Product Manual

ROS-ID® Total ROS Detection Kit

for fluorescence microscopy, flow cytometry and microplate assay

200 fluorescence microscopy assays or 50 flow cytometryassays or 2 x 96-well plates

Instruction Manual

Catalog Number: **ENZ-51011**

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Product Manual

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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.

I. INTRODUCTION

Free radicals and other reactive species play influential roles in many human physiological and pathophysiological processes, including cell signaling, aging, cancer, atherosclerosis, macular degeneration, sepsis, various neurodegenerative diseases (Alzheimer's and Parkinson's disease) and diabetes. Once produced within a cell, free radicals can damage a wide variety of cellular constituents, including proteins, lipids and DNA. However, at lower concentrations these very same agents may serve as second messengers in cellular signaling. Information-rich methods are required to quantify the relative levels of various reactive species in living cells and tissues, due to the seminal role they play in physiology and pathophysiology. The ROS-ID® Total ROS Detection Kit provides a simple and specific assay for the real-time measurement of global levels of reactive oxygen species (ROS), including peroxynitrite, in living cells.

This kit is designed to directly monitor real time reactive oxygen and/or nitrogen species (ROS/RNS) production in live cells using fluorescence microscopy and/or flow cytometry. The kit includes Oxidative Stress Detection Reagent (Green) as the major component. This non-fluorescent, cell-permeable total ROS detection dye reacts directly with a wide range of reactive species, such as hydrogen peroxide, peroxynitrite and hydroxyl radicals, yielding a green fluorescent product indicative of cellular production of different ROS/RNS types. The kit is not designed to detect superoxide and reactive chlorine or bromine species, as the fluorescent probe included is relatively insensitive to these analytes. Upon staining, the fluorescent product generated can be visualized using a wide-field fluorescence microscope equipped with standard green (490/525nm) filter set, or cytometrically using any flow cytometer equipped with a blue (488 nm) laser.

II. REAGENTS PROVIDED AND STORAGE

All reagents are shipped on dry ice. Upon receipt, the kit should be stored at -20°C, or -80°C for long term storage. When stored properly, these reagents are stable for at least twelve months. Avoid repeated freezing and thawing.

Reagents provided in the kit are sufficient for at least 200 microscopy assays or 50 flow cytometry assays or 2 x 96-well plates using live cells (adherent or in suspension)

Reagent	Quantity
Oxidative Stress Detection Reagent (Green)	300 nmoles
ROS Inducer (Pyocyanin)	1 µmole
ROS Inhibitor (N-acetyl-L-cysteine)	2 x 10 mg
Wash Buffer Salts	1 pack

III. OTHER MATERIALS NEEDED

- CO_2 incubator (37°C)
- Standard fluorescence microscope or flow cytometer equipped with a blue laser (488 nm)
- Calibrated, adjustable precision pipettes, preferably with disposable plastic tips
- 5ml round bottom polystyrene tubes for holding cells during induction of ROS/RNS (for suspension cells only) and during staining and assay procedure
- Adjustable speed centrifuge with swinging buckets
- Glass microscope slides
- Glass cover slips
- Deionized water
- Anhydrous DMF (100%)

IV. SAFETY WARNINGS AND PRECAUTIONS


Handle with care

- This product is for research use only and is not intended for diagnostic purposes.
- 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.


Protect from light

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

V. METHODS AND PROCEDURES

***NOTE**: Allow all reagents to warm to room temperature before starting with the procedures. Upon thawing of solutions, gently hand-mix or vortex the reagents prior to use to ensure a homogenous solution. Briefly centrifuge the vials at the time of first use, as well as for all subsequent uses, to gather the contents at the bottom of the tube.*

A. REAGENT PREPARATION

Reconstitution or dilution of any and all reagents in DMSO should be avoided, as this solvent inhibits hydroxyl radical generation in cells.

1. **Detection Reagent**

   The Oxidative Stress Detection Reagent (Green) is supplied lyophilized and should be reconstituted in 60 µL anhydrous DMF to yield a 5 mM stock solution. 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. Gently mix before use.

2. **Positive Control**

   The ROS Inducer (Pyocyanin) is supplied lyophilized and should be reconstituted in 100 µL anhydrous DMF to yield a 10mM stock solution. For use, a final concentration of 200-500 µM is recommended. 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 pyocyanin treatment and may decrease or disappear after that time. Plan accordingly.

3. **Negative Control**

   The ROS Inhibitor (N-acetyl-L-cysteine) should be reconstituted in 123 µL of deionized water to yield a 0.5 M stock concentration. N-acetyl-cysteine is not readily soluble and may require vortexing. For use, a final concentration of 5mM is recommended. However, the optimal final concentration is cell-dependent and should be determined experimentally for each cell line being tested.

   Endogenous fluorescence of untreated cells should be determined in advance or per assay.

4. **1X Wash Buffer**

   Prepare 1X Wash Buffer by dissolving the contents of the pack in 1 liter of deionized water. When not in use, the 1X Wash Buffer should be stored refrigerated. Warm to room temperature before use.

5. **ROS Detection Solution**

   Prepare the ROS Detection Solution as follows: To every 10 mL of 1X Wash Buffer (see step 4 above) or culture medium, add **2 µL** Oxidative Stress Detection Reagent (Green). Gently mix.

   To prepare smaller volumes of ROS Detection Solution, intermediate1:10 dilution of the Oxidative Stress Detection Reagent (Green) in 1X Wash Buffer or culture medium is recommended.

B. CELL PREPARATION

Cells should be maintained via standard tissue culture practices. Always make sure that cells are healthy and in the log phase of growth before using them for the experiment.

C. FLUORESCENCE/CONFOCAL MICROSCOPY (ADHERENT CELLS)

1. 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 important to have cells for both positive and negative controls.

   IMPORTANT: *Cells should be healthy and not overcrowded since results of the experiments will depend significantly on the cells' condition.*

2. On the day of the experiments, change media to fresh media and label the wells as needed.

3. Simultaneously treat the cells with an experimental test agent (or control) and load the cells with the same volume of the **ROS Detection Solution** (see step A-5).

   i. Treatment conditions and controls

   a) Positive control samples should be established by treatment with ROS Inducer (Pyocyanin) (see step A-2, page 4)

   b) Negative control samples should be established by treatment with the ROS Inhibitor (N-acetyl-L-cysteine (see step A-3, page 5)

   NOTE: *Cells should be pre-treated with the ROS Inhibitor at least 30 minutes prior to induction.*

   c) Untreated samples should use the vehicle

   d) Experimental samples

   IMPORTANT: *If the vehicle, experimental agent and ROS inducer (pyocyanin) will be added to the* ***ROS Detection Solution (2X),*** *their concentration needs to be 2X. For the negative control, 1X NAC needs to be compensated in the solution to maintain its final concentration at the same level.*

4. Carefully remove the ROS Detection Mix from the glass slides by gently tapping them against layers of paper towel, or from tissue culture plates.

5. Carefully wash cells twice with 1X Wash Buffer in a volume sufficient to cover the cell monolayer.

   Add a few drops of 1 x Wash Buffer on the top of the cells and immediately overlay the cells with a cover slip and observe them under a fluorescence/confocal microscope using standard excitation/emission filter sets. Oxidative

stress detection requires a filter set compatible with Fluorescein (Ex/Em: 490/525 nm). Make sure prepared samples are protected from drying. Dried out cells may present different fluorescence patterns.

D. FLUORESCENCE/CONFOCAL MICROSCOPY (SUSPENSION CELLS)

1. Cells should be cultured to a density not to exceed 1 x 10^6 cells/mL. Make sure that cells are in the log phase of growth before starting an experiment.

   IMPORTANT: *Cells should be healthy and not overcrowded since results of the experiments will depend significantly on the cells' overall condition. A sufficient volume of cells should be centrifuged at 400 x g for 5 minutes, yielding a working cell count of 1 x 10^5 cells/sample.*

2. Centrifuge the cells at 400 x g for 5 minutes to remove the supernatant.
3. Resuspend the cells in fresh media and aliquot 1 x 10^5 cells/sample at 1-5 x 10^5 cells/ml.
4. Simultaneously treat the cells with an experimental test agent (or control) and load the cells with the same volume of the **ROS Detection Solution** (see step A-5).
   - **i. Treatment conditions and controls**
     a) Positive control samples should be established by treatment with ROS Inducer (Pyocyanin) (see step A-2, page 4)
     b) Negative control samples should be established by treatment with the ROS Inhibitor (N-acetyl-L-cysteine (see step A-3, page 5)

        NOTE: *Cells should be pre-treated with the ROS Inhibitor at least 30 minutes prior to induction.*

     c) Untreated samples should use the vehicle
     d) Experimental samples

   IMPORTANT: *If the vehicle, experimental agent and ROS inducer (pyocyanin) will be added to the* ***ROS Detection Solution (2X),*** *their concentration needs to be 2X. For the negative control, 1X NAC needs to be compensated in the solution to maintain its final concentration at the same level.*

5. Centrifuge the cells at 400 x g for 5 minutes to remove the ROS Detection Solution.
6. Resuspend the cells in 5 mL of 1X Wash Buffer, centrifuge them at 400 x g for 5 minutes and remove the supernatant.
7. Resuspend the cells in 100 µL of 1X Wash Buffer and apply a 20 µL aliquot of the cell suspension, sufficient for 2 x 10^4 cells, onto a microscope slide. Immediately overlay the cells with a cover slip and analyze via fluorescence microscopy. Oxidative stress detection requires a filter set compatible with Fluorescein (Ex/Em: 490/525 nm). Make sure that prepared samples are protected from drying. Dried out cells may present different fluorescence patterns.

E. FLOW CYTOMETRY (ADHERENT CELLS)

1. The day before the experiment, seed the cells on appropriate tissue culture plates to ensure ~ 50-70% confluency on the day of the experiment.

   IMPORTANT: *Cells should be healthy and not overcrowded since results of the experiments will depend significantly on the cells' condition.*

2. Detach cells from the tissue culture plates using any appropriate method, collect cells in 5 mL round-bottom polystyrene tubes and wash them with 1X Wash Buffer. Centrifuge the cell suspension for 5 min. at 400 x g at room temperature. Discard the supernatant.
3. Simultaneously treat the cells with an experimental test agent (or control) and load the cells with the **ROS Detection Solution** (see step A-5).

   i. Treatment conditions and controls

   a) Positive control samples should be established by treatment with ROS Inducer (Pyocyanin) (see step A-2, page 4)

   b) Negative control samples should be established by treatment with the ROS Inhibitor (N-acetyl-L-cysteine (see step A-3, page 5)

   NOTE: *Cells should be pre-treated with the ROS Inhibitor at least 30 minutes prior to induction.*

   c) Untreated samples should use the vehicle

   d) Experimental samples

ii. Loading the cells with dye

Resuspend cell pellet in 500 µL of ROS Detection Solution, containing the treatment. Stain cells for 30 min. at 37°C in the dark. No washing is required prior to the analysis of the samples by flow cytometry.

Recommended controls for flow cytometry:

- Unstained untreated cells
- Positive control cells (pyocyanin-treated) stained with Oxidative Stress Detection Reagent

F. FLOW CYTOMETRY (SUSPENSION CELLS)

1. Cells should be cultured to a density not to exceed 1 x 10^6 cells/mL. Make sure that cells are in the log phase of growth before starting an experiment.

   IMPORTANT: *Cells should be healthy and not overcrowded since results of the experiments will depend significantly on the cell's overall condition. A sufficient volume of cells should be centrifuged at 400 x g for 5 minutes, yielding a working cell count of 1-5 x 10^5 cells/sample.*

2. Centrifuge cells for 400 x *g* for 5 minutes and remove the supernatant.
3. Resuspend cells in fresh media at a concentration of 1- 5 x 10^5/mL. Aliquot 0.5-1 mL per sample into flow tubes.
4. Simultaneously treat the cells with an experimental test agent (or control) and load the cells with the **ROS Detection Solution** (see step A-5).

   a. Treatment conditions and controls

   i. Positive control samples should be established by treatment with ROS Inducer (Pyocyanin) (see step A-2, page 4)

   ii. Negative control samples should be established by treatment with the ROS Inhibitor (N-acetyl-L-cysteine (see step A-3, page 5)

   NOTE: *Cells should be pre-treated with the ROS Inhibitor at least 30 minutes prior to induction.*

   iii. Untreated samples should use the vehicle

   iv. Experimental samples

   b. Loading the cells with dye

Resuspend cell pellet in 500 µL of ROS Detection Solution, containing the treatment. Stain cells for 30 min. at 37°C in the dark. No washing is required prior to the analysis of the samples by flow cytometry.

Recommended controls for flow cytometry:

- Unstained untreated cells
- Positive control cells (pyocyanin-treated) stained with Oxidative Stress Detection Reagent

G. FLUORESCENCE MICROPLATE ASSAY (ADHERENT CELLS)

1. The day before the experiment, seed the cells in 96-well black wall/clear bottom plates at a density of 1–2 x10^4 cells per well to ensure ~ 70-80% confluency on the day of the experiment. Leave several wells empty for the background fluorescence control measurements.

   ***Important**: Cells should be healthy and not overcrowded since results of the experiments will depend significantly on the cells' condition.*

2. Remove supernatant from cells.
3. Carefully wash cells with 1X Wash Buffer.
4. Simultaneously treat the cells with an experimental test agent (or controls) and load the cells with the **ROS Detection Solution** (see step A-5, page 5).

   i. Treatment conditions and controls

   For the highest quality data use at least 6 – 8 replicates for each condition tested.

   1. Positive control samples should be established by treatment with ROS Inducer (Pyocyanin) (see step A-2, page 4)
   2. Negative control samples should be established by treatment with the ROS Inhibitor (N-acetyl-L-cysteine) (see step A-3, page 5)

      ***NOTE:** Cells should be pre-treated with the ROS Inhibitor at least 30 minutes prior to induction.*

3. Untreated samples should use the vehicle
4. Experimental samples

ii. Loading the cells with dye

Add 100 µL/well of **ROS Detection Solution**, containing the treatment. Stain cells for 60 min. at 37°C in the dark.

5. Read the plates (bottom reading), without removing the detection mix, using a fluorescence microplate reader and a standard fluorescein (Ex=488 nm, Em=520 nm) filter sets.
6. If required, Z' factor may be calculated for each detection profile using the following formula:(5)

$$Z' = 1-[(3*SD_{sample}+3*SD_{control})/(|Mean_{sample} - Mean_{control}|)]$$

H. FLUORESCENCE MICROPLATE ASSAY (SUSPENSION CELLS)

***NOTE**: Perform all steps requiring centrifugation at 400 x g for 5 minutes using a centrifuge with swinging buckets.*

1. Collect the cells by centrifugation at 400 x *g* for 5 min, resuspend them in the appropriate cell culture medium at a density of 0.5 x 10^6 - 1.0 x 10^6 cells/mL, count and aliquot 100 µL of the suspension into wells of 96-well black wall plates. Leave several wells empty for the background fluorescence control measurements.

   ***Important**: Cells should be healthy and not overcrowded since results of the experiments will depend significantly on the cells' condition.*
2. Remove the medium by centrifugation of the plate.
3. Wash cells by centrifugation with 1X Wash Buffer.
4. Simultaneously treat the cells with an experimental test agent (or controls) and load the cells with the **ROS Detection Solution** (see step A-5, page 5).

iii. Treatment conditions and controls

For the highest quality data use at least 6 – 8 replicates for each condition tested.

a) Positive control samples should be established by treatment with ROS Inducer (Pyocyanin) (see step A-2, page 4)

b) Negative control samples should be established by treatment with the ROS Inhibitor (N-acetyl-L-cysteine) (see step A-3, page 5)

NOTE: *Cells should be pre-treated with the ROS Inhibitor at least 30 minutes prior to induction.*

c) Untreated samples should use the vehicle

d) Experimental samples

iv. Loading the cells with dye

Add 100 µL/well of **ROS Detection Solution**, containing the treatment. Stain cells for 60 min. at 37°C in the dark.

5. Read the plates (bottom reading), without removing the detection mix, using a fluorescence microplate reader and a standard fluorescein (Ex=488 nm, Em=520 nm) filter set.
6. If required, Z' factor may be calculated for each detection profile using the following formula:(5)

$$Z' = 1 - [(3*SD_{sample}+3*SD_{control})/(|Mean_{sample} - Mean_{control}|)]$$

VI. APPENDICES

A. FILTER SET SELECTION

For fluorescence microscopy, careful consideration must be paid to the selection of filters. Dichroic filters should be selected in which the "cut-off" frequency is optimally mid-way between the two emission bands that are desired (one reflected, the other transmitted). However, it is important to realize that dichroic filters have a somewhat limited reflectance range, *i.e.,* a 600 nm short-pass dichroic filter may actually reflect light <500 nm. When selecting filters, it is critical to discuss with the filter or microscope manufacturer exactly what wavelength specifications are required for both the transmitted and the reflected light.

In addition, filters should be obtained that have the highest possible transmission efficiency (typically requiring anti-reflection coating). Each optic that an emission beam must traverse will remove some fraction of the desired light. The difference between 80% transmission and 95%

transmission for each detector may result in a greater than three-fold difference in the amount of light available to the detector.

B. SETTING UP OPTIMAL EXPOSURE TIME FOR DETECTION OF THE DYE

Optimal exposure times should be established experimentally for each dye used in the experiment. Both negative and positive controls should be utilized. Start with the negative control (untreated stained cells) and set up the exposure time so the fluorescent background is negligible. Then switch to a positive control (pyocyanin-treated cells) and adjust the exposure time to record a bright fluorescent image. Avoid saturation of the signal (very bright spots on the image). If saturation of the signal occurs, decrease the exposure time. It is recommended to acquire 5-6 single color images for each sample.

C. FLUORESCENCE MICROSCOPY ANTICIPATED RESULTS

1. It is critical that positive (pyocyanin-induced) and control (untreated) samples be included in every experiment for every cell type. Negative (ROS Inhibitor-pretreated) sample is 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 give a uniform green cytoplasmic staining in the presence of the Oxidative Stress Detection Reagent (Green).
3. ROS positive control samples, induced with ROS Inducer (Pyocyanin), exhibit a bright green fluorescence in the cytoplasm.
4. Cells pretreated with the ROS Inhibitor (N-acetyl-L-cysteine) should not demonstrate any green or orange fluorescence upon induction.
5. Untreated samples should present only low autofluorescent background signal in any channel.

D. FLOW CYTOMETRY DATA ANALYSIS AND ANTICIPATED RESULTS

1. It is critical that positive (pyocyanin-induced) and control (untreated) samples be included in every experiment for every cell type. Negative (ROS Inhibitor-pretreated) sample is 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 FSC versus SSC dot plot.
3. Generate a log FL1 (X-axis) versus a log FL2 or SSC (Y-axis) dot plot and add quadrants to it. Adjust quadrants so the majority of control cells (80-90%) will fall into lower left quadrant. Keep the same quadrant gate throughout the assay. Alternatively, log FL1 histogram can be used, where the mean fluorescence of the peak for the untreated cells should fall within the first decade of a log FL1 scale.

   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 the cell lines.*
4. Cells with increased levels of oxidative stress demonstrate bright green fluorescence in the presence of the Oxidative Stress Detection Reagent and will be detected using FL1 channel. Such cells will appear in the two right quadrants of a log FL1 (X-axis) versus FSC or SSC dot plot. If log FL1 histogram is used, the peak generated by the ROS positive cells will have increased FL1 fluorescence compared to a control cells' fluorescence.
5. ROS positive control samples, induced with ROS Inducer (Pyocyanin), exhibit bright green fluorescence and appear to be positive in FL1 channel.
6. Cells pretreated with the ROS Inhibitor (N-acetyl-L-cysteine) should not demonstrate significant green fluorescence upon induction.
7. Control (untreated) samples should present only low autofluorescent background signal in any channel thus falling into the first decade on a log FL2 scale.

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

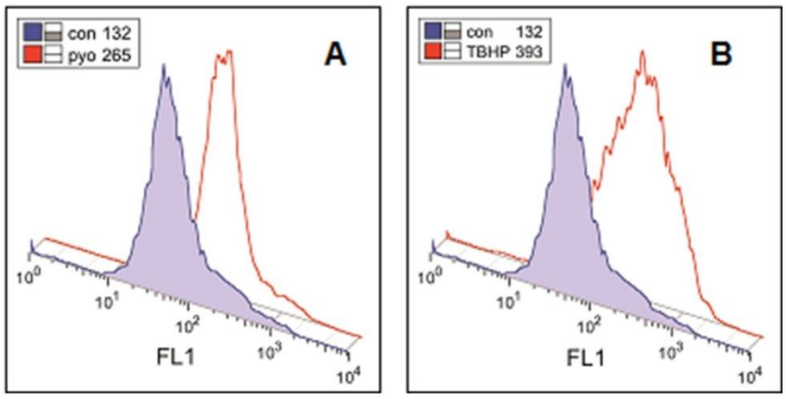

Figure 1. Jurkat cells were induced with 100 µM pyocyanin (general ROS inducer, panel A), or 1 µM of t-butyl-hydroperoxide (peroxide inducer, panel B), stained with ROS-ID® Total ROS Detection Reagent and analyzed using flow cytometry. Untreated cells were used as a control. Cell debris were ungated. The numbers in the inserts reflect the mean green fluorescence of the control and treated cells.

E. FLUORESCENCE MICROPLATE ASSAY DATA ANALYSIS AND ANTICIPATED RESULTS

1. It is critical that positive (pyocyanin-induced) and control (untreated) samples be included in every experiment for every cell type. Negative (NAC-pretreated) sample is optional, but very helpful. In preliminary experiments, it is important to establish appropriate doses of inducers and inhibitors for each cell type used.
2. Cells with increased levels of oxidative stress demonstrate a bright green staining in the presence of the Oxidative Stress Detection Reagent and the signal will be detected using standard fluorescein filter setting.
3. ROS positive control samples, induced with ROS Inducer (Pyocyanin), exhibit bright green fluorescence and can be detected in the green channel. Cells pretreated with the ROS Inhibitor (N-acetyl-L-cysteine) should not demonstrate significant green fluorescence upon induction.

4. Control (untreated) samples should present only low autofluorescence signal in the green channel.

   ***NOTE**: Remember that different cell types demonstrate different redox profiles. Therefore, the auto-fluorescence signal may vary significantly.*

5. Results of the experiments should be normalized using the background readings from empty wells (see step H1) and can be presented as ratios of the mean fluorescence of the induced samples versus control for the green channel.

6.

F. FLUORESCENCE MICROPLATE ASSAY DATA ANALYSIS AND ANTICIPATED RESULTS

i. It is critical that positive (pyocyanin-induced) and control (untreated) samples be included in every experiment for every cell type. Negative (NAC-pretreated) sample is optional, but very helpful. In preliminary experiments, it is important to establish appropriate doses of inducers and inhibitors for each cell type used.

ii. Cells with increased levels of oxidative stress demonstrate a bright green staining in the presence of the Oxidative Stress Detection Reagent and the signal will be detected using standard fluorescein filter setting.

iii. ROS positive control samples, induced with ROS Inducer (Pyocyanin), exhibit both bright orange and green fluorescence and can be detected in both green and orange channels. Cells pretreated with the ROS Inhibitor (N-acetyl-L-cysteine) should not demonstrate significant green or orange fluorescence upon induction.

iv. Control (untreated) samples should present only low autofluorescence signal in both channels.

   ***NOTE**: Remember that different cell types demonstrate different redox profiles. Therefore, the auto-fluorescence signal may vary significantly in both channels.*

v. Results of the experiments should be normalized using the background readings from empty wells (see step H1) and can be presented as ratios of the mean

fluorescence of the induced samples versus control for each channel.

G. REFERENCES

1. Tarpey, M. and Fridovich, I. Circ Res. **89** (2001), 224-236.
2. Batandier,C., *et al.* J Cell Mol Med. **6** (2002), 175-187.
3. Gomes, A., *et al*. J Biochem Biophys Meth. **65** (2005), 45-80.
4. Wardman, P. Free Rad Biol Med. **43** (2007), 995-1022.

H. TROUBLESHOOTING GUIDE

Problem	Potential Cause	Suggestion
Low or no fluorescent signal in 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 (fluorescence microscopy)	Multiple band pass filters sets provide less light than single band pass ones. Check Methods and Procedures section of this manual and Appendix A for recommendations.
	Insufficient fluorescent dye concentration	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.
	Species of interest may react with each other, thus attenuating the expected signal.	Check signaling pathways and all the components present in the cellular environment.
	Inappropriate time point of the detection	Make sure that time of detection is optimized and the samples are prepared immediately. 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.
	Cell density is too low in microplate well.	Check the cell count to confirm proper cell density. For suspension cells, careful removal of supernatant after washing steps is critical, as cells may be dislodged and washed away.

Problem	Potential Cause	Suggestion
High fluorescent background	Stressed (overcrowded) cells	Prepare new cell culture for the experiment. Make sure that the cells are in the log growth phase.
	Band pass filters are too narrow or not optimal for fluorescent probes.	Use correct filter for each fluorophore. Check Methods and Procedures section of this manual and Appendix A for the recommendations.
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 and the samples are prepared immediately.
	Inappropriate cell conditions	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.
No decrease in the fluorescent signal after using a specific inhibitor	Inappropriate inhibitor concentration (too low or too high)	Very low doses of inhibitor may not affect ROS production by inducer. Alternatively, very high doses of the inhibitors may cause oxidative stress itself and generate fluorescent signal. Optimize the concentration of the inhibitor and pretreatment time for each particular cell line.
	Inappropriate time point for detection	When cells are kept too long with the inhibitors or at very high inducer concentrations, after a certain time, the inhibitor becomes insufficient. Make sure that time of detection is optimized.
	Inappropriate filter set on the microscope	Use correct filter for excitation and emission. Check **Methods and Procedures** section of this manual and **Appendix A** for the recommendations.

NOTES

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