ab139476 ROS/Superoxide Detection Assay Kit (Cell-based)

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.

For overview, typical data and additional information please visit: www.abcam.com/ab139476
(use abcam.cn/ab139476 for China, or abcam.co.jp/ab139476 for Japan)

Materials Supplied 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. Avoid repeated freezing and thawing. Briefly centrifuge small vials at low speed prior to opening. Read the entire protocol before performing the experiment.

Item	Quantity	Storage Temperature
Oxidative Stress Detection Reagent (Green)	300 nmoles	-80°C
Superoxide Detection Reagent (Orange)	300 nmoles	-80°C
ROS Inducer (Pyocyanin)	1 µmoles	-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).

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.

1. Reagent Preparation

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

1.1 Detection Reagents

1.1.1 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.

1.1.2 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 week. Gently mix before use.

1.2 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.

1.3 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.

1.4 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.

1.5 2x ROS/Superoxide Detection Mix

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

- To every 10 ml of 1X Wash Buffer (see step 1.4) 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.

NOTE: To prepare smaller volumes of 2x ROS/Superoxide Detection Mix, first prepare a 1:10 intermediate dilution of both the Green and Orange Detection Reagents in 1X Wash buffer or culture medium. Complete the remaining 1:250 dilution separately in 1X wash buffer or culture medium to achieve the final 1:2500 (2x) diluted staining solution in the desired volume.

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.*

2. 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.

3. Assay Procedure

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. Use 1 x 10^5 cells per sample, choose the total assay volume for each sample at the range of 0.5-1 mL.
5. Negative control: add ROS inhibitor (N-acetyl-L-cysteine) in half of the total assay volume (ie 0.25-0.5mL) to pre-treat the cells for at least 30 min prior to induction. For other samples, please add 1X Wash Buffer instead.
6. 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 the dark with periodic shaking. No washing is required prior to the analysis of the samples by flow cytometry.
   Note: Alternatively, you can spin down the cells after NAC treatment, and resuspend with 0.5-1 mL of 1X ROS detection solution containing either ROS inducer (Pyocyanin), experimental agent or vehicle."

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 x 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. For the highest quality data use at least 6 – 8 replicates for each condition tested. Simultaneously treat the cells with an experimental test agent (or controls): Positive control (with ROS inducer, ie Pyocyanin), Negative control (with ROS inhibitor, ie N-acetyl-L-cysteine, Untreated samples (with vehicle), Experimental samples (with experimental agents).
5. Allow all the wells to be incubated 30 min at room temperature before the Induction step.
6. Induction: load 0.1 mL ROS/Superoxide Detection Solution (2X) with addition of either the vehicle, experimental agent, ROS inducer (pyocyanin) at a desirable working concentration. Incubate for 30 min – 1 hour at 37°C in the dark.
7. 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 at endpoint mode.
8. 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. For the highest quality data use at least 6 – 8 replicates for each condition tested. Simultaneously treat the cells with an experimental test agent (or controls): Positive control (with ROS inducer, ie Pyocyanin), Negative control (with ROS inhibitor, ie N-acetyl-L-cysteine, Untreated samples (with vehicle), Experimental samples (with experimental agents).
5. Allow all the wells to be incubated 30 min at room temperature before the Induction step.
6. Induction: load 0.1 mL ROS/Superoxide Detection Solution (2X) with addition of either the vehicle, experimental agent, ROS inducer (pyocyanin) at a desirable working concentration. Incubate for 60 min at 37°C in the dark.
7. 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 at endpoint mode.
8. If required, Z' factor may be calculated for each detection profile using the following formula:

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

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.

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.

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.*

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.

Technical Support

For all technical or commercial enquiries please go to:

www.abcam.com/contactus
www.abcam.cn/contactus (China)
www.abcam.co.jp/contactus (Japan)