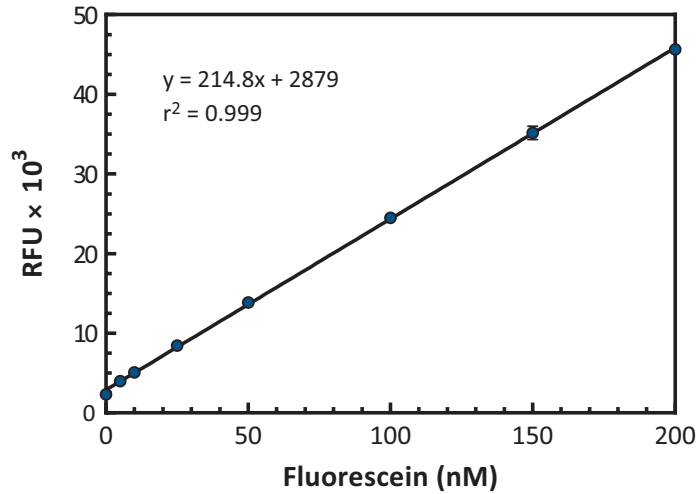


Figure 3. Fluorescein standard curve

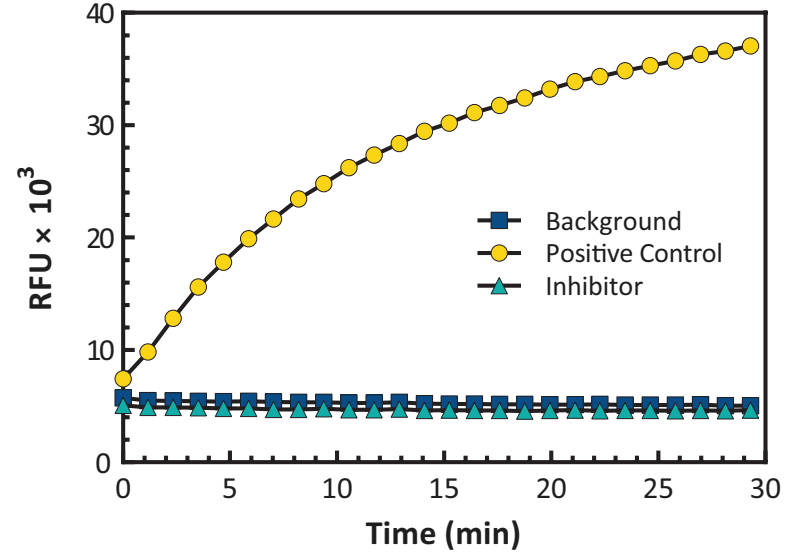


Figure 4. Human polymorphonuclear leukocyte MPO assayed with and without MPO inhibitor.

Performance Characteristics

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.

RESOURCES

Interferences

The following reagents were tested in the assay for interference in the assay:

| | Reagent | Will Interfere (Yes or No) |
|--|---------------------------|----------------------------|
| Buffers | Tris | Yes |
| | HEPES | Yes |
| | Phosphate | No |
| Detergents | Polysorbate 20 (0.1%) | Yes |
| | Polysorbate 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 |
| D Solvents | 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 a centrifugal concentrator with a 30 kDa cut-off |
| The plate reader 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 (see page 19) |

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.

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