

Myeloperoxidase Chlorination/Peroxidation Detection Kit

Contact Information

Address Cell Technology Inc
950 Rengstorff Ave
Suite D
Mountain View, CA 94043
USA

Telephone 650-960-2170
Toll Free 888 7ASSAYS (727-7297)
Fax 650-960-0367

General Information info@celltechnology.com
Sales sales@celltechnology.com
Technical Questions techsupport@celltechnology.com
Website www.celltechnology.com
Revised protocol 5/06

Notes

November 13th 2007: Lot # KAN21007 of FLMPO 100-3: Part 6015 : Store between +4-8 ° C .

Please contact techsupport@celltechnology.com or call us at 650-960-2170 should you have any questions



CELL TECHNOLOGY

Introduction

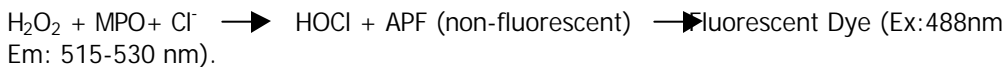
Myeloperoxidase (MPO) is a highly cationic glycosylated hemoprotein that has a molecular weight of 144kD. The hemoprotein consists of two dimers linked via a disulfide bridge. Each dimer is composed of a heavy (53kD) and light (15kD) subunit. Each heavy chain contains an independently acting protoporphyrin group containing a central iron⁽¹⁻⁵⁾. MPO is present in the azurophilic granules of polymorphonuclear leukocytes (PMNs) and is unique to neutrophils and monocytes. However, monocytes contain only one third of the MPO found in PMN's. MPO utilizes H₂O₂ produced by the neutrophils to oxidize a variety of aromatic compounds to give substrate radicals for bactericidal activity^(4 review). This enzyme is unique however in that it can oxidize chloride ions to produce a strong non-radical oxidant, HOCl. HOCl is the most powerful bactericidal produced by neutrophils^(4 review). Excessive production of these radicals can cause oxidative stress leading to oxidative tissue injury.

Applications:

- Detection of MPO chlorination activity in neutrophils and macrophages.
- Detection of PMN infiltration in tissue samples (inflammation and innate host defense mechanisms).
- Acute and chronic inflammatory disorders due to chlorination tissue damage.
- MPO chlorination/oxidation activity in acute and chronic manifestations of cardiovascular disease.

I. Chlorination Assay Principle:

The chlorination activity protocol utilizes a non-fluorescent detection reagent, APF, which is converted to its fluorescent analog in the presence of HOCl. Chlorination Reaction⁽¹⁵⁾:



II. Kits Supplied:

Cat # MPOHOCL 100-3 contains

1. Fluoro MPO Kit: Cell Technology Catalog # FLMPO100-3 (500 Assays)
2. hROS Kit: Cell Technology Catalog # FLAPF100-2 (290 Assays).

There is sufficient reagent for 500 peroxidation assays, and 290 chlorination assays.

III. Storage:

1. Kit Storage: 4-8°C and away from light.

IV. Warnings and Precautions:

1. For Research use only. Not for use in diagnostic procedures.
2. Practice safe laboratory procedures by wearing protective clothing and eyewear.
3. The fluorescent product of the detection reagent is not stable in the presence of thiols (DTT or 2-mercaptoethanol). Keep these reactants below 10µM. If you are using your own buffer, keep the reaction between pH 7.0-8.0 (optimal pH 7.4).
4. NADH and glutathione (reduced form: GSH) may interfere with the assay.

Materials required but not supplied:

1. Dimethyl sulfoxide (DMSO)
2. Black 96-well plates (clear bottom optional for bottom reading instruments).
3. Fluorescence plate reader
4. Deionized water

V. Preparation of reagent working solutions:

Fluoro MPO Kit : Catalog # FLMPO100-3

1. **Part # 3002. 10X Assay Buffer.** Make a 1X working solution of the assay buffer. For example dilute 4ml of 10X Assay buffer in 36ml of deionized water to make 1X Assay buffer. The 1X Assay buffer should be stored at 2-8⁰C up to 6 months.
2. **Part # 3012. Hydrogen Peroxide .** Make a 5mM H₂O₂ solution by diluting the stock H₂O₂ 1:176 in 1X Assay Buffer. This can be accomplished by adding 5.68 μL stock H₂O₂ to 994 μL 1X Assay buffer. Make enough H₂O₂ for one days work, discard remaining solution.
3. **Part # 4007. Detection reagent .** Not needed for chlorination assay.
4. **Part# 6015. Myeloperoxidase.** 1 vial. See vial label for units of activity and concentration. MPO enzyme should be diluted in 1X Assay buffer to construct a standard curve (see below Section VIII Assay Protocol).

hROS Chlorination Detection Kit – Cat # FLAPF100-2

1. The APF vial in solution at 5mM in DMF. Ready to use.

VI. Tissue Preparation

1. Tissue preparation: Prior to tissue extraction exsanguinate the animal to remove red blood cells from tissue. Homogenize tissue using standard techniques. Homogenization Buffer can be prepared as indicated below:
Dilute the 10X assay buffer 1:10 with distilled water to make a 1X solution.
Optional: Add NEM to the 1X buffer make a final concentration of 10mM NEM.
3. Centrifuge the homogenates at 8000 – 12000 g at 4⁰C for 15-20 minutes and remove the supernatant.
4. To the proteinaceous pellet add 1mL of solubilization buffer. Solubilization buffer can be prepared by making a 0.5% HTA-Br (w/v) solution in 1X assay buffer.
5. The samples are then homogenized and sonicated using the Sonic Dismembrator (Fisher Scientific, Pittsburgh) for 30 seconds and submitted to two cycles of freezing and thawing. Finally, the samples are centrifuged at 8000 X g for 20 minutes at 4^o C. The supernatant can be assayed or frozen at –70⁰C for later use.

VII. Cell Preparation.

1. Collect blood in heparin tubes and isolate PMN using standard techniques.
2. After PMN isolation, wash the PMS in PBS.
2. After the final wash, decant the supernatant and solubilize the cells in 1mL of ice cold 0.5% HTA-Br (w/v) (in 1X assay buffer).
3. Supernatants are ready to assay for MPO activity. It maybe necessary to further dilute the supernatants to optimize the assay.

VIII. Chlorination Assay Protocol:

1. Prepare 5ml **chlorination reaction cocktail** (for 100 assays) as follows:
12 μ L 5mM H_2O_2 (From above: V. Preparation of reagent working solutions step2).
12 μ L of 5mM APF (From above: V. Preparation of reagent working solutions step1).
4.976 ml of 1X assay buffer (From above: V. Preparation of reagent working solutions step 1).
2. Prepare standard curve of MPO by serially diluting in 1X assay buffer.
Refer to the MPO vial for units of activity and concentration. A sample standard curve is shown below.

MPO mU/mL	RFU
125	7015
62.5	7247.5
31.25	7166.5
15.625	4857.25
7.8125	2126.75
3.90625	745.25
1.953125	242.25
0.976563	56
0.488281	8.25

3. Pipette into a black opaque 96 well plate: 50uL of sample or standard curve and 50 uL of **chlorination reaction cocktail**.
4. Incubate at room temperature in the dark for 15-30 minutes.
Note: Each investigator should optimize the incubation time for their particular assay. Multiple readings may result in photo bleaching of the dye.
5. Measure the fluorescence at excitation: 488nm and emission at 515-530 nm in a fluorescent plate reader.

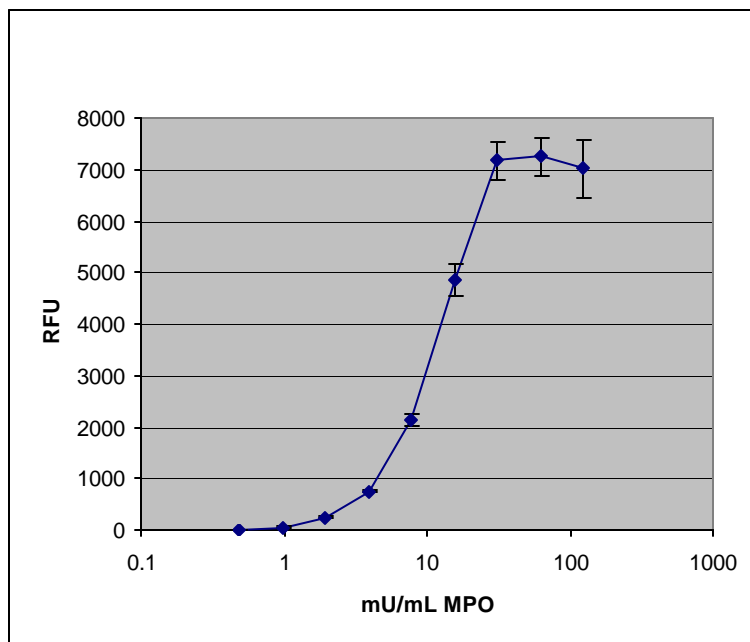
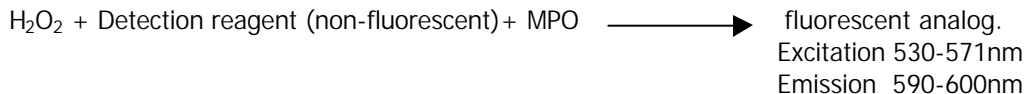


Figure 1. MPO standard was serially diluted in 1X reaction buffer. 50 μ L of the MPO standard and 50 μ L of the chlorination reaction cocktail was added to black clear bottom 96 well plates. The plate was incubated in the dark for 20 minutes and then read in a fluorescent plate reader (Ex: 488 nm Em: 515 nm).

I. Peroxidation Assay Principle:

The Fluoro MPO detection kit utilizes a non-fluorescent detection reagent, which is oxidized in the presence of hydrogen peroxide and MPO to produce its fluorescent analog ⁽⁶⁻¹³⁾

Reaction:



II. Storage:

1. Kit Storage: 4-8°C and away from light.
2. Once a vial of the Detection reagent is opened, it should be used promptly since it is subject to oxidation by air.

III. Warnings and Precautions:

1. For Research use only. Not for use in diagnostic procedures.
2. Practice safe laboratory procedures by wearing protective clothing and eyewear.
3. The fluorescent product of the detection reagent is not stable in the presence of thiols (DTT or 2-mercaptoethanol). Keep these reactants below 10 μ M. If you are using your own buffer, keep the reaction between pH 7.0-8.0 (optimal pH 7.4).
4. NADH and glutathione (reduced form: GSH) may interfere with the assay. See Technical note #5.

IV. Part # 5016. Kit contents (for 500 assays):

1. **Part # 3002. 10X Assay Buffer:** 60 mL
2. **Part # 4007. Detection reagent:** one dried vial for 500 assays.
3. **Part # 3012. Hydrogen Peroxide:** 1000 μ L of a stabilized 3% solution.
4. **Part# 6015. Myeloperoxidase:** 1 vial. See vial label for units of activity and concentration.

Materials required but not supplied:

5. Dimethyl sulfoxide (DMSO)
6. Ethanol
7. Black 96-well plates (clear bottom optional for bottom reading instruments).
8. Fluorescence plate reader
9. Deionized water
10. NEM : *N*-Ethylmaleimide (Sigma Cat# E1271)
11. HTA-Br: hexadecyltrimethylammonium (Sigma Cat# H9151).
12. Catalase Inhibitor: 3-Amino-1,2,4-triazole (Sigma Cat# A8056).
13. Eosinophil peroxidase inhibitor (Sigma Cat# R5645).

V. Preparation of reagent working solutions:

5. **Part # 3002. 10X Assay Buffer.** Make a 1X working solution of the assay buffer. For example dilute 4ml of 10X Assay buffer in 36ml of deionized water to make 1X Assay buffer. The 1X Assay buffer should be stored at 2-8⁰C up to 6 months.
6. **Part # 3012. Hydrogen Peroxide .** Make a 20mM H₂O₂: To 977 μ L of 1X Assay buffer, add 22.7 μ L of the 3% H₂O₂ (0.88M). Once diluted, the H₂O₂ should be used promptly as it degrades rapidly. Make enough H₂O₂ for one days work, discard remaining solution.
7. **Part # 4007. Detection reagent.** Make a 10mM stock solution of the Detection Reagent: Dissolve the contents of the vial in 500 μ L of DMSO. Once opened, should be used promptly and any remaining reagent should be aliquoted and refrozen at -70⁰C. Avoid repeated freeze thaw cycle.
8. **Part# 6015. Myeloperoxidase.** See vial label for units of activity and concentration. MPO enzyme should be diluted in 1X Assay buffer to construct a standard curve (see below Section VIII Assay Protocol).

VI. Tissue Preparation

Note: It is important to read the technical note section before you proceed with the setup and experiment

4. Tissue preparation: Prior to tissue extraction exsanguinate the animal to remove red blood cells from tissue. Homogenize tissue using standard techniques. Homogenization Buffer can be prepared as indicated below:
Dilute the 10X assay buffer 1:10 with distilled water to make a 1X solution.
Optional: Add NEM to the 1X buffer make a final concentration of 10mM NEM.

Note: See Technical note #1.

6. Centrifuge the homogenates at 8000 – 12000 g at 4^oC for 15-20 minutes and remove the supernatant.
7. To the proteinaceous pellet add 1mL of solubilization buffer. Solubilization buffer can be prepared by making a 0.5% HTA-Br (w/v) solution in 1X assay buffer.
8. The samples are then homogenized and sonicated using the Sonic Dismembrator (Fisher Scientific, Pittsburgh) for 30 seconds and submitted to two cycles of freezing and thawing. Finally, the samples are centrifuged at 8000 X g for 20 minutes at 4^o C. The supernatant can be assayed or frozen at –70^oC for later use.

Note: See Technical note #3.

VII. Cell Preparation.

Note: See Technical note #1 and 2.

3. Collect blood in heparin tubes and isolate PMN using standard techniques.
4. After PMN isolation, wash the PMS in PBS.
5. After the final wash, decant the supernatant and solubilize the cells in 1mL of ice cold 0.5% HTA-Br (w/v) (in 1X assay buffer).
6. Supernatants are ready to assay for MPO activity. It maybe necessary to further dilute the supernatants to optimize the assay.

VIII. Assay Protocol:

Note: Read technical note 6

6. Prepare 5ml **reaction cocktail** (for 100 assays) as follows:
50 µL of Detection Reagent (50 µM final)
5µL of 20mM Hydrogen peroxide
4.875ml of 1X assay buffer
Eosinophil peroxidase inhibitor at 0.1-1.0 µM (**optional see technical note 6**).
7. Prepare standard curve of MPO by serially diluting in 1X assay buffer. Refer to the MPO vial for units of activity and concentration. If you are adding the catalase inhibitor to your samples use the right column (MPO Standard with Catalase Inhibitor) as a guide to construct the standard curve.

MPO Standard without Catalase Inhibitor	MPO Standard with Catalase Inhibitor
0.0625 Units/mL	0.25 Units/mL
0.03125 Units/mL	0.125 Units/mL
0.0156 Units/mL	0.0625 Units/mL
0.00781 Units/mL	0.03125 Units/mL
0.00390 Units/mL	0.0156 Units/mL
0.00195 Units/mL	0.00781 Units/mL
0 Units/mL	0 Units/mL

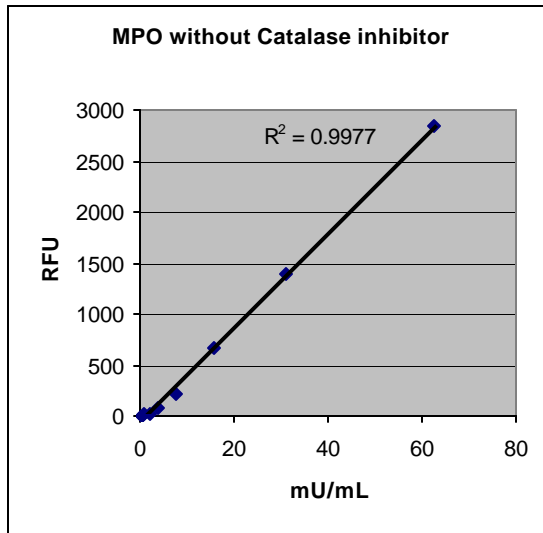
NOTE: When using 20mM catalase inhibitor 3-Amino-1,2,4-triazole in your samples, make the 1X assay buffer used to construct the MPO standard curve with 20mM 3-Amino-1,2,4-triazole. 3-Amino-1,2,4-triazole tends to slightly inhibit MPO activity. See Technical note 5.

8. Pipette into a black opaque 96 well plate: 50uL of sample or standard curve and 50 uL of **reaction cocktail**.
9. Incubate at room temperature in the dark for 30-60 minutes.
10. Measure the fluorescence at excitation: 530-570nm and emission at 590-600nm in a fluorescent plate reader.

IX. Technical Notes:

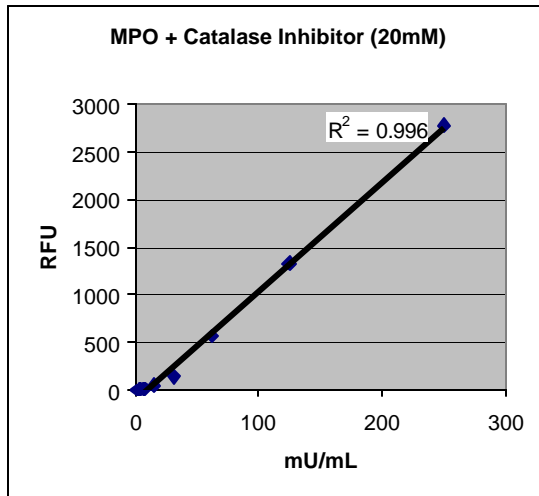
1. Tissue or cells that contain endogenous reductants (Glutathione: GSH) will interfere with the assay. Addition of NEM will block reductant interference and increase MPO yield.
2. If catalase activity is present in the cells, block by incubating cells, prior to solubilizing cells, with 20mM 3-Amino-1,2,4-triazole (Sigma Cat# A8056). Incubate cells with inhibitor for 30 minutes at 37°C and wash cells to remove excess inhibitor.
3. If catalase activity is present in the homogenates block by incubating homogenates with 20mM 3-Amino-1,2,4-triazole (Sigma Cat# A8056) for 60 minutes prior to running assay.
4. At NADH levels above 10µM, detection reagent oxidation results from side chain reaction between NADH and HRP. This could result in aberrant readings. To minimize this interference it is recommended to add superoxide dismutase (SOD) at 40U/mL to the reaction⁸.
At glutathione (reduced form GSH) above 300µM, detection reagent oxidation results from side chain reaction between GSH and HRP. This could result in aberrant readings. To minimize this interference it is recommended to add superoxide dismutase (SOD) at 40U/mL to the reaction⁸.
5. The catalase inhibitor should be used at a final concentration of 20mM. If using catalase inhibitor also add it to your MPO standard curve. This can be accomplished by adding the inhibitor, at a concentration of 40mM (final will be 20mM in the well) to the 1X Assay Buffer and then constructing your MPO standard curve. Note that the sensitivity will be lower and it is recommended to start your curve at a higher concentration (see below Fig.1 B).
6. If needed add the Eosinophil peroxidase inhibitor to a final concentration in the well of 0.1 to 1.0 µM. Make the inhibitor at double the concentration in the reaction cocktail (**VIII. Assay Protocol: Step 1.**) as this will be diluted 1:2 in the well. High concentrations of EPO inhibitor will slightly inhibit MPO activity. Use Eosinophil peroxidase inhibitor between 0.1 to 1.0 µM final concentration⁽¹⁴⁾.

A.



mU/mL	MPO RFU
62.5	2838
31.25	1401
15.625	668
7.8125	207
3.90625	71
1.953125	27
0.976563	14
0.488281	6
0.244141	4

B.



mU/mL	MPO +Inhib RFU
250	2783
125	1328
62.5	567
31.25	143
15.625	46
7.8125	12
3.90625	6
1.953125	2

Figure 1. MPO standard curve was serially diluted in 1X Assay buffer. Reaction cocktail (RC) was prepared as described **A**: without catalase inhibitor and **B**: with 20mM (final concentration) catalase inhibitor. Next 50 μ L of MPO standard and 50 μ L of RC was added to individual well of a 96 well black plates. The plate was incubated at room temperature in the dark for 30 minutes. Next the wells were read using Ex: 530nm and Em: 590nm. There is approximately 50% reduction in signal in the presence of 20mM catalase inhibitor.

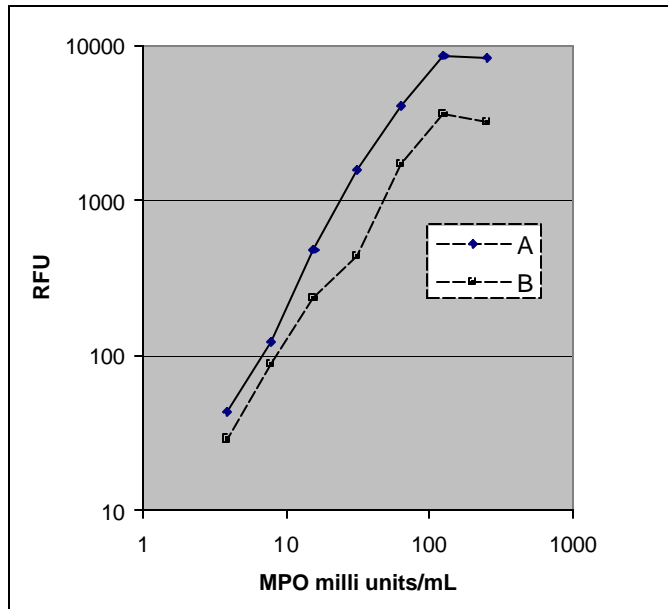


Figure 2. Reaction cocktail (RC) was prepared as described **A**: without EPO inhibitor and **B** with EPO inhibitor (100 μ M final). Next 50 μ L of MPO standard and 50 μ L of RC was added to individual well of a 96 well black plate. The plate was incubated at room temperature in the dark for 30 minutes. Next the wells were read using Ex: 530nm and Em: 590nm. There is approximately 50% reduction in signal in the presence of 100 μ M EPO inhibitor.

References:

- Olsen, R. L. & Little, c. (1983) *Biochem. J.* **209**, 781-787.
- Nauseef, W. M., and Malech, H. L. (1986) *Blood*, **67**, 1504-1507.
- Andrews, P. C., Parnes, C., and Krinsky, N. I. (1984) *Arch. Biochem. Biophys.*, **228**, 439-442.
- Mark B. Hampton, Anthony J. Kettle, and Christine C. Winterbourn. Inside the Neutrophil Phagosome: Oxidants, Myeloperoxidase, and Bacterial Killing. *Blood*, Vol. 92 No. 9 (November 1), 1998: pp. 3007-3017.
- Andrews, P.C., Parnes, C. & Krinsky, N.I. (1984) Comparison of myeloperoxidase and hemi-myeloperoxidase with respect to catalysis, regulation, and bacterial activity. *Arch. Biochem. Biophys.* 228, 439-442.
- Mingjie Zhou, Zhenjun Diwu, Nataliya Panchuk-Voloshina and Richard P. Haugland. A Stable Nonfluorescent Derivative of Resorufin for the Fluorometric Determination of Trace Hydrogen Peroxide: Applications in Detecting the Activity of Phagocyte NADPH Oxidase and Other Oxidases. *Anal Biochem* 253, 162 (1997).
- J. G. Mohanty, Jonathan S. Jaffe, Edward S. Schulman and Donald G. Raible. A highly sensitive fluorescent micro-assay of H₂O₂ release from activated human leukocytes using a dihydroxyphenoxazine derivative. *J. Immunol Methods* 202, 133 (1997).
- Tatyana V. Votyakova and Ian J. Reynolds. Membrane Potential dependent and -independent production of reactive oxygen species by rat brain mitochondria. *J Neurochem* 79, 266 (2001).
- Chun Song, Abu B. Al-Mehdi, and Aron B. Fisher. An immediate endothelial cell signaling response to lung ischemia. *Am J Physiol Lung Cell Mol Physiol* 281, L993 (2001).
- Samantha C. Richer and W.C.L. Ford. A critical investigation of NADPH oxidase activity in human spermatozoa. *Mol Hum Reprod* 7, 237 (2001).
- William G. Gutheil, Miglena E. Stefanova and Robert A. Nicholas. Fluorescent Coupled Enzyme Assays for Γ -Alanine: Application to Penicillin-Binding Protein and Vancomycin Activity Assays. *Anal Biochem* 287, 196 (2000).
- Dominik Peus, Remus A. Vasa, Astrid Beyerle, Alexander Meves, Carsten Krautmacher and Mark R. Pittelkow. UVB Activates ERK1/2 and p38 Signaling Pathways via Reactive Oxygen Species in Cultured Keratinocytes. *J Invest Dermatol* 112, 751 (1999).
- Tatyana V. Votyakova, Ian J. Reynolds. Detection of hydrogen peroxide with Amplex Red: interference by NADH and reduced glutathione auto-oxidation. *Archives of Biochemistry and Biophysics*, 431: 138-144 (2004).

14. Elizabeth Forbes, Tosei Murase, Ming Yang, Klaus I. Matthaei, *James J. Lee, Nancy A. Lee, Paul S. Foster, and Simon P. Hogan. Immunopathogenesis of Experimental Ulcerative Colitis Is Mediated by Eosinophil Peroxidase. *The Journal of Immunology*, 2004, 172:5664–5675.

15. Ken-ichi Setsukinai, Yasuteru Urano, Katsuko Kakinuma, Hideyuki J. Majima , and Tetsuo Nagano. Development of Novel Fluorescence Probes That Can Reliably Detect Reactive Oxygen Species and Distinguish Specific Species. *THE JOURNAL OF BIOLOGICAL CHEMISTRY* Vol. 278, No. 5, Issue of January 31, pp. 3170–3175, 2003