ab133080 –
Myeloperoxidase (MPO)
Inhibitor Screening
Assay Kit

Instructions for Use

The Myeloperoxidase (MPO) Inhibitor Screening Assay provides convenient fluorescence-based methods for screening inhibitors to both the chlorination and peroxidation activities of MPO.

This product is for research use only and is not intended for diagnostic use.

Version 3 Last Updated 15 September 2017
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1. Overview

Abcam’s 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. 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, where a single two electron oxidation of native enzyme (MPO) to compound I (MPO-I) is followed by two successive one electron reductions back to native enzyme by compound II (MPO-II). 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.
2. Background

Myeloperoxidase (MPO) is a member of the heme peroxidase superfamily and is stored within the azurophilic granules of leukocytes. MPO is found within circulating neutrophils, monocytes, and some tissue macrophages. 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. Recently, evidence has emerged that MPO-derived oxidants contribute to tissue damage and the initiation and propagation of acute and chronic vascular inflammatory diseases. 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. 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.
For best results, remove components and store as stated below.

<table>
<thead>
<tr>
<th>Item</th>
<th>Quantity</th>
<th>Storage</th>
</tr>
</thead>
<tbody>
<tr>
<td>MPO Assay Buffer</td>
<td>1 vial</td>
<td>4°C</td>
</tr>
<tr>
<td>MPO Chlorination Substrate</td>
<td>1 vial</td>
<td>4°C</td>
</tr>
<tr>
<td>ADHP assay reagent</td>
<td>2 vials</td>
<td>-20°C</td>
</tr>
<tr>
<td>Myeloperoxidase Assay reagent</td>
<td>1 vial</td>
<td>-20°C</td>
</tr>
<tr>
<td>MPO Inhibitor</td>
<td>1 vial</td>
<td>4°C</td>
</tr>
<tr>
<td>MPO Hydrogen Peroxide</td>
<td>1 vial</td>
<td>4°C</td>
</tr>
<tr>
<td>MPO DMSO</td>
<td>1 vial</td>
<td>4°C</td>
</tr>
<tr>
<td>96-Well Solid Plate (black)</td>
<td>2 plates</td>
<td>RT</td>
</tr>
<tr>
<td>96-Well Cover Sheets</td>
<td>2 covers</td>
<td>RT</td>
</tr>
</tbody>
</table>
Materials Needed But Not Supplied

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

1. 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.

2. 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.

3. MPO Peroxidation Substrate

Immediately prior to preparing the Peroxidation Working Solution, add 120 μl of MPO DMSO to one vial of ADHP Assay reagent and vortex until dissolved. Then add 470 μl of MPO Assay buffer for a final MPO Peroxidation substrate concentration of 1 mM. This is enough Substrate to assay 100 wells. Prepare additional vials as needed. The reconstituted Substrate is stable for 15 minutes. After 30 minutes, increased background fluorescence will occur.

4. 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.

5. MPO Inhibitor

The vial contains 300 μl of 50 mM 4-aminobenzhydrazide, a MPO inhibitor. 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.

6. 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.

7. MPO DMSO

The vial contains 1 ml of dimethylsulfoxide (DMSO). The reagent is ready to use as supplied.
5. Assay Protocol

A. Plate Setup

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. A typical layout of samples to be measured in duplicate is shown in Figure 2.

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 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.

B. Performing the Chlorination Assay

1. In a suitable tube, prepare the Chlorination Working Solution according to the table below. The solution will turn yellow.

<table>
<thead>
<tr>
<th>Component</th>
<th>50 wells</th>
<th>100 wells</th>
</tr>
</thead>
<tbody>
<tr>
<td>Assay Buffer</td>
<td>2.44 µl</td>
<td>4.88 µl</td>
</tr>
<tr>
<td>Chlorination Substrate (2.5 mM)</td>
<td>40 µl</td>
<td>80 µl</td>
</tr>
<tr>
<td>Hydrogen Peroxide (5 mM)</td>
<td>20 µl</td>
<td>40 µl</td>
</tr>
</tbody>
</table>

Table 1. Chlorination Working Solution preparation

2. **100% Initial Activity Wells** - add 10 µl of Assay Buffer and 50 µl of 250 ng/ml MPO to three wells.

3. **Background Wells** - add 60 µl of Assay Buffer to three wells.
4. **Sample (inhibitor) Wells** - add 10 μl of sample (inhibitor) and 50 μl of 250 ng/ml MPO to three wells.

5. Initiate the reactions by quickly adding 50 μl 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.

Sample (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 μl. 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.
C. Performing the Peroxidation Assay

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

<table>
<thead>
<tr>
<th>Component</th>
<th>50 wells</th>
<th>100 wells</th>
</tr>
</thead>
<tbody>
<tr>
<td>Assay Buffer</td>
<td>2.24 µl</td>
<td>4.48 µl</td>
</tr>
<tr>
<td>Peroxidation Substrate (1 mM)</td>
<td>250 µl</td>
<td>500 µl</td>
</tr>
<tr>
<td>Hydrogen Peroxide (5 mM)</td>
<td>10 µl</td>
<td>20 µl</td>
</tr>
</tbody>
</table>

Table 2. Peroxidation Working Solution preparation

2. **100% Initial Activity Wells** - add 10 µl of Assay Buffer and 50 µl of 250 ng/ml MPO to three wells.

3. **Background Wells** - add 60 µl of Assay Buffer to three wells.

4. **Sample (inhibitor) Wells** - add 10 µl of sample (inhibitor) and 50 µl of 250 ng/ml MPO to three wells.

5. Initiate the reactions by quickly adding 50 µl 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.

Sample (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 μl. 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.
6. Data Analysis

A. 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\textsubscript{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 ($IC_{50} = 0.4$ μM).
## 7. Troubleshooting

<table>
<thead>
<tr>
<th>Problem</th>
<th>Possible Causes</th>
<th>Recommended Solutions</th>
</tr>
</thead>
<tbody>
<tr>
<td>Erratic values; dispersion of duplicates/</td>
<td>A. Poor pipetting/technique</td>
<td>A. Be careful not to splash the contents of the wells</td>
</tr>
<tr>
<td>triplicates</td>
<td>B. Bubble in the well(s)</td>
<td>B. Carefully tap the side of the plate with your finger to</td>
</tr>
<tr>
<td></td>
<td></td>
<td>remove bubbles</td>
</tr>
<tr>
<td>No fluorescence above background is seen in</td>
<td>Inhibitor concentration is too</td>
<td>Reduce the concentration of the inhibitor and re-assay</td>
</tr>
<tr>
<td>the inhibitor wells</td>
<td>high and inhibited all of the</td>
<td></td>
</tr>
<tr>
<td></td>
<td>enzyme activity</td>
<td></td>
</tr>
<tr>
<td>The fluorometer exhibited ‘MAX’ values for</td>
<td>The GAIN setting is too high</td>
<td>Reduce the GAIN and re-read</td>
</tr>
<tr>
<td>the wells</td>
<td></td>
<td></td>
</tr>
<tr>
<td>No inhibition was seen with the MPO</td>
<td>A. The inhibitor concentration</td>
<td>Increase the inhibitor concentration and re-assay</td>
</tr>
<tr>
<td>inhibitor</td>
<td>is not high enough</td>
<td></td>
</tr>
<tr>
<td></td>
<td>B. The compound is not an</td>
<td></td>
</tr>
<tr>
<td></td>
<td>inhibitor of the enzyme</td>
<td></td>
</tr>
</tbody>
</table>
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