ab139476 – Cellular ROS/Superoxide Detection Assay Kit

Instructions for Use

Designed to detect ROS/Superoxide production in live cells using Fluorescence Microscopy, Flow Cytometry and microplate assay

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

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1. 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.

Cellular ROS/Superoxide Detection Assay Kit (ab139476) enables detection of comparative levels of total ROS and also allows determination of superoxide production in live cells. Through the combination of two specific fluorescent probes, the kit provides a simple and specific assay for the real-time measurement of global levels of reactive oxygen species (ROS), peroxynitrite and specifically superoxide in living cells.

2. Product Overview

Cellular ROS/Superoxide Detection Assay (ab139476) is designed to directly monitor real time reactive oxygen species (ROS) production in live cells using fluorescence microscopy and/or flow cytometry. The kit includes two fluorescent dye reagents as major components: Oxidative Stress Detection Reagent (Green) for ROS detection and Superoxide Detection Reagent (Orange). The non-fluorescent, cell-permeable ROS detection dye (green probe) reacts directly with a wide range of reactive species, such as hydrogen peroxide (H_2O_2), peroxynitrite ($ONOO^-$), hydroxyl radicals (HO), nitric oxide (NO), and peroxy radical (ROO), yielding a green fluorescent product indicative of cellular production of different ROS/RNS types. However, the green probe has a low sensitivity for superoxide (O_2^-), as well as hypochlorous acid (HClO) and nitric oxide (NO), relative to other free radicals. The superoxide detection dye (orange probe) is a cell permeable probe that reacts specifically with superoxide (O_2^-), generating an orange fluorescent product.

Upon staining, the fluorescent products generated by the two dyes can be visualized using a wide-field fluorescence microscope equipped with standard green (Ex/Em = 490/525 nm) and orange (Ex/Em = 550/620 nm) filter set, or by cytometry using any flow cytometer equipped with a blue laser (488 nm filter).

The kit is not designed to detect reactive chlorine or bromine species, as the fluorescent probes included are relatively insensitive to these analytes.

3. Assay Summary

Reconstitute detection reagents, inducers, inhibitors and controls and warm buffers to room temperature

Add ROS inhibitor to sample. Incubate for 30 minutes.

Load ROS/Superoxide Detection Mix +

+ vehicle/experimental agent/ROS inducer

onto the samples and incubate for 30 min – 1 hr.

Analyze

4. Kit Components

Item	Quantity	Storage Temperature
Oxidative Stress Detection Reagent (Green)	300 nM	-80°C
Superoxide Detection Reagent (Orange)	300 nM	-80°C
ROS Inducer (Pyocyanin)	1 µM	-80°C
ROS Inhibitor (N-acetyl-L-cysteine)	2 x 10 mg	-80°C
Wash Buffer Salts	1 pack	-80°C

Reagents provided in the kit are sufficient for at least 200 microscopy assays or 50 Flow Cytometry assays using live cells (adherent or in suspension).

5. Storage and Stability

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

Briefly centrifuge small vials at low speed prior to opening. Read the entire protocol before performing the experiment.

6. Materials Required, Not Supplied

- CO_2 incubator (37°C).
- Calibrated, adjustable precision pipetters, preferably with disposable plastic tips.
- 5 ml 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.
- Deionized water.
- Anhydrous DMF (100%).

For Fluorescence/Confocal Microscopy analysis:

- Standard fluorescence microscope equipped with a filter set compatible with Fluorescein (Ex/Em = 490/525nm) and Rhodamine (Ex/Em = 550/620nm).
- Glass microscope slides.
- Glass cover slips.

For Flow Cytometry analysis:

- Standard flow cytometer equipped with a blue laser (488nm)
- Flow cytometer tubes.

For Fluorescence Microplate analysis:

- Fluorescence microplate reader.
- 96-well black wall/clear bottom plates.

7. Pre-Assay Preparation

NOTE: Allow all reagents to thaw at room temperature before starting with the procedures. Once thawed, 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 reagent in DMSO should be avoided, as this solvent inhibits hydroxyl radical generation in cells.

1. Detection Reagents

1.1 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 for up to 1 week. Gently mix before use.

1.2 The Superoxide Detection Reagent (Orange) is supplied lyophilized and should be reconstituted in 60 µL anhydrous DMF to yield a 5 mM stock solution concentration. Upon reconstitution, the stock solution should be stored at -20°C for up to 1 month. 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 10 mM 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 solution. N-acetyl-cysteine is not readily soluble and may require vortexing. For use, a final concentration of 5 mM 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. **2x ROS/Superoxide Detection Mix**

   Prepare the 2x ROS/Superoxide Detection Mix as follows:

- To every 10 ml of 1X Wash Buffer (see step 7.A.4) or culture medium, add 4 µL Oxidative Stress Detection Reagent (Green) and 4 µL Superoxide Detection Reagent (Orange).
- Gently mix to make 1:2500 diluted staining solution. To prepare smaller volumes of 2x ROS/Superoxide Detection Mix, intermediate 1:10 dilution of both Green and Orange detection reagents in 1X Wash Buffer or culture medium is recommended.

NOTE: *For different cell lines, the staining efficiency may be different. 1:500 to 1:2500 (2x) is the suggested range for testing.*

NOTE: *To perform the compensation correction, single stained samples should be used. Make sure single component solutions (Green and Orange) are available. Depending on the experiments, dyes can be used separately according to a provided protocol.*

B. Cell Preparations

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.

8. Assay Protocol

A. 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.

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. For the negative control, add ROS inhibitor (N-acetyl-L-cysteine) to pre-treat the cells for at least 30 min prior to induction.
4. Induction: load the cells with the ROS/Superoxide Detection Mix (see 7.A.Step 5) with the addition of either the vehicle, experimental agent, ROS inducer (pyocyanin) at a desirable working concentration. Fully cover the cell monolayer. Incubate under normal tissue culture conditions for 30 min – 1 hour.

IMPORTANT: If the vehicle, experimental agent and ROS inducer (pyocyanin) will be added to the ROS/Superoxide 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. Carefully remove the 2x ROS/Superoxide Detection Mix from the glass slides by gently tapping them against layers of paper towel, or from tissue culture plates.
6. Carefully wash cells twice with 1X Wash Buffer in a volume sufficient to cover the cell monolayer.
7. Add a few drops of 1 x Wash Buffer on the top of the cells and immediately overlay the cells with a cover slip. Observe cells under a fluorescence/confocal microscope using standard excitation/emission filter sets. Oxidative stress detection requires a filter set compatible with Fluorescein (Ex/Em = 490/525nm). Make sure prepared samples are protected from drying. Dried out cells may present different fluorescence patterns.

Recommended filter sets:

- Oxidative stress (ROS) detection requires a filter set compatible with Fluorescein (Ex/Em = 490/525nm).
- Superoxide (O_2^-) detection requires a filter set compatible with Rhodamine (Ex/Em = 550/620nm).

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

B. 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.

2. Centrifuge a sufficient number of cells at 400 x g for 5 minutes and remove the supernatant. You should have a working cell count of 1 x 10^5 cells/sample.
3. Resuspend the cells in fresh media. Aliquot 1 x 10^5 cells/sample at 200 µL – 1 mL to achieve a cell density of 1 – 5 x 10^5 cells/mL.
4. For the negative control, add ROS inhibitor (N-acetyl-L-cysteine) to pre-treat the cells for at least 30 min prior to induction.
5. Induction: load the same volume of the ROS/Superoxide Detection Solution (2X) with addition of either the vehicle, experimental agent or ROS inducer (pyocyanin) at a desirable working concentration. Incubate under normal tissue culture conditions for 30 min - 1 hour with periodic shaking.

IMPORTANT: If the vehicle, experimental agent and ROS inducer (pyocyanin) will be added to the ROS/Superoxide 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.

6. Centrifuge the cells at 400x *g* for 5 minutes to remove the ROS Detection Solution.

7. Resuspend the cells in 5 ml of 1X Wash Buffer, centrifuge them at 400x *g* for 5 minutes and remove the supernatant.
8. 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 coverslip and analyze immediately in a fluorescence microscope. Make sure that prepared samples are protected from drying. Dried out cells may present different fluorescence patterns.

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

C. 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 5mL round-bottom polystyrene tubes and wash them with 1X Wash Buffer. Centrifuge the cell suspension for 5 min. at 400x *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/Superoxide Detection Solution.
4. Treatment conditions and controls:
    a) Positive control samples should be established by treatment with ROS Inducer (Pyocyanin).
    b) Negative control samples should be established by treatment with the ROS Inhibitor (N-acetyl-L-cysteine). 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.
5. Load the cells with dye: resuspend cell pellet in 500µL of ROS/Superoxide Detection Solution, containing the treatment. Incubate cells for 30 min at 37°C in the dark. No washing is required prior to the analysis of the samples by flow cytometry.

IMPORTANT: Compensation correction will be needed to avoid overlap between green and orange fluorescent signals (see Section 9.D).

Recommended controls for compensation correction:

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

- Positive control cells (pyocyanin-treated) stained with Superoxide Detection Reagent (Orange) only (”Orange” cells)

D. Flow Cytometry (Suspension Cells)

1. Cells should be cultured to a density less than $1x10^6$ 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.

2. Centrifuge a sufficient number of cells for 400x *g* for 5 minutes and remove the supernatant. You should have a working cell count of 1×10^5 cells/sample.
3. Resuspend cells in fresh media at a concentration of 1-5 x 10^5 cells/mL. Aliquot 0.5-1mL per sample into flow tubes.
4. Negative control: add ROS inhibitor (N-acetyl-L-cysteine) to pre-treat the cells for at least 30 min prior to induction.
5. Induction: load the same volume of the ROS/Superoxide Detection Solution (2X) with addition of either the vehicle, experimental agent, ROS inducer (pyocyanin) at a desirable working concentration. Incubate under normal tissue culture conditions for 30 min to 1 hour in ther dark with periodic shaking. No washing is required prior to the analysis of the samples by flow cytometry.

IMPORTANT: If the vehicle, experimental agent and ROS inducer (pyocyanin) will be added to the ROS/Superoxide Detection Solution (2X), their concentration need 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.

IMPORTANT: Compensation correction should be performed to avoid overlap between the green and orange fluorescent signals.

Recommended controls for Flow Cytometry:

- Unstained untreated cells.
- Positive control cells (pyocyanin-treated) stained with Oxidative Stress Detection Reagent (Green) only ("Green" cells).
- Positive control cells (pyocyanin-treated) stained with Superoxide Detection Reagent (Orange) only ("Orange" cells).

E. 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/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/Superoxide Detection Solution.
    i. 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).
        b) Negative control samples should be established by treatment with the ROS Inhibitor (N-acetyl-L-cysteine). 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:
    Add 100µL/well of ROS/Superoxide 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 standard fluorescein (Ex=488nm, Em=520nm) and rhodamine (Ex=550nm, Em=610nm) filter sets.

6. If required, Z' factor may be calculated for each detection profile using the following formula:

$$z' = 1 - \left[\frac{3 * SD\ sample + 3 * SD\ control}{Mean\ sample - Mean\ control}\right]$$

F. 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, re-suspend them in the appropriate cell culture medium at a density of 0.5 x 10^6 - 1 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/Superoxide Detection Solution. 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).
b) Negative control samples should be established by treatment with the ROS Inhibitor (N-acetyl-L-cysteine). 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

5. Loading the cells with dye: Add 100µL/well of ROS/Superoxide Detection Solution, containing the treatment. Stain cells for 60 min at 37°C in the dark.

6. If required, Z' factor may be calculated for each detection profile using the following formula:

$$z' = 1 - \left[\frac{3 * SD\ sample + 3 * SD\ control}{Mean\ sample - Mean\ control}\right]$$

9. Data Analysis

FLUORESCENCE MICROSCOPY

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.

B. Setting up optimal exposure time for detection of the dyes

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 (arginine or 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 dye for each sample.

C. Anticipated Results

- The Superoxide Detection Reagent (Orange) yields an evenly distributed, bright orange nuclear staining pattern in induced cells. Note the structural change in positively treated cells versus control untreated cells = diffuse, dim cytoplasmic structural pattern observed in the control cells is replaced with uniform cytoplasmic staining and bright nuclear staining in superoxide-positive cells.
- Increased levels of oxidative stress give a uniform green cytoplasmic staining in the presence of the Oxidative Stress Detection Reagent (Green).
- ROS positive control samples, induced with ROS Inducer (Pyocyanin), exhibit a bright orange fluorescence in the nucleus as well as a bright green fluorescence in the cytoplasm.
- Cells pretreated with the ROS Inhibitor (N-acetyl-L-cysteine) should not demonstrate any green or orange fluorescence upon induction.
- Untreated samples should present only low autofluorescent background signal in any channel.

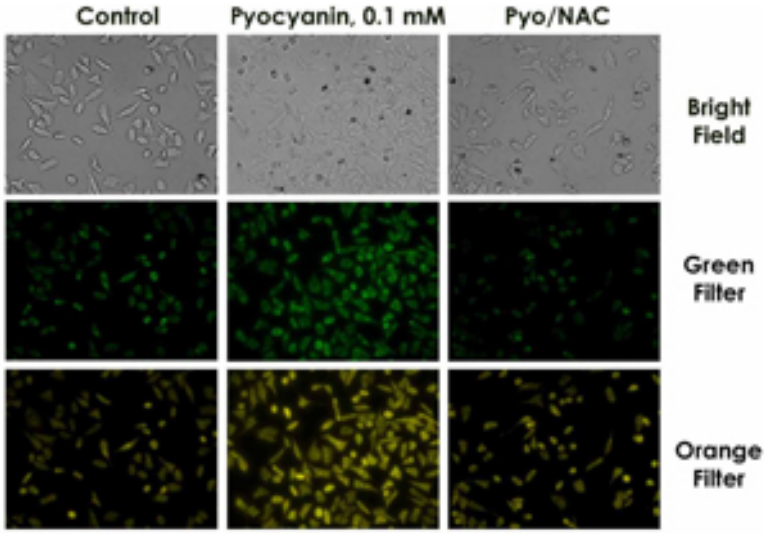

Figure 1. Profiling of reactive oxygen species formation by fluorescence microscopy was achieved in HeLa cells loaded with ROS/Superoxide detection reagents and treated with pyocyanin. General oxidative stress levels were monitored in the green channel, while superoxide production was detected in the orange channel. Pretreatment with NAC (N-acetyl-L-cysteine), a general ROS inhibitor prevents formation of ROS.

FLOW CYTOMETRY

D. Compensation Correction

Signals produced by peroxides, peroxynitrite and hydroxyl radicals will be detected in the FL1 channel. Superoxide production will be detected in the FL2 channel. To avoid overlap between green and orange fluorescent signals the following compensation procedure should be performed.

1. Run the unstained uninduced sample first. Generate a FSC versus SSC dot plot and gate out cell debris.
2. Generate a log FL1 (X-axis) versus a log FL2 (Y-axis) dot plot. Adjust PMT voltages for both channels so the signals from unstained cells should fall within the first log decade scale of FL1 and FL2 axes.
3. Run single stained “Green” positive control and adjust FL2-%FL1 compensation until the orange fluorescence signal will fall into the first decade of the log FL2 scale.
4. Repeat compensation procedure with the “Orange” single stained positive control and adjust FL1-%FL2 compensation until the green fluorescence signal will fall into the first decade of the log FL1 scale.

NOTE: *It is important to use the brightest positive single stained samples for proper compensation correction that allows distinguishing between negative and slightly positive (dim) cells.*

E. 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. Cell debris should be gated out using FSC/SSC dot plot.
3. Generate a log FL1 (X-axis) versus a log FL2 (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.

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 production of superoxide demonstrate bright orange fluorescence and will be detected using the FL2 channel. Such cells will appear in the two upper quadrants of a log FL1 (X-axis) versus a log FL2 (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 FL1 channel.

Such cells will appear in the upper and lower right quadrants of a log FL1 (X-axis) versus a log FL2 (Y-axis) dot plot.

6. ROS positive control samples, induced with ROS Inducer (Pyocyanin), exhibit both bright orange and green fluorescence and appear to be positive in FL1 and FL2 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 ROS Inhibitor (N-acetyl-L-cysteine) should not demonstrate significant green or orange fluorescence upon induction.
8. Control (untreated) samples should present only low autofluorescent background signal in any channel thus falling into the lower left quadrant on an FL1 versus FL2 dot plot.
9. 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.

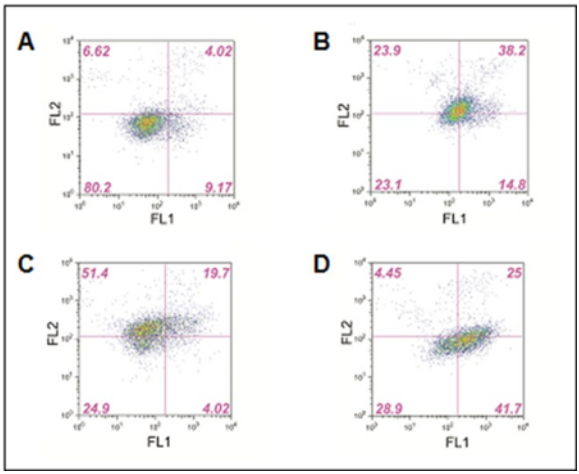

Figure 2. Jurkat cells were induced with 100 μM pyocyanin (general ROS inducer, **panel B**), 200 μM antimycin A (superoxide inducer, **panel C**) or 1 μM of t- butylhydroperoxide (peroxide inducer, **panel D**), stained with two color ROS Detection Kit and analyzed using flow cytometry. Untreated cells (**panel A**) were used as a control. Cell debris were ungated and compensation was performed using single stained pyocyanin-treated samples. Red numbers reflect the percentage of the cells in each quadrant.

FLUORESCENCE MICROPLATE READER

F. Data Analysis and Anticipated Results

1. It is critical that positive (pyocyanin-induced) and control (untreated) samples are 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 production of superoxide demonstrate bright orange fluorescence in the presence of the Superoxide Detection Reagent and the signal will be detected using standard rhodamine filter settings.
3. 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.
4. 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.
5. 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.*

6. 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.

10. Troubleshooting

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. Use correct filter for each fluorophore. Check Pre-Assay Preparation section of this manual and Section 9A for recommendations.
	Insufficient fluorescent dye concentration	Follow the procedures provided in this manual.

Problem	Potential Cause	Suggestion
Low or no fluorescent signal in positive control	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.
	Overcompensation of the signal (Flow Cytometry)	Change the values of compensation correction using single stained positive samples. Follow recommendation in Section 9.D.

Problem	Potential Cause	Suggestion
Low or no fluorescent signal in samples	Inappropriate time point of the detection	Make sure that time of detection is optimized and the samples are prepared immediately. Orange signal may disappear over time because of subsequent reactions of superoxide with other species like NO. 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.

Problem	Potential Cause	Suggestion
Low or no fluorescent signal in samples	Cell density is too low in microplate well.	Check the cell count to confirm proper cell density. For suspension cells, remove carefully supernatant after washing steps, as cells may be dislodged and washed away.
High fluorescence background	Stressed (overcrowded) cells	Prepare new cell culture for the experiment. Make sure that the cells are in the log growth phase.
	Wash step is necessary.	Make optional wash steps mandatory.
	Band pass filters are too narrow or not optimal for fluorescent probes (Fluorescence Microscopy).	Use correct filter for each fluorophore. Check Sections 7A and 9A for recommendations. Minimal spectral overlap should occur with the selected set of filters.

	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.

Problem	Potential Cause	Suggestion
	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 each fluorophore. Check Section 9A for the recommendations. Minimal spectral overlap should occur with the selected set of filters.

UK, EU and ROW
Email: technical@abcam.com | Tel: +44-(0)1223-696000

Austria
Email: wissenschaftlicherdienst@abcam.com | Tel: 019-288-259

France
Email: supportscientifique@abcam.com | Tel: 01-46-94-62-96

Germany
Email: wissenschaftlicherdienst@abcam.com | Tel: 030-896-779-154

Spain
Email: soportecientifico@abcam.com | Tel: 911-146-554

Switzerland
Email: technical@abcam.com
Tel (Deutsch): 0435-016-424 | Tel (Français): 0615-000-530

US and Latin America
Email: us.technical@abcam.com | Tel: 888-77-ABCAM (22226)

Canada
Email: ca.technical@abcam.com | Tel: 877-749-8807

China and Asia Pacific
Email: hk.technical@abcam.com | Tel: 108008523689 (中國聯通)

Japan
Email: technical@abcam.co.jp | Tel: +81-(0)3-6231-0940

www.abcam.com | www.abcam.cn | www.abcam.co.jp

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