

Fluoro EPO

Eosinophil peroxidase Detection Kit

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Notes



CELL TECHNOLOGY

Introduction

Eosinophil peroxidase (EPO) is the most abundant enzyme found in eosinophils. It is the major cytotoxic agent released by activated eosinophils and uses hydrogen peroxide to generate reactive oxidants from halides and pseudo halide thiocyanate [1,2]. Eosinophils peroxidase has been shown to have antimycobacterial activity [3], however it is also implicated in tissue damage that occurs in asthma and other diseases [4,5]. Currently, the function of eosinophil involvement in the immune response is being redefined. Once considered a cell involved in host protection of parasitic infection, eosinophils multiple functions as leukocytes involved in the initiation and propagation of diverse inflammatory responses is being investigated. Eosinophils are further involved as modulators of innate and adaptive immunity [6].

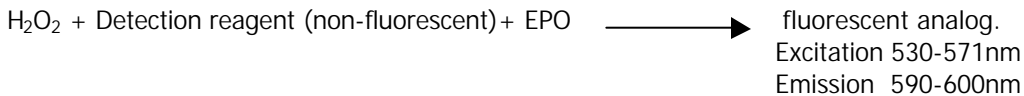
***Note: We do not recommend using this kit for eosinophil tissue infiltration experiments unless eosinophils are specifically isolated from tissue digests. This assay is subject to interference by myeloperoxidase (neutrophil) contamination in lysates from tissue infiltration experiments. See technical note 2.**

Applications:

- Readings: Fluorescence or absorbance
- Can monitor multiple time points to follow kinetics.
- One step no wash assay.
- Adaptable for High Throughput format.
- Detection of degranulation from isolated eosinophils.
- Sensitive

I. Assay Principle:

The Fluoro EPO detection kit utilizes a non-fluorescent Detection reagent, which is oxidized in the presence of hydrogen peroxide and EPO to produce its fluorescent analog ⁽⁷⁻¹³⁾
Reaction:



II. Storage:

1. Short term (several weeks): 4-8°C and away from light.
2. Long term: see individual components.
3. 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. **We do not recommend using this kit for eosinophil tissue infiltration experiments (unless eosinophils can be isolated from tissue digests) as myeloperoxidase (neutrophil) contamination will interfere with EPO measurement.**
2. **For Research use only. Not for use in diagnostic procedures.**
3. 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.
See Technical note #1.

IV. Part # 5016. Kit contents (for 100 assays):

1. **Part # 3002. 10 X Assay Buffer:** 60 mL
2. **Part # 4016. Detection reagent:** one dried vial for 100 assays.
3. **Part # 3012. Hydrogen Peroxide:** 1000 μ L of a stabilized 3% solution.
4. **Part# 6016. Eosinophil peroxidase:** 100 μ L 1 vial at 10 units/mL.

Materials required but not supplied:

1. Dimethyl sulfoxide (DMSO)
2. Ethanol
3. Black 96-well plates (clear bottom optional for bottom reading instruments).
4. Fluorescence plate reader
5. Deionized water
6. HTA-Br: hexadecyltrimethylammonium (Sigma Cat# H9151).

V. Preparation of reagent working solutions:

1. **Part # 3002. 10 X Assay Buffer.** Make a 1X working solution of the **Assay Buffer**. For example dilute 5mL of 10 X Assay Buffer in 45mL of deionized water. The 1 X Assay Buffer should be stored at 2-8^oC for up to 6 months.
2. **Part # 3012. Hydrogen Peroxide.** Make a 20mM H₂O₂: To 977.3 μ L of 1 X 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 and discard remaining solution after use.
3. **Part # 4016. Detection reagent.** Make a 10mM stock solution of the Detection Reagent: Dissolve the contents of the vial in 60 μ L of DMSO. Once opened, it should be used promptly and any remaining reagent should be aliquoted and refrozen at -70^oC. Avoid repeated freeze thaw cycle.
4. **Part# 6016. Eosinophil peroxidase** . The enzyme is at a stock concentration of 10U/mL. EPO enzyme can be diluted in 1 X Assay Buffer to construct a standard curve (see below). The stock enzyme should be aliquot into single use vials and frozen at -70^oC.

Note: The Donors are of US origin and have been tested and found to be NEGATIVE for HbsAg, HCV, HIV-1 & 2, Syphilis and HIV-1 Antigen by currently approved FDA methods. We certify this to be true to the best of our knowledge. However, because no test method can offer complete assurance that HIV, HBsAg, HCV, Syphilis or other infectious agents are absent, this material should be handled at the Bio-safety Level 2 as recommended for any potentially infectious human serum or blood specimen in the CDC/NIH manual "Biosafety in microbiological and Biomedical Laboratories", 1999.

VI. Cell Preparation.

Note:

1. Collect blood in heparin tubes and isolate eosinophils using standard techniques like density centrifugation or negative magnetic bead separation.

2. After eosinophil isolation, wash them in 1X Assay Buffer or media of your choice. Avoid high serum concentrations (above 2%) and phenol red in the media. Suspend cells to a concentration of $1-2 \times 10^6$ cells/mL. This concentration should be determined empirically. Cells are ready for the assay.

VII. Assay Protocol:

Summary of the Assay

1. Plate cells in a suitable tissue culture dish.

2. Stimulate cells and culture for 3-5 hours in a 37⁰C CO₂ incubator

3. After stimulation remove cell free supernatant. Supernatant is ready for assay or can be frozen at -70⁰C for later use.

4. Transfer cell free supernatant into appropriate 96 well plate. Add reaction cocktail and incubate. Sample size: 50mL

5. Measure fluorescence or absorbance

Assay Protocol Details

1. Plate cells into a suitable tissue culture dish (24 well plate) and stimulate them with your desired experimental protocol for 3-5 hours. The cells should be incubated in a 37°C CO₂ incubator.

Note: We recommend each investigator optimize the incubation time point for their particular experimental conditions.

Controls needed:

- A. Negative control should also be plated at this time point (no stimulant). This will measure spontaneous release of EPO.
 - B. Optional Control: Total peroxidase release. Plate cells at this time point to measure total peroxidase activity. After your incubation time point, these cells will be lysed to release total peroxidase activity.
2. After the 3-5 hour incubation time point the supernatant is ready to assay for EPO activity.

Optional: If total peroxidase release control has been included the cells must be lysed at this time point. Add HTA-Br to a final concentration of 0.02%. HTA-Br should be added at a high concentration to minimize volume changes relative to the other experimental samples. For example prepare a 2% solution of HTA-Br. This 2% solution will require a 1:100 dilution to make a final concentration of 0.02% in the total peroxidase release control (for example to a 1mL volume 10 µL of the 2% HTA-Br is added to make a final concentration of 0.02%). Add the HTA-Br and gently mix the sample and incubate at room temperature for several minutes to ensure complete lysis.

3. Next the supernatants should be centrifuged to remove cells. Supernatants are ready for the assay (**store on ice** and proceed to step 4) or they can be frozen at -70°C for later use.
4. Next prepare 5.5mL **reaction cocktail** (for 100 tests) as follows:

55 µL of Detection Reagent (50 µM final)
5.5µL of 20mM Hydrogen peroxide
5.440mL of 1 X Assay Buffer

Note: Keep it protected from direct light. Any unused reaction cocktail can be frozen for several days and reused. To make a smaller volume of reaction cocktail reduce components of reaction cocktail keeping the same ratios.

5. Optional: If you want to quantitate EPO activity: Prepare a standard curve of EPO by serially diluting it in 1 X Assay Buffer. The units/mL activity of stock EPO: refer to Part# 6016 above in step: **V. Preparation of reagent working solutions.**

Note: If you have used media, other than 1X Assay Buffer, to re-suspend the cells (in step VI. Cell Preparation.) use this same media to construct your standard curve.

The standard curve below represents the linear range of the assay.

Construct the standard curve in 1 X Assay Buffer.

EPO Standard
62.50 milli Units/mL
31.25 milli Units/mL
15.62 milli Units/mL
7.81 milli Units/mL
3.90 milli Units/mL

1.95 milli Units/mL
0.976 milli Units/mL
0 Units/mL

- To measure EPO activity: For fluorescence mode use 96 well black plates. Clear 96 well plates should be used to measure EPO activity when measuring in the absorbance mode.
- Into separate individual wells of the appropriate 96 well plate, pipette (in triplicates) 50µL of EPO standard curve and/or 50µL of stimulated cell free supernatant (from step 3 above) and 50µL of any controls you have set up.

Note: We recommend titrating out the supernatant using 1 X Assay Buffer so that their concentration falls within the linear range of the assay.

	1	2	3	4	5	6	7	8	9	10	11	12
A	Std	Std	Std	- Ctr	- Ctr	- Ctr	Samp	Samp	Samp	Samp	Samp	Samp
B	Std	Std	Std	TR	TR	TR	Samp	Samp	Samp	Samp	Samp	Samp
C	Std	Std	Std	Samp	Samp	Samp	Samp	Samp	Samp	Samp	Samp	Samp
D	Std	Std	Std	Samp	Samp	Samp	Samp	Samp	Samp	Samp	Samp	Samp
E	Std	Std	Std	Samp	Samp	Samp	Samp	Samp	Samp	Samp	Samp	Samp
F	Std	Std	Std	Samp	Samp	Samp	Samp	Samp	Samp	Samp	Samp	Samp
G	Std	Std	Std	Samp	Samp	Samp	Samp	Samp	Samp	Samp	Samp	Samp
H	Std	Std	Std	Samp	Samp	Samp	Samp	Samp	Samp	Samp	Samp	Samp

Figure1. Assay Set up: This figure is for demonstration purposes only; each investigator should determine their assay set up according to their experimental protocol. Std= EPO standard, -Ctr= Negative control, TR= Total peroxidase release control and Samp= experimental stimulated cell free supernatant.

- Next add 50uL of reaction cocktail to all the wells and incubate a further 30-60 minutes at room temperature in the dark.
- Measure the fluorescence at excitation: 530-570nm and emission at 590-600nm in a fluorescent plate reader or measure absorbance at: 570nm.

milli units/mL	RFU
62.50	8461
31.25	5547
15.62	2470
7.81	1115
3.90	536
1.95	261
0.976	122

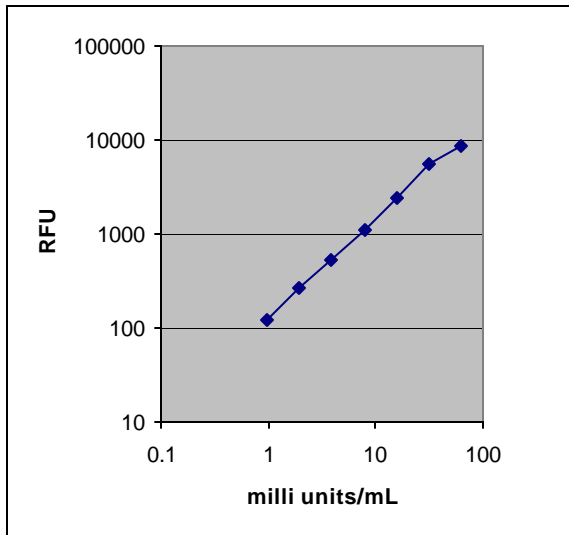


Figure 2. Eosinophil peroxidase was titrated in 1 X Assay Buffer and 50 μ L of each point was added in triplicate to 96 black wells. 50 μ L of reaction cocktail was added and the reaction was incubated in the dark at room temperature for 30 minutes. Fluorescence was read at 530nm excitation and emission detected at 590nm.

VIII. Technical Notes:

- At NADH levels above 10 μ M, 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 reaction¹⁴.
At glutathione (reduced form GSH) above 300 μ M, detection reagent (ADHP) 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 reaction¹⁴.
- Eosinophils can be purified via density centrifugation or through negative selection by immunomagnetic beads.

References:

- Mayeno, A. N., Curran, A. J., Roberts, R. L. and Foote, C. S. (1989) Eosinophils preferentially use bromide to generate halogenating agents. *J. Biol. Chem.* 264, 5660 \pm 5668
- Slungaard, A. and Mahoney, J. R. (1991) Thiocyanate is the major substrate for eosinophil peroxidase in physiologic fluids. *J. Biol. Chem.* 266, 4903 \pm 4910 (Pruitt, K. M. and Tenovuo, J. V., eds.), pp. 31 \pm 53, Marcel Dekker, New York
- Human Eosinophil Peroxidase Induces Surface Alteration, killing, and lysis of *Mycobacterium tuberculosis*", *Infection and Immunity*, Feb. 2003, p. 605-613 by The American Society for Microbiology.
- Bousquet, J., Chanez, P., Lacoste, J. Y., Barneon, G., Ghavanian, N., Enander, I., Venge, P., Ahlstedt, S., Simony-Lafontaine, J., Godard, P. and Michel, F. (1990) Eosinophilic inflammation in asthma. *New Engl. J. Med.* 323, 1033 \pm 1039
- Parrillo, J. E., Borer, J. S., Henry, W. L., Wolff, S. M. and Fauci, A. S. (1979) The cardiovascular manifestations of the hypereosinophilic syndrome. Prospective study of 26 patients, with review of the literature. *Am. J. Med.* 67, 572-582

6. Marc E. Rothenberg and Simon P. Hogan. THE EOSINOPHIL Annual Review of Immunology Vol. 24: 147-174 (Volume publication date April 2006.)
7. 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).
8. 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).
9. 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).
10. 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).
11. Samantha C. Richer and W.C.L. Ford. A critical investigation of NADPH oxidase activity in human spermatozoa. *Mol Hum Reprod* 7, 237 (2001).
12. 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).
13. 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).
14. 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).