



Neutrophil Myeloperoxidase Activity Assay Kit

Item No. 600620

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

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

Item Number	Item	Quantity/Size	Storage
10009322	Cell-Based Assay Buffer Tablet	1 vial/1 tablet	RT
601077	RBC Lysis Buffer (10X)	1 vial/10 ml	4°C
600612	Cell-Based Assay Neutrophil Isolation Histopaque®	1 vial/25 ml	4°C
400145	PMA (1 mM) Assay Reagent	1 vial/50 µl	-20°C
600622	Cell-Based Assay Myeloperoxidase Positive Control	1 vial/25 µl	-20°C
400074	TMB Substrate Solution	1 vial/12 ml	4°C
600621	Cell-Based Assay Myeloperoxidase Inhibitor	1 vial/50 µl	-20°C
400014	96-Well Solid Plate (Colorimetric Assay)	2 plates	RT
400012	96-Well Cover Sheet	2 covers	RT

NOTE: Histopaque® is a product of Sigma-Aldrich Co.

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

The kit should be stored at -20°C. Once it is opened, remove the PMA (1 mM) Assay Reagent and Cell-Based Assay Myeloperoxidase Inhibitor from the kit and store at -20°C. Store the TMB Substrate Solution at 4°C. The rest of the components may be stored at room temperature. The kit will perform as specified if used before the expiration date indicated on the outside of the box.

Materials Needed But Not Supplied

1. RPMI cell culture medium
2. Bovine Serum Albumin
3. EDTA Blood collection Tubes (can be obtained from Becton Dickinson, Product Number 366643)
4. A plate reader capable of measuring absorbance at 650 nm
5. Adjustable pipettes and a repeating pipettor
6. A source of pure water; glass distilled water or HPLC-grade water is acceptable

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)hypochlorous acids and radicals, including hypochlorous acid (HOCl), using hydrogen peroxide (H₂O₂) for chlorination or peroxidation.¹ The use of H₂O₂ 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 Neutrophil Myeloperoxidase Activity Assay kit utilizes 3,3',5,5'-tetramethyl-benzidine (TMB) as a chromogenic substrate, which upon reacting with MPO, yields a blue color detectable by its absorbance at 650 nm. The color intensity is proportional to the amount of MPO in the sample. Reagents needed to isolate neutrophils from whole blood are included in the kit, as is PMA, which is known to stimulate MPO release from neutrophils. A specific inhibitor for MPO, 4-aminobenzhydrazide (ABH), is also included in the kit for verifying the specificity of the assay.

Reagent Preparation

1. Cell-Based Assay Buffer

Dissolve one Cell-Based Assay Buffer Tablet (Item No. 10009322) in 100 ml of distilled water. Filter through a 0.2 µM filter before using to dilute the whole blood. Use the filtered Cell-Based Assay Buffer in a cell culture hood. This buffer should be stable for approximately one year at room temperature.

2. RBC Lysis Buffer (10X) - (Item No. 601077)

On the day of use, combine 5 ml of RBC Lysis Buffer (10X) (Item No. 601077) with 45 ml distilled water. Warm to room temperature prior to use. Discard the combined reagent after 48 hours. The remaining unused RBC Lysis Buffer (10X) can be stored at 4°C for one year.

3. Cell-Based Assay Neutrophil Isolation Histopaque® - (Item No. 600612)

The vial contains 25 ml of Histopaque®. It is ready to use for isolation of neutrophils from whole blood. Use the Cell-Based Assay Neutrophil Isolation Histopaque® in a cell culture hood.

4. PMA (1 mM) Assay Reagent - (Item No. 400145)

The vial contains 50 µl of 1 mM PMA in DMSO. For stimulating neutrophil MPO release, dilute this PMA 1:10,000 in the culture medium used for your cells.

5. TMB Substrate Solution

The vial contains 12 ml TMB Substrate Solution. It is ready to use. The substrate is stable at 4°C for at least one year.

NOTE: TMB is very sensitive to light. Avoid direct exposure to light.

6. Cell-Based Assay Myeloperoxidase Inhibitor - (Item No. 600621)

The vial contains 50 µl of 25 mM 4-aminobenzhydrozide (4-ABH) in DMSO. Prior to use, dilute the Cell-Based Assay Myeloperoxidase Inhibitor 1:100 in Assay Buffer. The inhibitor is stable at -20°C for at least one year.

7. Cell-Based Assay Myeloperoxidase Positive Control - (Item No. 600622)

This vial contains 25 µl human polymorphonuclear leukocyte myeloperoxidase at 0.1 mg/ml. To use the enzyme as a Positive Control, add 2 µl of the Cell-Based Assay Myeloperoxidase Positive Control (Item No. 600622) to 0.2 ml of the diluted Assay Buffer. Mix well. Add 50 µl of this diluted enzyme into corresponding wells in the assay plate.

Sample Preparation

Neutrophil Isolation

1. Collect 15 ml of whole blood in an EDTA blood collection tube.
2. Transfer the blood to a 50 ml conical tube. Rinse the blood collection tube with 15 ml of filtered Cell-Based Assay Buffer prepared as described on page 8. Add the rinsed solution to the 50 ml conical tube.
3. Pipet 10 ml of Cell-Based Assay Neutrophil Isolation Histopaque to a different 50 ml conical tube. Slowly add 30 ml of the diluted blood on the top of Cell-Based Assay Neutrophil Isolation Histopaque.
4. Centrifuge at 500 x g for 20-30 minutes at 18-26°C.
5. Carefully aspirate the yellowish and clear top layers and leave the reddish pellet containing neutrophils and red blood cells in the tube.
6. Pipette 30 ml of the Cell-Based Assay Red Blood Cell Lysis Buffer into the tube. Vortex to ensure mixing of the cells with the Lysis Buffer. Rock the tube on a rocker for 10-15 minutes to lyse the red blood cells.
7. Centrifuge at 1,200 x rpm for 10 minutes to pellet the neutrophils.
8. Carefully aspirate the reddish supernatant.
9. Add 5 ml of RPMI containing 1% BSA to the tube and mix well.
10. Centrifuge at 1,200 x rpm for five minutes to pellet the neutrophils.
11. Repeat steps 9 and 10 one more time.
12. Resuspend the cells in 20 ml RPMI containing 1% BSA. Mix well to ensure sufficient separation of the cells. The cells are now ready to be seeded and should be sufficient for two 96-well cell culture plates at a density of 1×10^5 - 5×10^5 cells/well.

Treatment of Cells

The following protocol is designed for a 96-well plate. For other sizes of plates the volume of medium/solution to apply to each well should be adjusted accordingly.

1. Seed a 96-well plate with the cells prepared above at 100 μ l/well. Be sure to include two wells containing only culture medium for background controls.
2. Treat the cells with experimental compounds or vehicle for two to four hours, or for the period of time used in your typical experimental protocol, in a cell culture hood. To use the PMA (1 mM) Assay Reagent (Item No. 400145), dilute the PMA solution 1:10,000-1:50,000 into the culture medium. PMA at these concentrations causes a significant activation of neutrophil MPO release.
3. At the end of treatment, centrifuge the plate at 1,200 rpm for 10 minutes at 18-25°C.
4. The culture supernatant in each well is now ready for use in the MPO assay.

Plate Set Up

There is no specific pattern for using the wells on the plate. We suggest that two wells contain culture medium without cells to be designated as background wells. Each sample should be assayed at least in duplicate. We suggest you record the contents of each well on the template sheet provided (see page 18).

Pipetting Hints

- It is recommended that a repeating pipettor be used to deliver reagents to the wells. This saves time and helps 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.

Performing the Assay

Use the plates included in the kit to perform the assay described below.

1. **Positive Control Wells** - add 50 μl of positive control per well in the corresponding wells on the assay plate.
2. **Sample Wells** - Transfer 25 μl of culture supernatant from each well of the experimental plate to a corresponding well on the assay plate. Add 25 μl of the diluted Assay Buffer to each of the sample wells. The final volume for each sample well before addition of the substrate should be the same as that for the Positive Control Wells, which is 50 μl . Lesser amounts, for example 10 μl of culture supernatant, can be used when the concentration of myeloperoxidase released from stimulated cells is too high and has an absorbance >2 at 650 nm. If less sample is used, be certain to increase the amount of Assay Buffer accordingly.
3. **Sample Plus Inhibitor Wells** - Transfer 25 μl of culture supernatant from each well of the experimental plate to a corresponding well on the assay plate. Add 25 μl of the Cell-Based Assay Myeloperoxidase Inhibitor solution prepared above to each of the sample plus inhibitor wells.
4. **Addition of the MPO Substrate** - add 50 μl of TMB Substrate Solution to each well. Cover the plate with one of the plate covers included in the kit.
5. Incubate the plate at room temperature for 5-10 minutes.
6. Read the plate at absorbance 650 nm. *NOTE: If calculating enzyme activity using the optional method on page 14, measure the absorbance at one minute and five minutes after addition of the MPO substrate.*

Calculations (optional)

*The following is an optional calculation that can be performed to convert A_{650} to units of enzyme activity (Units/ml; $\mu\text{moles}/\text{min}/\text{ml}$). You may also choose to use the A_{650} value to compare enzyme activity between sample types (see Figure 1 on page 15).

1. Determine the change in absorbance (ΔA_{650}) per minute by reading the absorbance at one minute and five minutes after addition of the MPO Substrate. The change in absorbance during that time can be determined using the following equation:

$$\Delta A_{650} = \left[\frac{A_{650} (\text{Time 5 min.}) - A_{650} (\text{Time 1 min.})}{4 \text{ min.}} \right]$$

2. Use the formula below to determine the myeloperoxidase activity. The reaction rate at 650 nm can be determined using an extinction coefficient of $3.9 \times 10^4 \text{M}^{-1}\text{cm}^{-1}$.

Myeloperoxidase activity ($\mu\text{moles}/\text{min.}/\text{ml}$) =

$$\frac{\Delta A_{650}/\text{min.}}{(3.9 \times 10^4 \text{M}^{-1}\text{cm}^{-1}) \times 0.4^* \text{ cm}} \times \frac{0.1 \text{ ml}}{0.025^{**} \text{ ml}} \times \text{Sample dilution}$$

*Pathlength of sample in the well.

**This value corresponds to the amount of sample added to the well. Adjust this value as necessary if a sample volume other than 25 μl was used.

Performance Characteristics

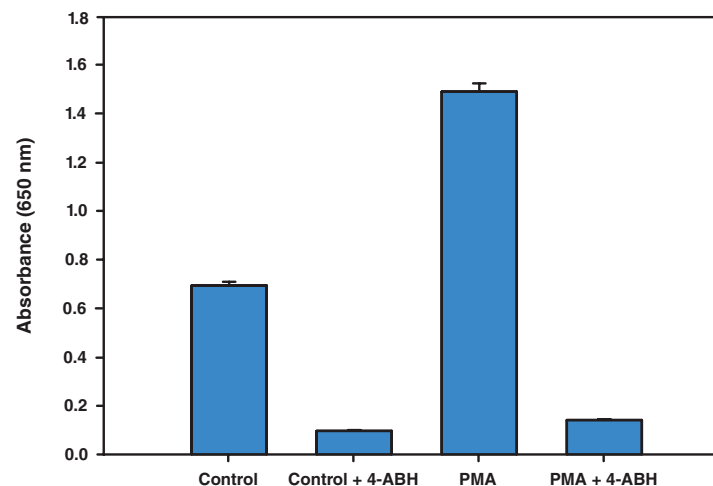


Figure 1. PMA stimulates neutrophil myeloperoxidase release from human neutrophils. Human neutrophils were isolated from freshly collected human blood according to the procedure described in the booklet. The cells were then plated in RPMI containing 1% BSA in a 96-well cell culture plate. Cells were treated with either vehicle or 25 nM PMA for 2.5 hours. The supernatant from each well (25 μl) was sampled and assayed for neutrophil myeloperoxidase according to the procedure described in the booklet.

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
Erratic response curve of compound treatments	Unequal number of cells in each well	Make sure each well contains the same number of cells
High level of myeloperoxidase in control samples	Lysis of red blood cells is too long	Reduce the incubation time for red blood cell lysis

Reference

1. Arnhold, J. and Flemmig, J. Human myeloperoxidase in innate and acquired immunity. *Arch. Biochem. Biophys.* **500(1)**, 92-106 (2010).
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Warranty and Limitation of Remedy

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