Product Manual

OxiSelect™ Myeloperoxidase Peroxidation Activity Assay Kit (Fluorometric)

Catalog Number

STA- 805 **192 assays**

FOR RESEARCH USE ONLY
Not for use in diagnostic procedures

Introduction

Myeloperoxidase (MPO) is a heme-based peroxidase enzyme responsible for antimicrobial activity against a wide range of organisms. MPO is found in neutrophils, monocytes, and some soft tissue macrophages. MPO is copiously expressed in stimulated neutrophil granulocytes, where it catalyzes the production of hypoalous acids, such as hypochlorous acid (HOCl), from hydrogen peroxide (H_2O_2) and chloride ion (Cl^-), or other halides. HOCl is the most powerful bactericidal compound produced by neutrophils and excessive production can lead to oxidative stress and tissue damage.

Myeloperoxidase has been implicated in many disease states including arthritis, cancer, renal dysfunction, and cystic fibrosis. While myeloperoxidase deficiency is a hereditary disease that results in immune deficiency, antibodies against MPO have been linked to various types of vasculitis. The reactive species it creates may also damage normal tissues, thereby contributing to inflammation. An association of elevated MPO levels and coronary artery disease has been demonstrated and it has been reported that the enzyme could serve as a predictor of myocardial infarction in certain patients. The numerous roles MPO plays in the innate immune response and disease states make it a target for potential therapies. MPO exhibits both chlorination and peroxidation activities (see Figure 1).

Cell Biolabs' OxiSelect™ Myeloperoxidase Peroxidation Activity Assay Kit is a quantitative fluorescence assay for measuring myeloperoxidase peroxidation activity within cell lysates, tissue samples, and purified enzyme samples. The assay is not applicable for use in serum samples. Myeloperoxidase activity is defined as follows: 1 unit of MPO enzyme is the amount of enzyme that will oxidize the MPO substrate to generate 1 µmole of fluorescein per minute at 25ºC. Each kit provides sufficient reagents to perform up to 192 assays, including standard curve and unknown samples.

Assay Principle

The OxiSelect™ Myeloperoxidase Peroxidation Activity Assay Kit is a quantitative fluorescence-based assay for measuring the myeloperoxidase peroxidation activity within a sample. Ferric, or native, MPO enzyme reacts with hydrogen peroxide (H_2O_2) to form the active redox intermediate compound MPO-I. Subsequent electron reduction reactions transition MPO-I back to native MPO via intermediate compound MPO-II. In the presence of MPO, hydrogen peroxide oxidizes the non-fluorescent probe, ADHP (10-Acetyl-3, 7-dihydroxyphenoxazine), to the highly fluorescent Resorufin (λ_{ex} = 530nm/λ_{em} = 590nm). The myeloperoxidase activity in unknown samples is determined by comparison with the predetermined resorufin standard curve.

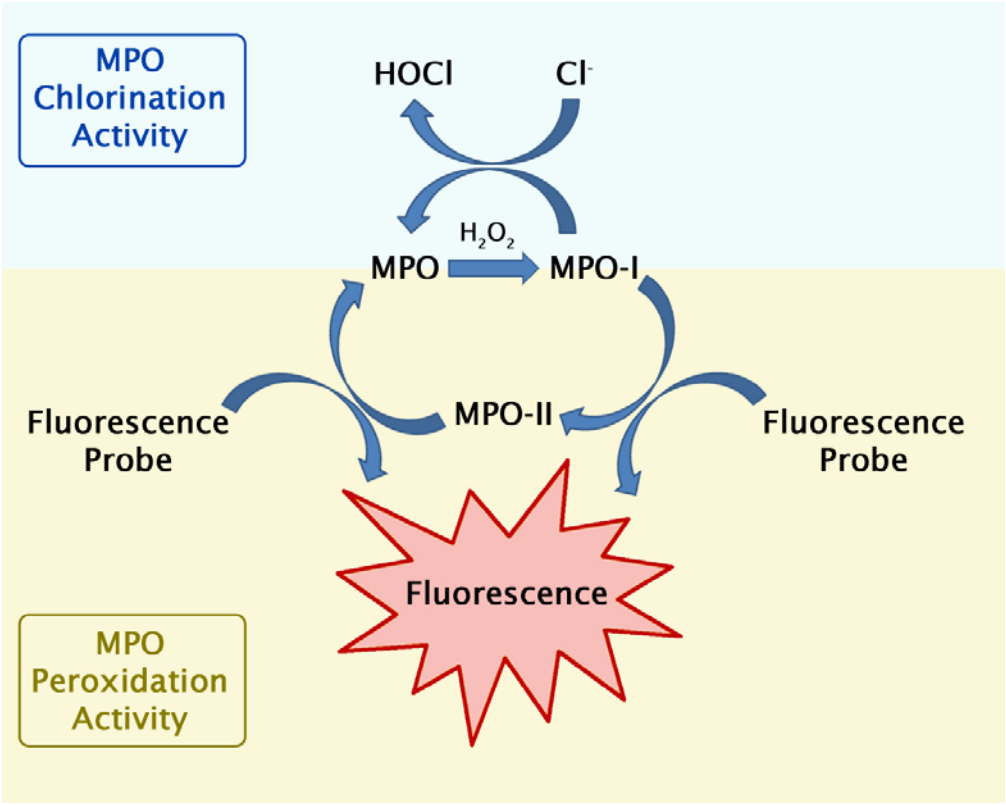

Figure 1. Chlorination and Peroxidation Activities of Myeloperoxidase (MPO). To measure the chlorination activity of myeloperoxidase, use one of the OxiSelect™ Myeloperoxidase Chlorination Activity Assays (Cat. #STA-803 or #STA-804).

Related Products

1. STA-312: OxiSelect™ Total Glutathione Assay Kit
2. STA-330: OxiSelect™ TBARS Assay Kit (MDA Quantitation)
3. STA-340: OxiSelect™ Superoxide Dismutase Activity Assay
4. STA-341: OxiSelect™ Catalase Activity Assay Kit
5. STA-342: OxiSelect™ Intracellular ROS Assay Kit (Green Fluorescence)
6. STA-347: OxiSelect™ *In Vitro* ROS/RNS Assay Kit (Green Fluorescence)
7. STA-802: OxiSelect™ *In Vitro* Nitric Oxide (Nitrite / Nitrate) Assay kit
8. STA-803: OxiSelect™ Myeloperoxidase Activity Assay Kit (Colorimetric)
9. STA-804: OxiSelect™ Myeloperoxidase Chlorination Activity Assay Kit (Fluorometric)
10. STA-832: OxiSelect™ MDA Competitive ELISA Kit

Kit Components

1. 96-well Microtiter Plate (Part No. 234501): Two 96-well clear bottom black plates.
2. Hydrogen Peroxide (Part No. 234102): One 100 µL amber vial of an 8.82 M solution
3. Fluorescence Probe (Part No. 237502): One 200 µL amber tube in DMSO
4. Assay Buffer (5X) (Part No. 280402): One 25 mL bottle

5. Resorufin Standard (Part No. 280501): One 50 µL amber tube of a 5 mM solution
6. HTAB Extraction Reagent (10X) (Part No. 280305): One 20 mL bottle

Materials Not Supplied

1. Distilled or deionized purified water
2. 1X PBS
3. 10 µL to 1000 µL adjustable single channel micropipettes with disposable tips
4. 50 µL to 300 µL adjustable multichannel micropipette with disposable tips
5. Conical tubes, microcentrifuge tubes, and bottles for sample and buffer preparation
6. Centrifuge and/or microfuge
7. Sonicator or tissue homogenizer
8. Multichannel micropipette reservoirs
9. Catalase Inhibitor: 3-amino-1,2,4-triazole (optional)
10. NEM: N-ethylmaleimide (optional)
11. Fluorescence microplate reader equipped with a 530-570 nm excitation filter and 590-600 nm emission filter

Storage

Upon receipt, store the Fluorescence Probe and the Resorufin Standard at -20ºC. The Fluorescence Probe is light sensitive and must be stored accordingly. Avoid multiple freeze/thaw cycles. Store the remaining kit components at 4ºC.

Preparation of Reagents

- 1X Assay Buffer: Prepare 1X Assay Buffer by adding deionized water to the 5X Assay Buffer (e.g. add 25 mL of 10X Assay Buffer to 100 mL of deionized water). Mix thoroughly until homogeneous. Use this buffer for preparing kit reagents. Store at 4ºC when not in use.
- Working Solution: Prepare this solution just before use and prepare only enough for immediate applications. The Working Solution is used to prepare the MPO Peroxidation Solution and the resorufin standards. First, dilute the provided 8.82 M Hydrogen Peroxide in 1X Assay Buffer to 5 mM. (e.g. add 4 µL of Hydrogen Peroxide stock to 7 mL 1X Assay Buffer). Vortex thoroughly. Next, dilute the 5 mM Hydrogen Peroxide Solution 1:250 in 1X Assay Buffer for a 20 µM final concentration. (e.g. add 40 µL of 5 mM Hydrogen Peroxide Solution to 9.960 mL 1X Assay Buffer). Vortex thoroughly.
- MPO Peroxidation Solution: Immediately prior to use, prepare by diluting the Fluorescence Probe 1:50 in Working Solution (e.g. Add 50 µL to 2.450 mL of Working Solution for 50 assays). Vortex thoroughly. Prepare only what is needed for immediate applications and do not store diluted solutions. Protect from light until needed.

  Note: The Fluorescence Probe is light sensitive and must be stored accordingly.

Preparation of Samples

These preparation protocols are intended as a guide for preparing unknown samples. Optimal experimental conditions for samples must be determined by the investigator. The user may need to

adjust the sample treatment accordingly. All samples should be assayed immediately or stored for up to 2 months at -80°C. The following recommendations are only guidelines and may be altered to optimize or complement the user's experimental design. A set of serial dilutions is recommended for samples to achieve optimal assay results and minimize possible interfering compounds. A trial assay with a representative test sample should be performed to determine the sample compatibility with the dynamic range of the standard curve. Samples may be diluted in 1X Assay Buffer or 1X PBS, pH 7.4, or concentrated with an Amicon centrifuge concentrator with a molecular weight cut-off of 30,000 as necessary before testing. High levels of interfering substances may cause variations in results. Run proper controls as necessary. Always run a standard curve with samples.

- Non-Neutrophil Cell Lysates: Collect cells (~1 x 10^7) by centrifugation at 1000-2000 x g for 10 minutes at 4ºC. For adherent cells, do not use proteolytic enzymes. Use a rubber policeman instead. Sonicate cell pellet in 0.5-1 mL ice-cold 1X PBS, pH 7.4 on ice. Centrifuge at 13,000 x g for 10 minutes at 4ºC. Collect the supernatant and store on ice if testing immediately or freeze at -80ºC for up to 1-2 months.
- Neutrophil Lysates: Collect whole blood in EDTA blood collection tubes. Isolate neutrophils using density-gradient centrifugation. Resuspend the neutrophil pellet in cold 100 mM Phosphate Buffer, pH 6.0, 1 mM EDTA containing 0.5% HTAB at 1-2 x 10^7 cells/mL. Stimulate myeloperoxidase activity with PMA or other stimulants (ref. 3). Lyse cells by sonication or multiple freeze-thaw cycles. Collect neutrophil extract in the supernatant following centrifugation at 12,000 rpm for 15 minutes at 4ºC.
- Tissue Lysates/Homogenates: Wash or perfuse tissues with cold sterile saline or cold 100 mM Phosphate Buffer, pH 6.0 prior to homogenization to eliminate MPO from blood. Homogenize approximately 100 mg of tissue in 1-2 mL cold 100 mM Phosphate Buffer, pH 6.0, containing 0.5% HTAB. Centrifuge the homogenate at 12,000 rpm for 15 minutes at 4ºC. Collect the supernatant. Store on ice if using immediately or freeze at -80ºC for up to 1-2 months.

  Notes:
  - *Samples with endogenous reductants, (eg. NADH concentrations above 10 µM and glutathione (GSH) concentrations above 50 µM), will interfere with the assay and result in erroneous readings. To minimize this interference, it is recommended that NEM (N-ethylmaleimide) be added at 10 mM final concentration to freshly prepared homogenates. NEM will block reductant interference and increase MPO yield.*
  - *Tissue homogenates with catalase activity may be blocked by adding the catalase inhibitor 3-amino-1,2,4-triazole at 20 mM final concentration for 60 minutes prior to performing the assay. For cells with catalase activity, incubate cells for 30 minutes at 37ºC with 20 mM final catalase inhibitor 3-amino-1,2,4-triazole. Wash cells prior to solubilizing.*
  - *Avoid samples containing DTT or β-mercaptoethanol since Resorufin is not stable in the presense of thiols (above 10 µM).*

Preparation of Standard Curve

Use microcentrifuge tubes to prepare a series of standards according to Table 1 below. Prepare standards immediately prior to each assay performed. Vortex tubes thoroughly. Do not store or reuse the resorufin standard preparations.

Standard Tubes	5 mM Resorufin Standard (µL)	1X Assay Buffer (µL)	Final Resorufin Concentration (nM)	Final Resorufin (pmol/well)*
1	5	4995	5000	250
2	250 of Tube #1	250	2500	125
3	250 of Tube #2	250	1250	62.5
4	250 of Tube #3	250	625	31.3
5	250 of Tube #4	250	313	15.6
6	250 of Tube #5	250	156	7.8
7	250 of Tube #6	250	78	3.9
8	250 of Tube #7	250	39	2
9	250 of Tube #8	250	20	1
10	0	250	0	0

Table 1. Preparation of resorufin standards.

**Based on 50 µL volume/well.*

Assay Protocol

I. Standard Curve Generation

Note: Resorufin standards should be prepared immediately prior to the assay.

1. Prepare and mix all reagents thoroughly before use. Standards should be assayed in duplicate or triplicate.
2. Add 50 µL of the resorufin standards to unused wells within the 96-well microtiter plate.
3. Add 50 µL of Working Solution to each standard well and mix briefly.
4. Read absorbance of each microwell on a fluorometer using 530-570 nm excitation filter and 590-600 nm emission filter

II. Myeloperoxidase Activity

Note: The assay is continuous, thereby allowing for readings at multiple time points. This may be necessary in order to ensure the values of unknowns fall within the linear range of the standard curve.

1. Add 50 µL of unknown samples or controls to the microtiter plate. Samples and controls should be assayed in duplicate or triplicate.
2. Add 50 µL of MPO Peroxidation Solution to each sample well to be read. Mix briefly.
3. Immediately read the fluorescence of each sample microwell on a fluorometer using 530-570 nm excitation filter and 590-600 nm emission filter. This is the initial time point plate reading ($T_{initial}$). Cover plate to protect from light and continue to incubate at room temperature for up to 30-60 minutes.

   Note: If measuring multiple time points, begin reading samples after adding the MPO Peroxidation Solution at every set time point (eg. 5 minutes) until complete. Continue taking measurements until the value of the most active sample is greater than the standard curve values. At this point, the most active sample value exceeds the end of the linear range of the standard curve. The final fluorescence value prior to exceeding the highest standard curve value within the linear range of the curve is the penultimate reading, and is used to determine MPO activity. It is important that the final measured value fall within the linear range of the standard curve.

4. Once the assay is complete, read the fluorescence of each microwell on a fluorometer using 530-570 nm excitation filter and 590-600 nm emission filter. This is the final time point plate reading (T_{final}).

Calculation of Results

Determine the average fluorescence values for every myeloperoxidase sample, control, and resorufin standard. Subtract the average zero standard value from itself and all standard and sample values. This is the background correction. Graph the standard curve (see Figure 2).

1. Calculate the change in sample fluorescence values (ΔF) between the initial fluorescence $(F_{initial})$ and the final fluorescence (F_{final}):

$$(\Delta F) = (F_{final}) - (F_{initial})$$

2. Compare the change in fluorescence (ΔF) of each sample to the resorufin standard curve to determine the amount of resorufin produced within the assay. Only use values within the linear range of the standard curve.
3. Determine the myeloperoxidase activity in microunits/mL (µU/mL) of a sample using the equation:

Q = Quantity (in pmole/well) of resorufin produced as determined from graph
T = Reaction time (in minutes) determined by $T_{final} - T_{initial}$

$$\text{MPO Activity (µU/mL)} = \left[\frac{Q}{T \times 0.050\ mL^*} \right]$$

**50 µL sample volume. Be sure to account for any dilution factors made on unknown samples prior to the assay.*

MPO activity is quantified as pmole/min/mL = microunit/mL (µU/mL)

Example Calculation

MPO Sample $F_{initial} = 60$

MPO Sample $F_{final} = 335$
Zero Resorufin Standard F = 10
Time = 30 minutes
Sample Volume = 0.050 mL

1. Subtract the zero standard fluorescence from MPO sample fluorescence:
   MPO Sample $F_{initial} = 60 – 10 = 50$
   MPO Sample $F_{final} = 335 – 10 = 325$

   Calculate the change in fluorescence (ΔF): 325 - 50 = 275

2. Using the equation of the trendline of the graphed standard curve, extrapolate the amount of pmoles consumed.
   (E.g. The calculated value using the standard curve in Figure 2 is Q = 120 pmoles)

3. Solve for MPO activity: $\left[\frac{(120 \text{ pmoles})}{(30 \text{ minutes})(0.050 \text{ mL})}\right] = 80 \ \mu\text{U/mL}$

Example of Results

The following figures demonstrate typical Myeloperoxidase Peroxidation Activity Assay results. One should use the data below for reference only. This data should not be used to interpret actual results.

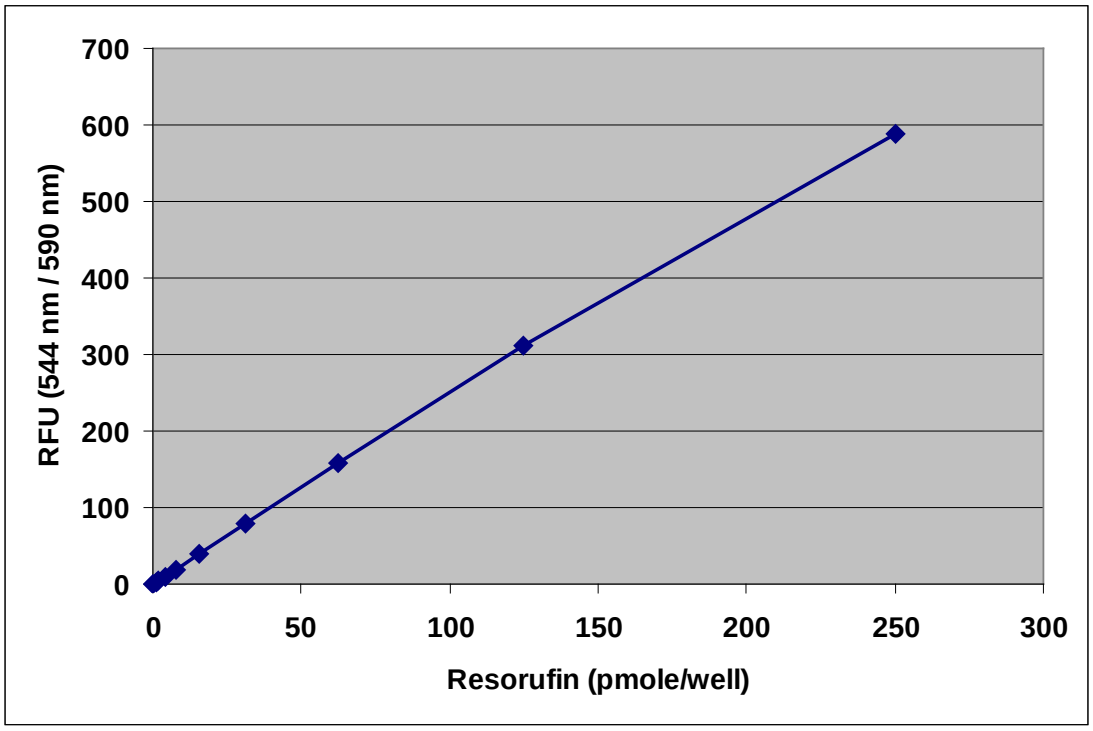

Figure 2. Resorufin Standard Curve.

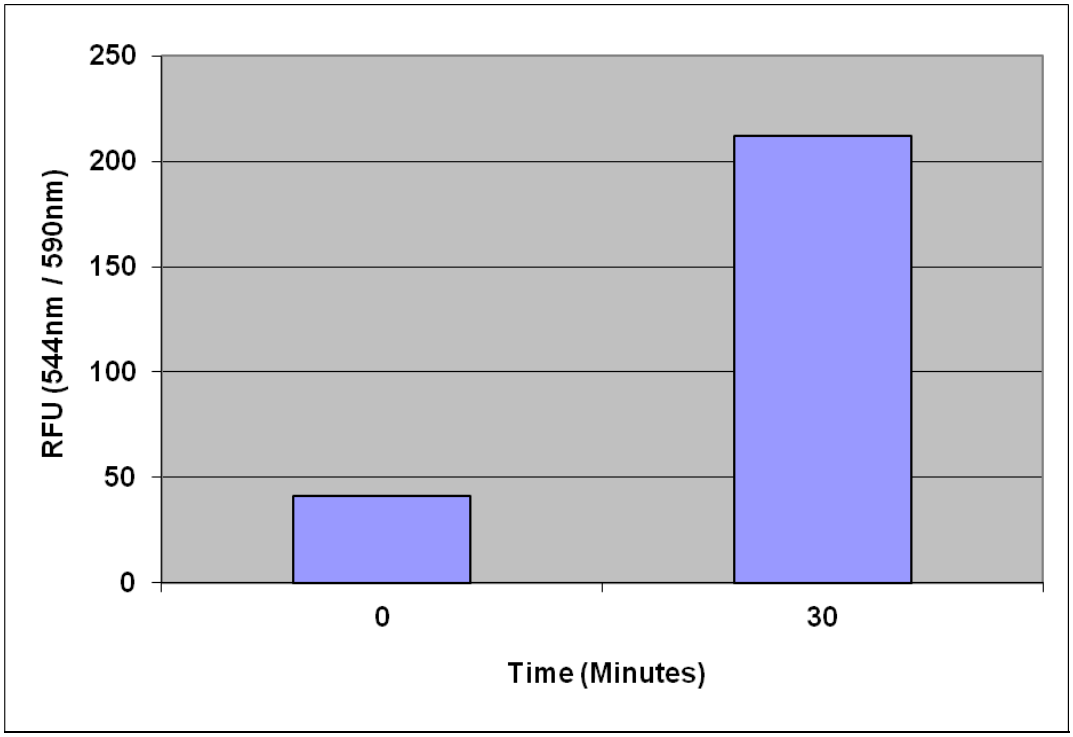

Figure 3: Purified Human MPO. 650 pM of purified human myeloperoxidase was tested according to the assay protocol. The activity was determined to be 42 µU/mL.

References

1. Hampton, M.B., et al. Blood (1998) 92: 3007-3017.
2. Nauseef, W. (2007) Isolation of Human Neutrophils From Venous Blood. In *Neutrophil Methods and Protocols* (Quinn M. T. et. al. eds.), Humana Press Totowa, NJ, 15-21.
3. Shih, J., et. al. *Clin.Chem.* (2008) 54: 1076-1079.
4. Tatyana, V., et al. *Neurochem.* (2001) 79: 266-277.

Warranty

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