

## **ROS-ID<sup>®</sup> Hypoxia/Oxidative Stress Detection Kit**

*Catalog Number: ENZ-51042*

### **ENZ-51042-K500**

*For 500 fluorescence microscopy or 100 flow cytometry assays*

### **ENZ-51042-0125**

*For 125 fluorescence microscopy or 25 flow cytometry assays*

**NOTE:** This version contains a change to preparation of **ROS-ID<sup>®</sup> Hypoxia/Oxidative Stress Detection Mix**

For the latest product information, including support documentation, visit us  
online:

[www.enzolifesciences.com](http://www.enzolifesciences.com)

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## TABLE OF CONTENTS

Introduction .....	2
Safety Warnings and Precautions .....	3
Reagents Provided and Storage.....	4
Additional Materials Required.....	4
Methods and Procedures .....	5
Reagent Preparation .....	5
Fluorescent/confocal microscopy (Adherent cells).....	7
Fluorescent/confocal microscopy (Suspension cells).....	9
Flow Cytometry (Adherent Cells).....	11
Flow Cytometry (Suspension Cells).....	13
Appendices .....	15
Spectral Characteristics.....	15
Technical Hints.....	15
Anticipated Results (Fluorescence Microscopy) .....	16
Anticipated Results (Flow Cytometry) .....	17
References.....	19
Troubleshooting .....	20
Contact Information.....	22



Please read entire booklet before proceeding with the assay.



Carefully note the handling and storage conditions of each kit component.



Please contact Enzo Life Sciences Technical Support if necessary.

## INTRODUCTION

The ability of cells to detect and respond to a decrease in oxygen tension has fundamental importance for maintaining oxidative metabolism and tissue homeostasis. Both hypoxia (lack of oxygen relative to metabolic needs) and reoxygenation (reintroduction of oxygen to hypoxic tissue) are important in human pathophysiology because they occur in a wide variety of important clinical conditions including cancer, cardiopathy, ischemia and vascular diseases. A correlation exists between the percentage of hypoxic cells in the solid tumor and cancer treatment prognosis, since hypoxic cells are refractory to radiation therapy and resistant to toxic drugs used in chemotherapy. As a result, detection and analysis of hypoxic cell fractions in tumors can provide invaluable information about cancer status, its prognosis and the specific treatment options. Many of the cellular responses to hypoxia are now known to be mediated by the production of reactive oxygen species at mitochondrial complex III. While the mechanism by which cytosolic oxidant concentration is increased during hypoxia is unknown, the importance of the maintenance of cellular oxygen supply requires further investigation into the role of reactive oxygen species (ROS) as hypoxia signaling molecules.

Since diseases due to ischemia (e.g., myocardial infarction and stroke) are exceedingly common causes of morbidity and mortality and organ transplantation is becoming increasingly frequent, understanding the role of ROS and reactive nitrogen species (RNS) in reoxygenation injury has the potential to lead to therapies that could improve public health. Cellular models of hypoxia-reoxygenation have provided useful tools for the study of reactive species-mediated mechanisms of cellular dysfunction in ischemia-reperfusion injury.

Enzo Life Sciences' ROS-ID<sup>®</sup> Hypoxia/Oxidative Stress Detection Kit is designed for functional detection of hypoxia and oxidative stress levels in live cells (both suspension and adherent) using fluorescent microscopy or flow cytometry. The kit includes fluorogenic probes for hypoxia (red) and for oxidative stress levels (green) as two major components. Red Hypoxia Detection Reagent (probe) is a non-fluorescent or weakly fluorescent aromatic compound containing a nitro (NO<sub>2</sub>) moiety. Due to a nitroreductase activity present in hypoxic cells, the nitro group is converted in a series of chemical steps to hydroxylamine (NHOH) and amino (NH<sub>2</sub>) group, the original molecule then degrades releasing the fluorescent probe. The Oxidative Stress Detection Reagent is a non-fluorescent, cell-permeable total ROS detection dye which reacts directly with a wide range of reactive species, such as hydrogen peroxide, peroxyxynitrite and hydroxyl radicals, yielding a green fluorescent product indicative of cellular production of different ROS/RNS types. Note that this probe is relatively insensitive to superoxide, reactive chlorine or bromine species. Upon staining, the generated fluorescent products can be visualized using a wide-field fluorescence microscope equipped with standard fluorescein (490/525 nm) and Texas Red (596/670) filters, confocal microscopy, or cytometrically using any flow cytometer equipped with a blue (488 nm) laser.

The ROS-ID<sup>®</sup> Hypoxia/Oxidative Stress Detection Kit is a member of the CELLESTIAL<sup>®</sup> product line, reagents and assay kits comprising fluorescent molecular probes that have been extensively benchmarked for live cell analysis applications. CELLESTIAL reagents and kits are optimal for use in demanding cell analysis applications involving confocal microscopy, flow cytometry, microplate readers and HCS/HTS, where consistency and reproducibility are required.

## SAFETY WARNINGS AND PRECAUTIONS

### FOR RESEARCH USE ONLY. NOT FOR USE IN DIAGNOSTIC PROCEDURES.

- Reagents should be treated as possible mutagens and should be handled with care and disposed of properly.
- Observe good laboratory practices. Gloves, lab coat, and protective eyewear should always be worn. Never pipet by mouth. Do not eat, drink or smoke in the laboratory areas. All blood components and biological materials should be treated as potentially hazardous and handled as such. They should be disposed of in accordance with established safety procedures.



Handle with  
care

- To avoid photobleaching, perform all manipulations in low light environments or protected from light by other means.

## REAGENTS PROVIDED AND STORAGE

All reagents are shipped on dry ice. Upon receipt, the kit should be stored upright at  $\leq -20^{\circ}\text{C}$ , protected from light. When stored properly, these reagents are stable for at least twelve months. **Avoid repeated freezing and thawing.**

Reagents provided in the –K500 kit are sufficient for 500 fluorescence microscopy assays or 100 flow cytometry assay using live cells (adherent or suspension cells)

Reagents provided in the –0125 kit are sufficient for 125 fluorescence microscopy assays or 25 flow cytometry assay using live cells (adherent or suspension cells)

Reagent	ENZ-51042-K500	ENZ-51042-0125
<b>Hypoxia Red Detection Reagent (red cap)</b>	25 $\mu\text{L}$	7 $\mu\text{L}$
<b>Oxidative Stress Detection Reagent (green cap), Lyophilized</b>	300 nmoles	80 nmoles
<b>Hypoxia Inducer (Deferoxamine, DFO), Lyophilized</b>	1 $\mu\text{mole}$	1 $\mu\text{mole}$
<b>ROS Inducer (Pyocyanin), Lyophilized</b>	1 $\mu\text{mole}$	1 $\mu\text{mole}$

## ADDITIONAL MATERIALS REQUIRED

- $\text{CO}_2$  incubator ( $37^{\circ}\text{C}$ ), tissue culture plastic ware
- Standard fluorescence microscope or flow cytometer equipped with a blue laser (488 nm)
- Calibrated, adjustable precision pipettors, preferably with disposable plastic tips
- 5ml round bottom polystyrene tubes for holding cells during staining and assay procedure
- Adjustable speed centrifuge with swinging buckets
- Anhydrous dimethylformamide (DMF) (100%)
- Glass microscope slides, glass cover slips
- 1X PBS (optional, for washing procedures)
- Deionized water

## METHODS AND PROCEDURES

The procedures described in this manual assume that the user is familiar with the basic principles and practices of fluorescence microscopy and flow cytometry and that the investigator is able to run samples according to the operator's manual pertaining to the instrument being used.



Important

**NOTE: PLEASE READ THE ENTIRE PROCEDURE BEFORE STARTING.** Allow all reagents to thaw at room temperature. Upon thawing, ensure that the solutions are homogenous by gently hand-mixing or vortexing the reagents. Prior to any use, recover all contents provided by briefly centrifuging vials.

## REAGENT PREPARATION

### Hypoxia Red Detection Reagent Stock Solution

The Hypoxia Red Detection Reagent (red cap) is supplied in DMSO at a stock concentration of 1 mM. The working concentration is 0.5  $\mu$ M, *i.e.* 1:2000 dilution from stock when making the ROS-ID<sup>®</sup> Hypoxia/Oxidative Stress Detection Mix.

### Oxidative Stress Detection Reagent (Green) Stock Solution

The Oxidative Stress Detection Reagent (green cap) is supplied lyophilized. The –K500 size should be reconstituted in 60  $\mu$ L anhydrous DMF to yield a 5 mM stock solution concentration. The –0125 size should be reconstituted in 15  $\mu$ L anhydrous DMF to yield a 5mM stock solution concentration. The working concentration is 1-6  $\mu$ M, *i.e.* 1:800 – 1:5000 dilution from stock when making the ROS-ID<sup>®</sup> Hypoxia/Oxidative Stress Detection Mix. Optimized dilution factor is dependent on microscope settings by end user. Upon reconstitution, the stock solution should be stored at -20°C. The shelf life of the reconstituted reagent is about 1 week at -20°C.

### ROS-ID<sup>®</sup> Hypoxia/Oxidative Stress Detection Mix

Prepare the ROS-ID<sup>®</sup> Hypoxia/Oxidative Stress Detection Mix by combining appropriate volumes of Oxidative Stress Detection Reagent, Hypoxia Red Detection Reagent and 1X PBS or culture medium. As an example when 10 ml of ROS-ID<sup>®</sup> Hypoxia/Oxidative Stress Detection Mix is needed (approximately for 100 tests), **Table 1** can be used. Actual volume of each component shall be adjusted accordingly depending on tests to perform at one time.

**NOTE:** Depending on the experiments, dyes should be used simultaneously or separately according to a provided protocol.

Table 1. ROS-ID® Hypoxia/Oxidative Stress Detection Mix		
Reagent	Vol Req'd per 10 mL Detection Mix	
	Microscopy Assay	Flow Cytometry Assay
Oxidative Stress Reagent (green cap)	2-12 µL*	2-12 µL*
Hypoxia Detection Reagent (red cap)	5 µL	10 µL
1X PBS or culture medium	10 mL	10 mL
<b>Total Volume</b>	10 mL	10 mL

\*Volume of Oxidative Stress Reagent is dependent on microscope settings.

### Positive Controls

**The ROS Inducer** (Pyocyanin) is supplied lyophilized and should be reconstituted in 20 µL of anhydrous DMF to yield a 50 mM stock concentration. A final concentration of 200 µM – 500 µM is recommended to be used as a positive control for ROS induction. However, the optimal final concentration is cell-dependent and should be determined experimentally for each cell line being tested. ROS induction generally occurs within 20-30 minutes upon treatment and may decrease or disappear after that time.

**The Hypoxia Inducer** (Deferoxamine, DFO) is supplied lyophilized and should be reconstituted in 20 µL of deionized water to yield a 50 mM stock solution. A 3-4 hrs treatment at a final concentration of 200 µM is recommended to be used as a positive control for inducing hypoxia. The optimal final concentration is cell-dependent and should be determined experimentally for each cell line being tested.

**IMPORTANT:** In the proceeding sections, the recommended positive ROS Inducer control (pyocyanin) and positive Hypoxia Inducer (DFO) should follow the short treatment protocol, regardless of the application (microscopy or flow cytometry).

Long treatment is only required if the experimental agent induces oxidative stress or hypoxia in more than 5 hours.

**NOTE:** The procedures described below were developed and optimized using HeLa cells. Cells should be maintained via standard sterile tissue culture practices. Always make sure that cells are healthy and in the log phase of growth before using them for an experiment. Results of the experiments will depend on the cells' well-being.

Optimization might be required based on the cell line used and time required for ROS and Hypoxia induction.

## Fluorescent/Confocal Microscopy (Adherent Cells)

1. Grow a cell line of choice in an appropriate media. The day before the experiment, seed the cells directly onto glass slides or polystyrene tissue culture plates to ensure 50-70% confluency on the day of the experiment. It is recommended to setup separate positive controls for both oxidative stress and hypoxia (refer to Reagent Preparation section, pg. 5). A negative control is required.

**IMPORTANT:** Proceed to step 2 if experimental agent requires short treatment (less than 5 hours) for ROS and hypoxia induction. Proceed to step 3 if greater than 5 hours is required for induction.

### 2. Short Treatment

- a. Remove media from the appropriate well(s).
- b. Load a sufficient volume (approximately 100  $\mu$ L) of the ROS-ID<sup>®</sup> Hypoxia/Oxidative Stress Detection Mix (refer to Reagent Preparation section, pg. 5) that contains either the vehicle, experimental agent, ROS Inducer Control (pyocyanin) or Hypoxia Inducer Control (DFO) at a desirable working concentration. Fully cover the cell monolayer.
- c. Incubate under normal tissue culture conditions (37°C at 5% CO<sub>2</sub>). ROS Inducer (pyocyanin) and Hypoxia Inducer (DFO) should be incubated for 30mins and 3.5 hrs, respectively.
- d. **Post Treatment** - Carefully remove the ROS-ID<sup>®</sup> Hypoxia/Oxidative Stress Detection Mix that contains the vehicle, experimental agent or positive controls from the glass slides or tissue culture plates. This can be done by gently tapping the plate or slide against layers of paper towel.
- e. Wash the cells twice with sterile 1X PBS or any other compatible buffer to full cover the cell monolayer. **Do not allow the cells to dry and proceed to step 4.**

### 3. Long Treatment

**NOTE:-** Provided controls for ROS Induction and Hypoxia Induction should use the short treatment protocol.

- a. Treat the appropriate well(s) with the experimental agent (directly into the media) at a desirable concentration.
- b. **Post Treatment-** Remove the media with the experimental agent(s) by tapping the liquid off the slide.
- c. Wash the cells twice with 1X PBS or an appropriate buffer in a volume sufficient to cover the monolayer.

- d. Using a sufficient volume (approximately 100µL), cover the cell monolayer with the ROS-ID® **Hypoxia/Oxidative Stress Detection Mix** (refer to Reagent Preparation section, pg. 5). Incubate under normal tissue culture conditions (37°C at 5% CO<sub>2</sub>) for 30 min.
- e. Carefully remove the ROS-ID® **Hypoxia/Oxidative Stress Detection Mix** from the glass slides or tissue culture plates by gently tapping them against layers of paper towel.
- f. Wash the cells twice with sterile 1X PBS or any other compatible buffer using a sufficient volume to cover the cell monolayer.
- g. Overlay the cells with a coverslip and observe them under a fluorescence/confocal microscope using standard excitation/emission filter sets. Make sure prepared samples are protected from drying. Dried out cells may present different.

#### **Recommended Filter Sets**

- Oxidative stress detection requires a filter set compatible with Fluorescein (Ex/Em: 490/525 nm).
- Hypoxia detection requires a filter set compatible with Texas Red (Ex/Em: 596/670 nm).

***NOTE: Different exposure times may be required for optimal visualization.***

## Fluorescence/Confocal Microscopy (Suspension Cells)

1. Cells should not exceed a density of ( $1 \times 10^6$  cells/mL) when they are being grown in an appropriate media. It is recommended to setup separate positive controls for both oxidative stress and hypoxia (see Reagent Preparation, pages 5-6). A negative control is required.

**IMPORTANT:** Proceed to step 2 if experimental agent requires short treatment (less than 5 hours) for ROS and hypoxia induction. Proceed to step 3 if greater than 5 hours is required for induction.

### 2. Short Treatment

- a. Centrifuge cells at  $400 \times g$  for 5 minutes.
- b. Treat the cell pellet(s) with  $\sim 100 \mu\text{L}$  of the ROS-ID<sup>®</sup> **Hypoxia/Oxidative Stress Detection Mix** (refer to Reagent Preparation section, pg. 5) that contains either the vehicle, experimental agent, ROS Inducer Control (pyocyanin) or Hypoxia inducer control (DFO) at their desirable working concentration. A cell count of ( $1 \times 10^5$  cells/sample) should be used. Incubate under normal tissue culture conditions ( $37^\circ\text{C}$  at 5%  $\text{CO}_2$ ). ROS Inducer Control (pyocyanin) and Hypoxia Inducer Control (DFO) should be incubated for 30 mins and 3.5 hrs., respectively.
- c. **Post Treatment** - Centrifuge the cells at  $400 \times g$  for 5 minutes to remove the ROS-ID<sup>®</sup> **Hypoxia/Oxidative Stress Detection Mix** containing the vehicle, experimental agent, ROS Inducer Control (pyocyanin) or Hypoxia Inducer Control (DFO).
- d. Resuspend the cells in 5 mL 1X PBS or any other appropriate buffer. Centrifuge again at  $400 \times g$  for 5 minutes and remove supernatant.
- e. Resuspend the cells in  $100 \mu\text{L}$  of 1X PBS. Aliquot  $20 \mu\text{L}$  of the cell suspension, sufficient for  $\sim 2 \times 10^4$  cells, onto a microscope slide. Proceed to step 4.

### 3. Long Treatment

**NOTE:** Provided controls for ROS induction and Hypoxia induction should use the short treatment protocol.

- a. Cells should be centrifuged at  $400 \times g$  for 5 minutes, and resuspended to yield a working cell count of  $1 \times 10^5$  cells/sample in an appropriate media.
- b. Treat the cells with the desired vehicle or experimental agent. Incubate under normal tissue culture conditions ( $37^\circ\text{C}$  and 5%  $\text{CO}_2$ ). Incubation time will vary based on experimental agent used.

- c. **Post Treatment** - Centrifuge cells at 400 x g. Carefully remove the media that contains the experimental agent. Wash the cells twice with PBS or any other appropriate buffer.
  - d. Resuspend the cell pellet in 100  $\mu$ L ROS-ID<sup>®</sup> **Hypoxia/Oxidative Stress Detection Mix** (refer to Reagent Preparation section, pg. 5) and incubate under normal tissue culture conditions (37°C at 5% CO<sub>2</sub>) for 30 mins.
  - e. Centrifuge the cells at 400 x g for 5 minutes to remove the ROS-ID<sup>®</sup> **Hypoxia/Oxidative Stress Detection Mix**. Wash the cells in 5 mL 1X PBS or any other appropriate buffer. Centrifuge at 400 x g for 5 minutes and remove the supernatant.
  - f. Resuspend the cells in 100  $\mu$ L of 1X PBS. Aliquot 20  $\mu$ L of the cell suspension, sufficient for 2 x 10<sup>4</sup> cells, onto a microscope slide.
4. Overlay the cells with a coverslip and analyze immediately via fluorescence microscopy. Make sure that prepared samples are protected from drying. Dried out cells may present different fluorescence patterns than healthy, hydrated cells.

#### **Recommended Filter Sets**

- Oxidative stress detection requires a filter set compatible with Fluorescein (Ex/Em: 490/525 nm).
- Hypoxia detection requires a filter set compatible with Texas Red (Ex/Em: 596/670 nm).

***NOTE: Different exposure times may be required for optimal detection of the two dyes used in the kit.***

## Flow Cytometry (Adherent Cells)

1. Grow a cell line of choice in an appropriate media. The day before the experiment, seed the cells directly onto glass slides or polystyrene tissue culture plates to ensure ~50-70% confluency on the day of the experiment. It is recommended to setup separate positive controls for both oxidative stress and hypoxia induction (refer to Reagent Preparation section, pg. 5). A negative control is required.

**IMPORTANT:** Proceed to step 2 if experimental agent requires short treatment (less than 5 hours) for ROS and hypoxia induction. Proceed to step 3 if greater than 5 hours is required for induction.

### 2. Short Treatment

- a. Remove media from the appropriate well(s). Detach cells from the tissue culture plates using any appropriate method.
- b. Collect cells in a 5 mL round-bottom polystyrene tube. Centrifuge the cells for 5 mins at 400 x g and wash pellet using sterile 1X PBS. Repeat once.
- c. Resuspend cells (1 to 5 x 10<sup>5</sup> cells/sample) in 200 µL of ROS-ID<sup>®</sup> **Hypoxia/Oxidative Stress Detection Mix** (refer to Reagent Preparation section, pg. 5) that contains either the vehicle, experimental agent, ROS Inducer Control (Pyocyanin) or Hypoxia Inducer Control (DFO) at a desirable working concentration. Incubate under normal tissue culture conditions (37°C at 5% CO<sub>2</sub>). ROS Inducer Control (pyocyanin) and Hypoxia Inducer Control (DFO) should be incubated for 30 mins and 3.5 hrs, respectively.
- d. **Post Treatment** - Centrifuge the cells at 400 x g for 5 minutes to remove the ROS-ID<sup>®</sup> **Hypoxia/Oxidative Stress Detection Mix** that contains the vehicle, experimental agent or positive inducer controls.
- e. Resuspend the cells in 5 mL 1X sterile PBS and centrifuge for 5 minutes at 400 x g. Remove supernatant. **Proceed to step 4.**

### 3. Long Treatment

**NOTE:** *Provided controls for ROS Induction and Hypoxia Induction should use the short treatment protocol.*

- a. Treat well(s) with the experimental agent at a desirable working concentration. Incubate under normal tissue culture conditions (37°C at 5% CO<sub>2</sub>). Incubation time will vary based on experimental agent used.
  - b. **Post Treatment-** Carefully remove the media from the cells by aspiration. Wash the cells twice with 1X sterile PBS or any other appropriate buffer, by covering the cell monolayer completely.
  - c. Detach cells from the tissue culture plates using any appropriate method. Collect cells in a 5 mL round-bottom polystyrene tube.
  - d. Centrifuge the cell suspension for 5 min at 400 x *g* at room temperature and wash with sterile 1x PBS. Discard supernatant.
  - e. Resuspend cells (1 to 5 x 10<sup>5</sup> cells/ sample) in 200 µL of ROS-ID® **Hypoxia/Oxidative Stress Detection Mix** (see Table 1, pg. 6). Incubate under normal tissue culture conditions for 30 min.
  - f. Centrifuge the cells at 400 x *g* for 5 minutes to remove the ROS-ID® **Hypoxia/Oxidative Stress Detection Mix**. Resuspend the cells in 5 mL 1X sterile PBS and centrifuge at 400 x *g* for 5 minutes to remove supernatant.
4. Resuspend the cells in 500 µL of 1X sterile PBS and analyze using flow cytometry. The green fluorescence signal of the Oxidative Stress Detection Reagent should be measured using an FL1 detection channel. The red fluorescence signal of the Hypoxia Detection Reagent should be measured using an FL3 detection channel. No compensation correction is required.

## Flow Cytometry (Suspension Cells)

1. Cells should not exceed a density of ( $1 \times 10^6$  cells/mL) when they are being grown in an appropriate media. It is recommended to setup positive controls for ROS and hypoxia induction (refer to Reagent Preparation section, pg. 5). A negative control is required.

**IMPORTANT:** A sufficient volume of cells should be centrifuged at  $400 \times g$  for 5 minutes, yielding a work cell count of  $1$  to  $5 \times 10^5$  cells/sample. Proceed to step 2 if experimental agent requires short treatment (less than 5 hours) for ROS and hypoxia induction. Proceed to step 3 if greater than 5 hours is required for induction.



Centrifuge

### 2. Short Treatment

**NOTE:** Provided controls for ROS Induction and Hypoxia Induction should use the short treatment protocol.

- a. Centrifuge cells at  $400 \times g$  for 5 minutes and remove supernatant.
- b. Resuspend cells in 200  $\mu$ L of ROS-ID<sup>®</sup> **Hypoxia/Oxidative Stress Detection Mix** (refer to Reagent Preparation section, pg. 5) that contains either the vehicle, experimental agent, ROS Inducer Control (Pyocyanin) or Hypoxia Inducer (DFO) at a desirable working concentration. Concentration of the cells should fall within the range of ( $1$  to  $5 \times 10^5$ ) cells/sample.
- c. Incubate under normal tissue culture conditions ( $37^\circ\text{C}$  at 5%  $\text{CO}_2$ ). ROS Inducer Control (pyocyanin) and Hypoxia Inducer Control (DFO) should be incubated for 30 min and 3.5 hrs., respectively.
- d. **Post Treatment** - Centrifuge the cells at  $400 \times g$  for 5 minutes to remove the ROS-ID<sup>®</sup> **Hypoxia/Oxidative Stress Detection Mix** which contains the vehicle, experimental agent or positive inducer controls. Aspirate the supernatant.
- e. Resuspend the cells in 5 mL 1X sterile PBS and centrifuge at  $400 \times g$  for 5 minutes to remove supernatant. **Proceed to step 4.**

### 3. Long Treatment

- a. Cells should be at a concentration of (1 to 5 x 10<sup>5</sup> cells/sample) in the appropriate media.
  - b. Treat cells with the vehicle or experimental agent at a desirable working concentration. Incubate under normal tissue culture conditions (37°C and 5% CO<sub>2</sub>). Incubation time will vary based on experimental agent used.
  - c. **Post Treatment** - Centrifuge cells for 5 min at 400 x g. Carefully remove the supernatant containing the experimental agent or vehicle.
  - d. Wash the cells twice by covering the cell pellet completely with PBS or any other appropriate buffer. Remove wash following centrifuging cells at 400xg.
  - e. Resuspend the cell pellet in 200 µL (1 to 5 x 10<sup>5</sup> cells/sample) of the ROS-ID® **Hypoxia/Oxidative Stress Detection Mix** (refer to Reagent Preparation section, pg. 5).
  - f. Incubate under normal tissue culture conditions (37°C and 5% CO<sub>2</sub>) at the desired time.
  - g. Centrifuge the cells at 400 x g for 5 minutes and remove the ROS-ID® **Hypoxia/Oxidative Stress Detection Mix** by aspirating the supernatant.
  - h. Resuspend the cells in 5 mL 1X sterile PBS and centrifuge at 400 x g for 5 minutes. Remove supernatant.
4. Resuspend the cells in 500 µL of 1X sterile PBS and analyze using flow cytometry.

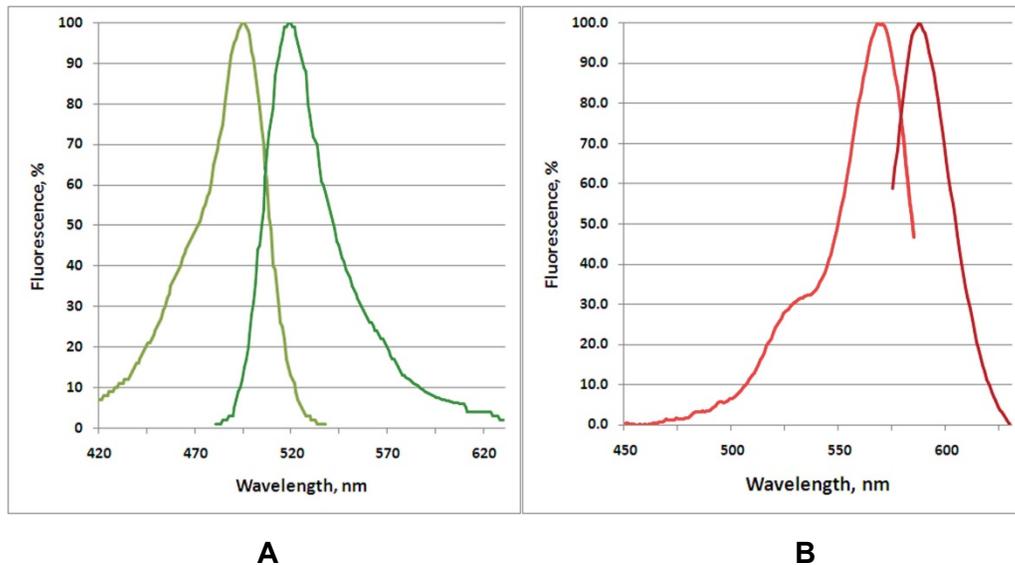
The green fluorescence signal of the Oxidative Stress Green Detection Reagent should be measured using an FL1 detection channel. The red fluorescence signal of the Hypoxia Detection Reagent should be measured using an FL3 detection channel. No compensation correction is required.

#### Filter Set Selection

The selection of optimal filter or monochromator settings for a fluorescence microplate application requires matching the optical specifications to the spectral characteristics of the dyes employed in the analysis. Consult the microplate reader or filter set manufacturer for assistance in selecting optimal filter sets for your microplate reader.

## APPENDICES

### Spectral Characteristics



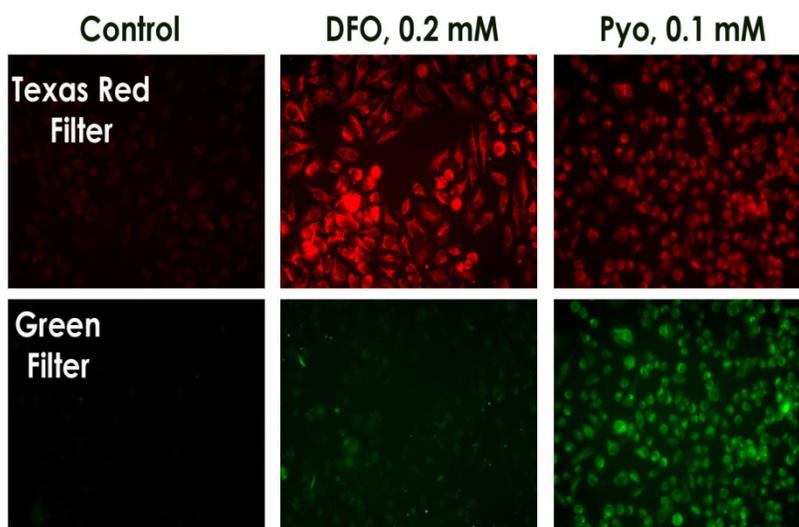
**Figure 1.** The absorption and emission peaks for the Oxidative Stress (A) and Hypoxia Red (B) detection dyes are 504 nm/524 nm and 580 nm/595 nm, respectively. The dyes can be excited with an argon ion laser at 488 nm, and detected in the FL3 channel (Hypoxia Red dye) and FL1 Channel (Oxidative Stress dye) on most bench flow cytometers.

### Technical Hints

The ROS-ID<sup>®</sup> Hypoxia/Oxidative Stress Assay is a functional test that requires living cells in good condition. The cells should always be kept in the appropriate culture medium, containing all the essential components and at the appropriate cell density (confluence) to avoid artificial hypoxic and/or oxidative stress conditions.

### Anticipated Results (Fluorescence Microscopy)

1. It is critical that positive (pyocyanin-induced and hypoxia-induced) and control (untreated) samples be included in every experiment for every cell type. Negative (pre-treated with ROS or hypoxia inhibitors) samples are optional but very helpful. In preliminary experiments, it is important to establish appropriate doses of inducers and inhibitors for each cell type used.
2. Increased levels of oxidative stress provide a uniform green cytoplasmic staining in the presence of the Oxidative Stress Detection Reagent.
3. ROS positive control samples, induced with ROS Inducer (pyocyanin), exhibit a bright green fluorescence in the cytoplasm.
4. Increased hypoxia levels provide a bright red cytoplasmic staining in the presence of Hypoxia Detection Reagent.
5. Hypoxia positive control samples induced with DFO exhibit a bright red fluorescence in the cytoplasm. Typical results of the assay are presented in Figure 2.



**Figure 2.** HeLa cells were seeded on microscope slides and next day treated with DFO (chemical inducer of hypoxia) or pyocyanin (oxidative stress inducer) for 4 h at 37°C as described in the manual. Post-treatment, slides were washed with PBS, cover-slipped and visualized using an Olympus BX-51 fluorescence microscope.

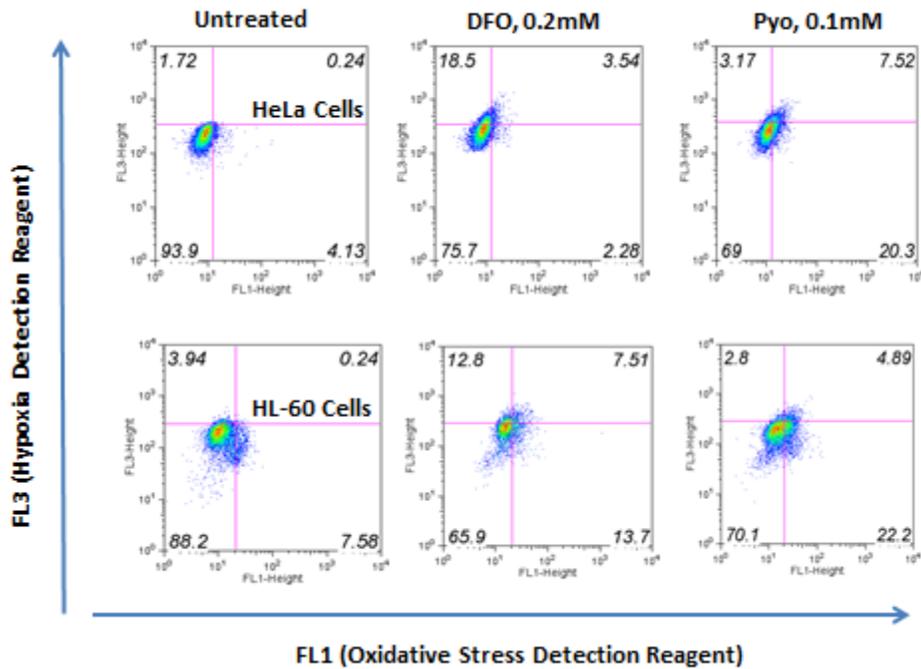
### Anticipated Results (Flow Cytometry).

1. It is critical that positive (pyocyanin-induced and hypoxia-induced) and control (untreated) samples be included in every experiment for every cell type. Negative (inhibitor pre-treated) samples are optional but very helpful. In preliminary experiments it is important to establish appropriate doses of inducers and inhibitors for each cell type used.
2. Cell debris should be gated out using an FSC versus SSC dot plot.
3. Generate a log FL1 (X-axis) versus a log FL3 (Y-axis) dot plot and add quadrants to it. Adjust quadrants so the majority of control cells (80-90%) will fall into the lower left quadrant of the plot. Keep the same quadrant gate throughout the assay.

**Note:** Remember that different cell types demonstrate different redox profiles therefore the number of the cells in the lower left quadrant may vary significantly between cell lines.

4. Cells with increased hypoxia demonstrate bright red fluorescence in the presence of the Hypoxia Red Detection Reagent and will be detected using the FL3 channel. Such cells will appear in the two upper quadrants of a log FL1 (X-axis) versus a log FL3 (Y-axis) dot plot.
5. Cells with increased levels of oxidative stress demonstrate a bright green staining in the presence of the Oxidative Stress Detection Reagent and can be registered in the FL1 channel. Such cells will appear in the upper and lower right quadrants of a log FL1 (X-axis) versus a log FL3 (Y-axis) dot plot.
6. ROS positive control samples, induced with the ROS Inducer (pyocyanin), exhibit both bright red and green fluorescence and appear to be positive in both FL1 and FL3 channels. The increase of the cell population in the upper left, upper right and lower right quadrants will be registered.
7. Cells pretreated with the hypoxia inducer (DFO) will demonstrate significant red fluorescence upon induction, compared to untreated cells. The increase of the cell population in the two upper quadrants will be registered.
8. Control (untreated) samples should present only low autofluorescent background signal in either channel thus falling into the lower left quadrant on an FL1 versus FL3 dot plot.

Results of the experiments can be presented as the percentage of cells with increased ROS production or as the increase in the mean fluorescence of the induced samples versus control.



**Figure 3.** Detection of hypoxia and oxidative stress levels in cultured human HeLa and HL-60 cells. Cells were treated and stained as described in the present manual and subjected to flow cytometry assay. Numbers on the plots reflect the percentage of the cells in each quadrant.

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## TROUBLESHOOTING

Problem	Potential Cause	Suggestion
Low or no fluorescent signal in the positive control.	Dead or stressed (overcrowded) cells.	Prepare fresh cell culture for the experiments. Make sure that the cells are in the log growth phase.
	Band pass filters are too narrow or not optimal for fluorescent probes.	Multiple band pass filters sets provide less light than single band pass ones. Use correct filter for each fluorophore.
	Insufficient concentration of the Hypoxia and/or Oxidative Stress Detection Reagents	Check the concentration of the reagent. Follow the procedures provided in this manual.
	Insufficient inducer concentration.	Determine an appropriate concentration of inducer for the cell line(s) used in the study.
	Inappropriate time point of the detection.	Make sure that time of detection is optimized. Green signal may quench if concentration of product becomes too high (due to long exposure to the inducer). Otherwise, oxidized product may eventually leak out of the cells when left for a prolonged period.
High fluorescent background.	Stressed (overcrowded) cells	Prepare new cell culture for the experiment. Make sure that the cells are in the log growth phase.
	Quality of the reagents is compromised.	Check storage, stability and freshness of reconstituted reagents.
	Band pass filters are too wide or not optimal for fluorescent probes.	Use correct filter for each fluorophore. Check Methods and Procedures section of this manual for the recommendations. Minimal spectral overlap should occur with the selected set of filters.

Problem	Potential Cause	Suggestion
High fluorescent background.	Cell viability is low.	Make sure that you have viable cells at the beginning of the experiment, and that the inducer treatment does not kill the cells during the time frame of the experiment.
High fluorescent background.	Wash step is necessary.	Follow the procedures provided in this manual, making optional wash steps mandatory.
	Inappropriate time point for detection	Make sure that time of detection is optimized.

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