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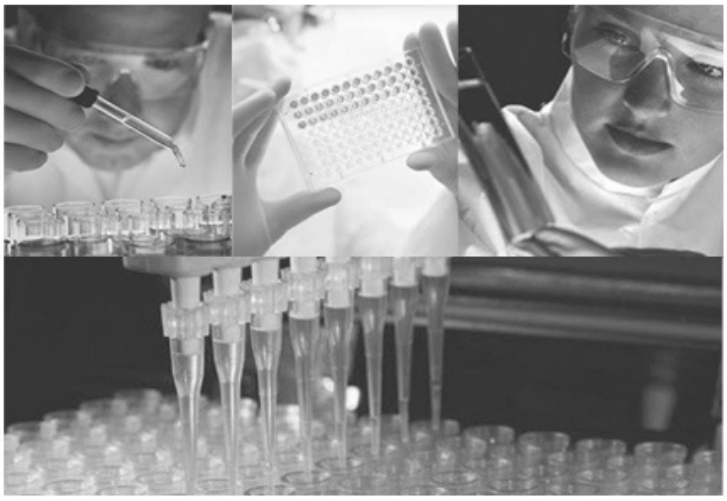


Reactive Oxygen Assays

Diogenes

- Enhanced Chemiluminescent Superoxide Detection System

Hydrogen Peroxide Assay Kit

- Quantitative Colorimetric Assay

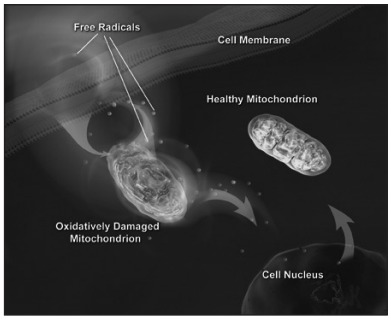
Introduction

The cellular metabolism of oxygen generates deleterious reactive oxygen species. The first reduction product of oxygen is the superoxide radical (O_2^-). Carrying an unpaired electron, superoxide is a potent oxidizing agent. It has been found to react with numerous cellular structures, such as iron-sulfur clusters, unsaturated lipids, and nucleic acids. Superoxide can also cause biological damage by acting as a reductant.

Reducing superoxide yields hydrogen peroxide (H_2O_2). Hydrogen peroxide is also a powerful oxidant and is commonly used in first aid as a biocide to cleanse wounds or in high concentrations as a bleaching agent. While superoxide carries a charge—and is thus unable to freely cross biological membranes—H_2O_2 is uncharged and can diffuse across membranes as easily as water. Hydrogen peroxide is also more stable than superoxide and can diffuse through a cell or tissue, causing damage at a distance from its point of origin.

Accurate measurement of superoxide and hydrogen peroxide is critical to research into the causes of many disease states. A long-standing contributor to the art of assaying for reactive oxygen, National Diagnostics is pleased to offer the Hydrogen Peroxide Assay Kit and the Diogenes system for enhanced chemiluminescent detection of superoxide.

A healthy mitochondrion and an oxidatively stressed and damaged one. The arrows indicate the movement of free radicals, which can spread easily from damaged mitochondria to other parts of the cell.



Hydrogen Peroxide Assay Kit

- Sensitive, Quantitative Assay for Hydrogen Peroxide
- Detects as Little as 15ng/ml
- Easy-to-use, Colorimetric System

National Diagnostics' Hydrogen Peroxide Assay Kit is a rapid, sensitive and quantitative method for the determination of hydrogen peroxide in chemical or biological systems. The assay is based upon formation of a complex between xylenol orange and ferric iron, which is produced by the peroxide-dependent oxidation of ferrous iron. This reaction is quantified colorimetrically, detecting as little as 15ng/ml of peroxide. Each kit provides reagents sufficient for 100 assays.

Assay Reagent Preparation

Prepare 1.8ml of reagent per sample to run tests in duplicate.

1. To prepare 20ml: Combine 19.8ml of Component A with 0.2ml Component B.
2. The mixture is stable at room temperature for one working day.

Assay Procedure

1. Mix 0.9ml of Assay Reagent with up to 0.1ml of sample.
2. Incubate at room temperature for at least 30 minutes to allow for complete color development.
3. Read absorbance at 560nm.

For Absolute Quantitation

Diluted hydrogen peroxide may be used as a standard for absolute quantitation, provided it is standardized by UV absorption measurement prior to dilution. Hydrogen peroxide solutions can be unstable so standardization is essential for accurate results. To determine the concentration of a hydrogen peroxide solution, measure the absorbance at 240nm and use a molar extinction coefficient of $43.6 M^{-1}cm^{-1}$ (JBC 245, pp2409-13, 1970) A standard 3% solution is 0.88M, and at a 1:100 dilution would have an A_{240} of 0.388.

Frequently Asked Questions

Will the peroxide detection kit also detect organic peroxides (ROOH)?

Yes, color will be developed by organic peroxides, as they will also oxidize iron. In order to be sure that the signal is due to H_2O_2, a control should be run in the presence of catalase, which will eliminate the H_2O_2 signal. The absorbance which is eliminated by catalase is due to H_2O_2.

What components of my system might interfere with the Peroxide Assay?

Chelating agents such as EDTA, radical scavengers like ethanol, DMSO or BHT, or sulfonated buffers like MES or HEPES will interfere with color development. Common components such as SDS, phosphate, Tris, glucose, mannitol, glutathione or BSA do not interfere with the assay in moderate concentrations.

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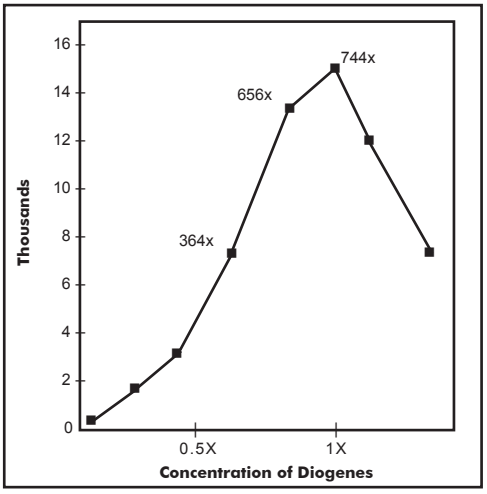
Diogenes Enhanced Superoxide Detection

- Sensitive, linear, selective and non-cytotoxic
- Up to 600-fold enhancement
- Requires fewer cells
- Easy to use

National Diagnostics' Diogenes System is a superoxide chemiluminescence enhancer that is non-denaturing to living cells. Superoxide radical (O_2^-) is produced intracellularly as a consequence of aerobic metabolism and extracellularly by leukocytes in response to infection. The extent of "oxidative burst" produced by white blood cells (WBCs) when stimulated by f-met-leu-phe, phorbol esters, anti-Fc receptor antibodies or LPS is a partial indicator of the immunocompetence of the cells tested. Diogenes is ideally suited to the detection of cell-mediated superoxide production. The intensity of light produced by Diogenes in the presence of superoxide is directly proportional to the O_2^- concentration, but is much higher than that achieved by using luminol. Therefore, Diogenes is ideal for monitoring cellular immunocompetence, utilizing a luminometer to quantify the light output.

Diogenes enhances signal output

The Diogenes Cellular Luminescence Enhancement System contains the luminescent compound luminol plus the enhancer complex of luminol. Diogenes has been shown to routinely increase chemiluminescent milllivolt (mV) output from 100-fold to greater than 600-fold.



At optimum concentration, Diogenes can increase output by greater than 600-fold.

Diogenes improves existing technology

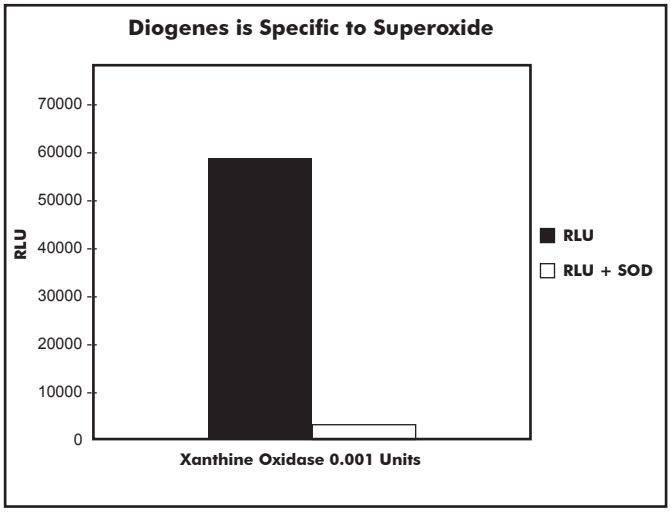
Traditional methods of detecting superoxide include (1) the reduction of exogenously supplied cytochrome C; (2) measurement of oxygen uptake using a Clark electrode; (3) lucigenin (which is sensitive but can trigger O_2^- production); and (4) luminol-mediated chemiluminescence. With a signal range spanning over three orders of magnitude, luminol-mediated chemiluminescence offers the greatest sensitivity, is the most accessible and requires the least number of cells. However, until now, chemiluminescence signal output has been limited. Diogenes overcomes this handicap by increasing the photon-to-superoxide release ratio.

Diogenes enhances sensitivity

The Diogenes System enhances the sensitivity of the chemiluminescence assay to more accurately detect lower concentrations of superoxide anion. It is estimated that under non-enhanced conditions, only one (1) photon is released for each one thousand (1000) molecules of superoxide produced. By substantially increasing the photon release ratio, Diogenes improves sensitivity by at least two (2) orders of magnitude. This means that Diogenes is capable of detecting at least 100-fold lower concentrations of superoxide. This translates to fewer needed cells and greater monitoring ability of targeted cells. Diogenes is linear over at least two orders of magnitude.

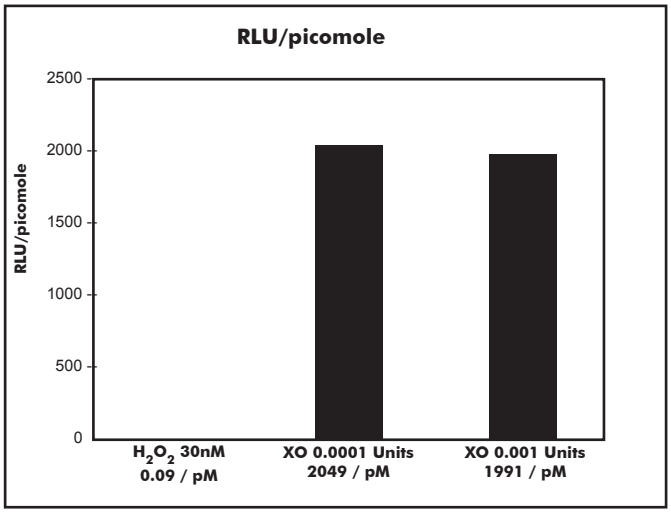
Diogenes is specific for superoxide

The response of the Diogenes Cellular Luminescence Enhancement System to superoxide is at least 10^4 greater than that for hydrogen peroxide (H_2O_2). Backgrounds are kept low and results may be interpreted with confidence.



Xanthine oxidase produces approximately 20% superoxide and 80% hydrogen peroxide. The Diogenes signal virtually disappears in the presence of superoxide dismutase (SOD).*

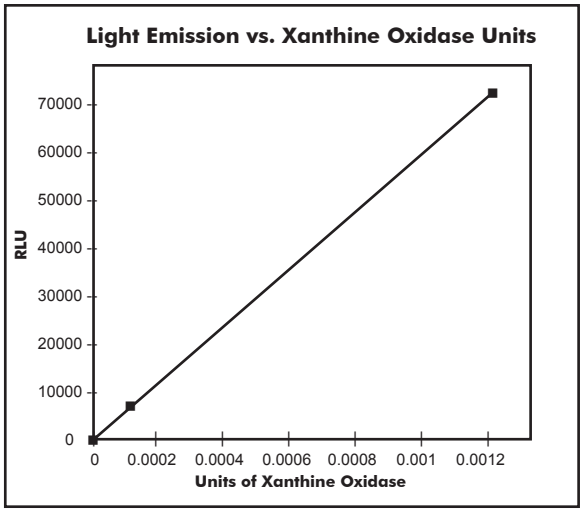
*Ryosuke Asai et al (2007). Two Mutations Convert Mammalian Xanthine Oxidoreductase to Highly Superoxide-productive Xanthine Oxidase. Journal of Biochemistry. 141(4):525-534; doi:10.1093/jb/mvm054.



Diogenes is both quantitative and specific for superoxide.

Diogenes is quantitative

Diogenes signal output is in direct proportion to the amount of superoxide present in cell culture suspension.

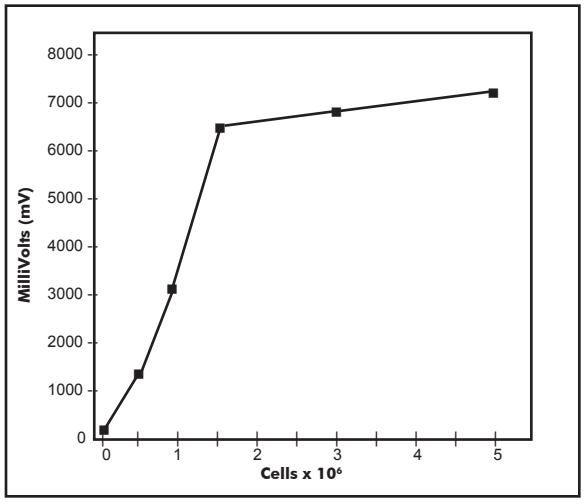


Diogenes maintains cell sample integrity

The Diogenes Cellular Luminescence Enhancement System is non-toxic to living cells and does not interfere with the normal cellular superoxide response. Diogenes is highly selective for superoxide and does not induce up-regulation of superoxide production. In the absence of superoxide, Diogenes will not cause luminol cleavage, and Diogenes itself does not stimulate superoxide production. Therefore, there is virtually no background.

Diogenes requires fewer cells

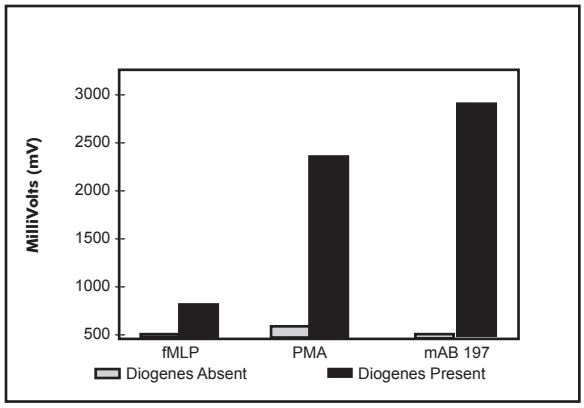
A minimum number of cells are required for the Diogenes Cellular Luminescence Enhancement System. In fact, increasing cell concentrations over the suggested number of cells has only limited effect on the sensitivity of the assay.



Increased cell concentrations yield only a limited increase in sensitivity. Inhibition at higher concentrations is due to a self-quenching by cells absorbing emitted photons.

Diogenes detects response to multiple stimuli

The Diogenes Cellular Luminescence Enhancement System will respond to any extracellular superoxide regardless of the means by which the cells are stimulated. Any signal that activates an oxidase enzyme complex will result in enhanced chemiluminescence when Diogenes is present. The stimulatory signals may be either physiologic or mimetic of the physiologic pathway. Known stimulants of superoxide production include any ligand that cross-links Fc receptors (e.g. mAb197), phorbol myrisate acetate (PMA), and f-met-leu-phe (fMLP).



Diogenes will detect superoxide regardless of the source.

Diogenes may be used to assay SOD

Diogenes may be used to assay superoxide dismutase (SOD), which is routinely assayed using a standardized O_2^--generating system (usually xanthine/xanthine oxidase) coupled to a superoxide detection system (McCord & Fridovich, *J.Biol.Chem*. 244**:**6049, 1969). Diogenes can be substituted for any superoxide detection system with a resulting increase in sensitivity. This will translate to less O_2^- needed per assay and thus a greater sensitivity for SOD detection. Typically, 10^{-5} units or less of xanthine oxidase (XO) in 0.5 mM xanthine gives a convenient baseline signal of O_2^-, depending upon the sensitivity of the luminometer utilized.

Frequently Asked Questions

What is the time course of the light output of Diogenes?
Diogenes responds immediately to the presence of superoxide. Therefore the time course of light emission is determined by the response of the cells to the chosen stimulant. Of course, extremely high levels of superoxide will eventually consume all of the luminol in the system- this will result in a loss of signal which cannot be compensated by the addition of fresh cells.

What types of cells can be assayed using Diogenes?
Diogenes is not specific for cell type or stimulant. Any cell which produces superoxide will activate the Diogenes reagent system.

Does Diogenes detect superoxide inside the cell?
Diogenes is not designed to penetrate the cell membrane. In addition, some metabolism of the Diogenes Reagent would be expected once it entered the cell. Most studies to date suggest only extracellular superoxide is detected.

I am not detecting a signal. Now what?
Lack of signal indicates either the cells are not producing superoxide or that there is some interference with the Diogenes system. To control for interference add the xanthine/xanthine oxidase system described above to your samples—the light output should not be diminished by the presence of the sample. If interference is detected it may indicate the presence of SOD or other superoxide scavengers in the sample. Feel free to call Technical Services for advice on how to handle such samples.

Diogenes is Easy to Use

The Diogenes Cellular Luminescence Enhancement System is easy to use and requires only a luminometer and no lengthy laboratory procedures. The Diogenes Kit is a two-component system consisting of the Diogenes Reagent and the Diogenes Activator.

Preparing the Solution

1. Add 1.0 ml of deionized water to the Diogenes Reagent that is contained in the vial. Mix until completely dissolved.

2. Add 9.0 ml of deionized water to the Diogenes Activator that is contained in the bottle. Mix until completely dissolved.

3. Add vial contents (Diogenes Reagent) to bottle contents (Diogenes Activator), recap the bottle and shake vigorously to mix.

4. The combined solution (10 ml) comprises the Diogenes Complete Enhancer in its ready-to-use, final working strength, and will yield up to 100 assays.

Cellular Assay

1. Add $5X10^4$ - $5X10^5$ cells contained in glucose media into a luminometer cuvette or microtiter plate.

2. Add 100 μl of Diogenes Complete Enhancer Solution.

3. Add 20 μl of stimulant (PMA, mAb197, fMLP, etc.).

4. Read the results. Time to peak output will depend on cell and stimulant used. The response of Diogenes to superoxide is instantaneous.

Conducting a Positive Control Using Xanthine - Xanthine Oxidase

1. Prepare xanthine substrate: Dissolve xanthine to 0.5mM in 50mM potassium phosphate buffer, pH 7.8. This may require heating.

2. Prepare xanthine oxidase enzyme: Commercial xanthine oxidase preparations can vary widely in activity, and will change over time as well. As a result, the exact amount of XO to be added must be determined empirically. A good starting point is to dilute the enzyme into 50mM potassium phosphate buffer (pH 7.8) to give 10^{-3} units per milliliter. Keep this solution cold. Addition of 10 microliters to a reaction will give a final concentration of 10^{-5} units. The optimal amount to add can then be determined by adjusting this initial amount until the light output falls in the linear range of the detector used.

3. For each assay, mix 180 microliters Diogenes Activator, 20 microliters Diogenes Reagent, 100 microliters xanthine solution, and 10 microliters xanthine oxidase solution.

4. Record the initial light output, then add the test sample. SOD activity will be reflected in a decrease in the light emitted, and can be calibrated against purified SOD of known activity.

Storing Diogenes

1. The shelf-life of the non-reconstituted reagents in original packaging is one (1) year.

2. The prepared Diogenes Complete Enhancer Solution can be stored at 4°C for up to 5 days.

Ordering Information

Product Name	Cat. No.	Size	Price
Diogenes Kit	CL-202	1 Kit - 100 Assays	
Hydrogen Peroxide Assay Kit	CL-204	1 Kit - 100 Assays	$115.00

Contact Technical Services

National Diagnostics' Technical Service department is staffed by individuals with extensive practical and theoretical expertise in molecular and cell biology. Our technical staff acts as a partner in your research efforts, offering not only clear concise answers to your questions, but also helpful suggestions and technical insights that both our own laboratories and our customers have found useful. We encourage you to call our Technical Service department with any questions about National Diagnostics products or about any molecular biology application.

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