# Oxidative Burst: Amplex® Red Detection of Hydrogen Peroxide Release from Activated Human Neutrophils and HL-60 Cells using FlexStation™II Microplate Reader

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## Abstract

**The release of reactive oxygen species by activated human neutrophils plays an important role in the body's defense against infection. This process of oxidative burst is correlated with many disease states, and is a primary component of phagocytosis and apoptosis in neutrophils. Here, we have activated human neutrophils or differentiated HL-60 cells with phorbol 12-myristate 13-acetate (PMA) to undergo oxidative burst. In the presence of horseradish peroxidase, Amplex Red reagent (10-acetyl-3,7dihydroxyphenoxazine) is oxidized by extracellular $H_2O_2$ to produce red-fluorescent resorufin. Capabilities for reagent addition, fluorescent signal detection, and data analysis by FlexStation™ II Microplate Reader are used to optimize a cell-based medium throughput kinetic screen. HL-60 cells typically perform oxidative burst at about 50% the capacity of normal human neutrophils. A comparison will be performed to determine whether the HL-60 assay has acceptable sensitivity and is a viable alternative to the use of neutrophils.**

## Introduction

Cellular response to infection and injury as well as the ability to measure subsequent hydrogen peroxide production is a useful drug discovery tool. Amplex Red reagent (10-acetyl-3,7-dihydroxyphenoxazine) in combination with horseradish peroxidase (HRP) has been described to detect hydrogen peroxide release from activated human leukocytes[1,2]. HL-60 cells, derived from a myelocytic cell line developed from the peripheral blood leukocytes of a patient with acute promyelocytic leukemia, can be differentiated from a suspended cell line by treatment with dimethylsulfoxide to display many of the characteristics of phagocytic white blood cells such as neutrophils or macrophages.[3] Among these is Phorbol 12-myristate 13 acetate (PMA) induced phagocytosis-associated oxidative burst.[4] Hydrogen peroxide released by cells catalyzes the oxidation, in the presence of Horseradish Peroxidase, of non-fluorescent N-Acetyl-3,7-Dihydroxyphenoxazine(Amplex Red) to 7-Hydroxy-3H-Phenoxazine-3-one, a highly fluorescent compound known as Resorufin.[1] The FlexStation II measures the fluorescence. The detection parameters for this conversion are 530 nm excitation and 590 nm emission.

| Parameter | Setting |
|---|---|
| Read Mode | FLEX |
| Read Type | Fluorescence, Bottom Read |
| Wavelengths | Excitation 530-560nm, Emission detection 590 nM, no cut off |
| PMT sensitivity and number of readings | High/2 readings |
| Timing | 600 seconds, Read every 3 |
| Automix | Off |
| Autocalibrate | Once |
| Assay Plate Type | 96 well BD Bio-Coat |
| Wells to Read | Read Area A1-H12 |
| Compound Source | Polyfiltronics 96 well 2ml |
| Compound Transfer | Initial volume 50µl, Pipette Height 95, Volume 50µl, Rate 5, Addition Time point 15 sec |
| Triturate | Off |
| Pipette Tip Layout | A1-H12 |
| Compound and Tip Columns | Varies according to plate layout |
| Auto Read | Off |

**Table 1. FlexStation II Setup Parameters**

## Materials and Methods

**Instrument**: FlexStation II Microplate Reader, (Molecular Devices, Sunnyvale, CA) Optimal assay parameters are listed in Table 1.

**Cell Line:** HL-60 Promyelocytic Leukemia cell line (ATCC, Manassas,VA) or human neutrophils derived from fresh blood donation from human volunteers.

**Cell Culture Media and Reagents**: RPMI 1640, no Phenol Red, 1% Penicillin Streptomycin, Glutamine (Gibco, Carlsbad, CA), 10% Cosmic Calf Serum (low retinoic acid lot) (Hyclone, Logan, UT) Krebs Ringer's phosphate glucose buffer (KRPG) 145 mM NaCl, 5.7 mM Sodium Phosphate, 4.86 mM KCl ,0.54 mM $CaCl_2$, 1.22 mM $MgSo_4$, 5.5 mM Glucose (All from Sigma, St. Louis, MO) and Sterile water (Irvine Scientific,Santa Ana, CA) pH 7.4.

**Cell Line Culture**: HL-60 cells are non-adherent and are grown in RPMI Culture Media. Calf Serum with a low concentration of retanoic acid will prevent the cells from spontaneous differentiation. Split cells to 5x10e5/ml 1-2x per week. Incubate at 37ºC in 5% C02.

**Cell Line Differentiation**: Culture cells in a T-75 flask in RPMI Culture Media at $0.5x10^6$/ml. Add 1.75% DMSO and incubate for 3 days. Cell growth rate will decrease as the cells differentiate. Adjust cell volumes and numbers to meet experimental needs. Control cells are set up at $0.25x10^6$/ml as they will continue to grow.

**Neutrophil Culture**: Isolation of primary human neutrophils is done by a Dextran /Ficoll method from whole blood as described previously. [6] Purified neutrophils are seeded at $0.5x10^6$/ml for assay in a proprietary buffer similar to KRPG. Cells are used within 6 hr of isolation.

**Assay Reagents**: Phorbol 12-myristate 13-acetate, (PMA) (Sigma), Rolipram, (Sigma), Diphenyliodonium chloride (DPI) (Fluka, St. Paul, MN), Horseradish Peroxidase (HRP) (Sigma), 10mM Stock Sol'n Amplex Red (Molecular Probes, Eugene, OR) in DMSO (Sigma), Hydrogen Peroxide ($H_2O_2$) 3% (Sigma). The assay reagents are also available from Molecular Probes in the Amplex Red Hydrogen Peroxide Assay Kit (A-22188)[5]. Poly-d-lysine coated Black And Clear 96 or 384 well plates (Beckton-Dickinson, Bedford, MA)

**Assay preparation**: Serum Starve the cells by setting them up in RPMI plus 1% Cosmic Calf serum overnight. Two to four hours before the experiment is to be performed, wash cells by centrifugation in KRPG buffer 2X and set up at 20-30 $x10^5$ cells/well in a volume of 50µl KRPG buffer in a Black and Clear Poly-d-lysine coated 96 well plate.

**Reaction Mixture**: Just prior to running the experiment, prepare 2X reaction buffer in KRPG with 0.2U/ml Horseradish Peroxidase and 100µM Amplex Red. For simplicity, PMA is added simultaneously with known or novel inhibitors of choice.

**Activators and Inhibitors**: Prepare a 2X dose response of PMA in the reaction buffer. Determine an $EC_{80}$ concentration of PMA to be added with the reaction buffer and oxidative burst inhibitors and controls. Include 2x dose responses of Rolipram and Diphenyliodonium Chloride. FlexStation II is used to add RXN mixture to the cell plates. Volume varies with plate size. The assay is read for 500 seconds. The excitation is 560nm and the emission is 590nm.

## Results

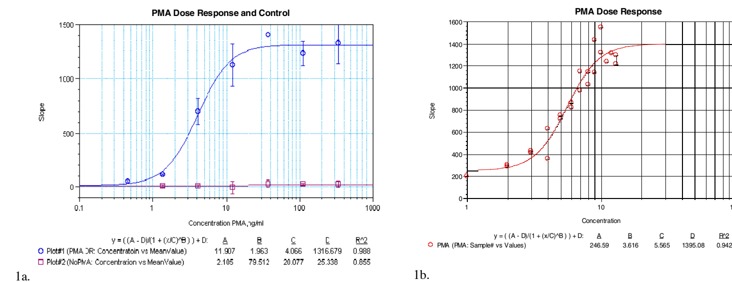


Figure 1. Dose Response to Phorbol 12-myristate 13 acetate and control (PMA) in HL-60 Cells (1a.) and human neutrophils(1b.).

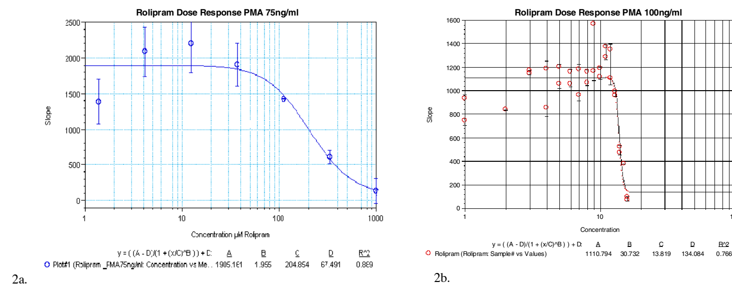


Figure 2. Rolipram Inhibition of response to PMA in HL-60 Cells(2a.) and human neutrophils(2b.).

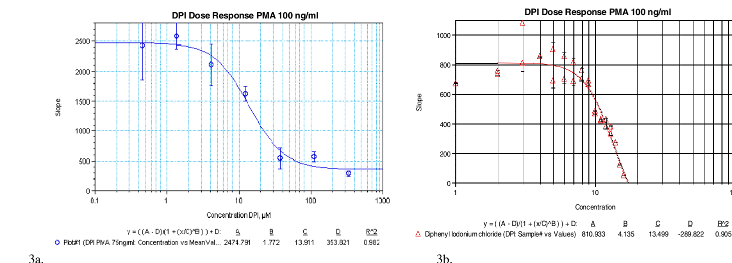


Figure 3. Diphenhyiodonium Chloride Inhibition of response to PMA in HL-60 Cells(3a.) and human neutrophils(3b.).

## Discussion

The FlexStation II Microplate Reader is used to measure oxidative burst in two cell types. PMA was used as the stimulator of oxidative burst (Figure 1) We measured oxididative burst using a fluorescent indicator of hydrogen peroxide production (Amplex Red, Mol. Probes) in HL-60 cells differentiated into neutrophils (blue graphs), and also in primary human neutrophils isolated from healthy human volunteers (red graphs). Two inhibitors of oxidative burst, Rolipram (Figure 2) and also Diphenyl iodonium Chloride (Figure 3), were used as controls in this experimental system.[7,8] HL-60 cells performed as well as neutrophils in responding to PMA, and had lower standard deviation than the primary human neutrophils in inhibitory response curves. Differences were seen in response, with neutrophils being more dramatic and erratic in their response to Rolipram, the PDE IV inhibitor. Differences were also seen with DPI, the flavoprotein inhibitor, which inhibits the cytochrome component of the oxidative complex within an activated cell. Oxidative burst of neutrophils was inhibited more rapidly by increasing DPI concentrations in comparison to HL-60s. The data is summarized in Table 2 below. These global differences were seen regardless of 96 or 384 well plate usage, and regardless of blood donor.

| | HL-60 Cells | Neutrophils |
|---|---|---|
| $IC_{50}$ Rolipram | 180µM | 15µM |
| $IC_{50}$ Diphenyliodonium Chloride | 15.9µM | 12µM |
| $EC_{80}$ PMA | 10ng/ml | 75ng/ml |

Table 2. $IC_{50}$ and $EC_{80}$ comparison curves between HL-60 cells and Neutrophils in Oxidative Burst Assay with Amplex Red as the indicator.

## Conclusions

**The FlexStation II Microplate Reader provides an easy and flexible way to assay inhibitors of oxidative burst in both HL-60 cells and primary human neutrophils.**

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