ab139473

Cellular ROS/RNS Detection Assay Kit

Instructions for Use

Designed to directly monitor real time reactive oxygen and/or nitrogen species (ROS/RNS) production in live cells using fluorescence microscopy.

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/RNS Detection Assay Kit (ab139473) enables detection of comparative levels of ROS/RNS in cells and also distinguishes between different reactive species in live cells. Through the combination of three specific fluorescent probes, the kit provides a simple and specific assay for the real-time measurement of free nitric oxide (NO) and by extension nitric oxide synthase (NOS) activity, as well as global levels of reactive oxygen species (ROS), and specifically superoxide in living cells.

2. Product Overview

Cellular ROS/RNS Detection Assay Kit (ab139473) is designed to directly monitor real time reactive oxygen and/or nitrogen species (ROS/RNS) production in live cells using fluorescence microscopy. The kit includes three fluorescent dye reagents as major components: nitric oxide (NO) Detection Reagent (red fluorescent), Oxidative Stress Detection Reagent (green fluorescent) for total ROS detection reagent and Superoxide Detection Reagent (orange fluorescent). The non-fluorescent, cell-permeable NO detection dye reacts with NO in the presence of O_2 with high specificity, sensitivity and accuracy, yielding a water-insoluble red fluorescent product. The non-fluorescent, cell-permeable total ROS detection dye reacts directly with a wide range of reactive species, such as hydrogen peroxide (H_2O_2), peroxynitrite ($ONOO^-$) and hydroxyl radicals (HO), yielding a green fluorescent product indicative of cellular production of different ROS/RNS types. The superoxide detection dye is a cell-permeable probe that reacts specifically with superoxide, generating an orange fluorescent product. The kit is not designed to detect reactive chlorine or bromine species, as the fluorescent probes included are relatively insensitive to these analytes. Upon staining, the fluorescent products generated by the three dyes can be visualized using a wide-field fluorescence microscope equipped with standard green (Ex/Em = 490/525 nm), orange (Ex/Em = 550/620 nm), and red (Ex/Em = 650/670 nm) fluorescent cubes.

3. Assay Summary

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

Pipette Detection Mix onto the cell monolayers or resuspended cells and incubate under tissue culture conditions for 2 hours.

Remove detection reagent

Treat cells with test agent. Set up controls.

Wash

Overlay cells with coverslip

Observe cells

Analyze

4. Kit Components

A. Kit Contents

Item	Quantity	Storage Temperature
NO Detection Reagent (Red)	60 µL	-80°C
Oxidative Stress Detection Reagent (Green)	300 nmoles	-80°C
Superoxide Detection Reagent (Orange)	300 nmoles	-80°C
NO Inducer (L-Arginine)	100 µL	-80°C
ROS Inducer (Pyocyanin)	1 µmole	-80°C
NO Scavenger (c-PTIO)	400 nmoles	-80°C
ROS Inhibitor (N-acetyl-L-cysteine)	2 x 10 mg	-80°C
10X Wash Buffer	15 mL	-80°C

Reagents provided in the kit are sufficient for approximately 200 assays using either live, adherent cells or cells 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

- Standard Fluorescent microscope.
- CO2 incubator (37°C).
- Calibrated, adjustable precision pipetters, preferably with disposable plastic tips.
- Adjustable speed centrifuge with swinging buckets.
- Glass microscope slides.
- Glass cover slips.
- Deionized water.
- Anhydrous DMF (100%).

For suspension cells only:

- Tubes appropriate for holding cells during induction of ROS/RNS.

7. Pre-Assay Preparation

NOTE: Allow all reagents to thaw at room temperature before starting with the procedures. Upon thawing, 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. Endogenous fluorescence of untreated cells should be determined in advance or per assay.

1. Detection Reagents

1.1. The Oxidative Stress Detection Reagent (Green) is supplied lyophilized and should be reconstituted in 60 µl DMF to yield a 5 mM stock solution concentration. Upon reconstitution, stock solution should be stored at -20°C.

1.2. The Superoxide Detection Reagent (Orange) is supplied lyophilized and should be reconstituted in 60 µl DMF to yield a 5 mM stock solution concentration. Upon reconstitution, stock solution should be stored at -20°C.

2. Positive Controls

2.1. The NO Inducer (L-Arginine) is supplied at a stock concentration of 100 mM in deionized water. A final concentration of 1 mM is recommended. However, the

optimal final concentration is cell-dependent and should be determined experimentally for each cell line being tested. NO induction generally occurs within 15-20 minutes upon treatment with NO Inducer (L-Arginine) and may drastically decrease or disappear after that time. Plan accordingly.

2.2. 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 Controls

3.1. The NO Scavenger (c-PTIO) is supplied lyophilized and should be reconstituted in 100 µl DMF to produce a stock solution of 4 mM. For use, a final concentration of 20-80 µM is recommended. However, the optimal final concentration is cell-dependent and should be determined experimentally for each cell line being tested.

NOTE: *Adherent cells pre-treated with c-PTIO may become weakly adherent and/or detach from the cell culture substratum.*

3.2. The ROS Inhibitor (N-acetyl-L-cysteine) should be reconstituted in 170 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 5mM is recommended. However, the optimal final concentration is cell-dependent and should be determined experimentally for each cell line being tested.

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

4. 1X Wash Buffer

Allow the 10X Wash Buffer to warm to room temperature. Make sure that the reagent is free of any crystallization before dilution. Prepare enough 1X Wash Buffer for the number of samples to be washed by diluting each milliliter (ml) of the 10X Wash Buffer with 9 ml of deionized water.

5. ROS/RNS 3-Plex Detection Mix

5.1. Pre-dilute the reconstituted Oxidative Stress Detection Reagent (Green) stock 1:10 using pre-warmed tissue culture media.

5.2. Pre-dilute the reconstituted Superoxide Detection Reagent (Orange) stock 1:10 using pre-warmed tissue culture media.

5.3. Prepare the ROS/RNS 3-Plex Detection Mix by combining appropriate volumes of the pre-diluted Oxidative Stress Detection Reagent (step 5.1), pre-diluted Superoxide Detection Reagent (step 5.2) and NO Detection Reagent (Red) using the volumes specified in the following table.

NOTE: *The NO Detection Reagent (Red) is ready-to-use and does not require a pre-dilution step. Important: Pre-warmed media should be used to avoid precipitation of the NO Detection Reagent (Red) in the mix.*

ROS/RNS 3-Plex Detection Mix

Item	Reagent volume per 1ml
NO Detection Reagent (Red)	2.5 µL
Diluted (1:10) Oxidative Stress Detection Reagent (Green)	2 µL
Diluted (1:10) Superoxide Detection Reagent (Orange)	2 µL
Tissue culture medium	993.5 µL
Total volume	1mL

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. Load the cells with the ROS/RNS 3-Plex Detection Mix (see 5.3) using a volume sufficient to cover the cell monolayer and incubate under normal tissue culture conditions for 2 hours.

3. Carefully remove the ROS/RNS 3-Plex Detection Mix from the glass slides by gently tapping them against layers of paper towel or from tissue culture plates. Optional: Cells may be washed with the 1X Wash Buffer.

4. Treat the cells with an experimental test agent. Separate positive control samples should be treated with the NO Inducer (L-Arginine) and the ROS Inducer (Pyocyanin). Negative control samples should be established by treatment with the ROS Inhibitor (N-acetyl-L-cysteine) or NO Scavenger (c-PTIO) correspondingly, for the same length of time as the inducer.

NOTE: *Cells should be treated with the NO Scavenger or ROS Inhibitor 30 minutes prior to induction.*

All treatments should be performed under normal tissue culture conditions. It is recommended to perform a pre-treatment by adding the NO Scavenger or ROS Inhibitor to the aliquots of ROS/RNS 3-Plex Detection Mix for the last 30 minutes of the reagent loading. Treatment with an experimental test agent or ROS/NO inducers included with the kit should be performed in the cell culture media without dyes.

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

   NOTE: *Adherent cells pre-treated with NO Scavenger (c-PTIO) may become loose and/or detach from the cell culture substratum.*

6. Overlay the cells with a cover slip 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 fluorescence patterns.

   Recommended filter sets:

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

- NO detection requires a filter set compatible with Cyanine 5 (650/670nm).

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

C. 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. Resuspend the cell pellet in 100 µl of ROS/RNS 3-Plex Detection Mix (see 5.3) and incubate under normal tissue culture conditions for 2 hours with periodic shaking.

3. Centrifuge the cells at 400 x g for 5 minutes to remove the ROS/ RNS 3-Plex Detection Mix. Optional: Resuspend the cells in 5 ml 1X Wash Buffer, centrifuge them at 400 x g for 5 minutes and remove the supernatant.

4. Treat the cells with an experimental test agent. Separate positive control samples should be treated with the NO Inducer

(L-Arginine) and the ROS Inducer (Pyocyanin). Negative control samples should be established by treatment with the ROS Inhibitor (N-acetyl-L-cysteine) or NO Scavenger (c-PTIO) correspondingly, for the same length of time as the inducer.

***NOTE**: Cells should be treated with the NO Scavenger or ROS Inhibitor 30 minutes prior to induction. All treatments should be performed under normal tissue culture conditions. It is recommended to perform a pre-treatment by adding the NO Scavenger or ROS Inhibitor to the aliquots of ROS/RNS 3-Plex Detection Mix for the last 30 minutes of the reagent loading. Treatment with an experimental test agent or ROS/NO inducers included with the kit should be performed in the cell culture media without dyes.*

5. Centrifuge the cells at 400 x g for 5 minutes.

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. Overlay the cells with a cover slip and analyze immediately via fluorescence microscopy. Make sure that prepared samples are protected from drying. Dried out cells may present different fluorescence patterns.

Recommended filter sets:

- Oxidative stress detection requires a filter set compatible with Fluorescein (Ex/Em: 490/525nm).
- Superoxide detection requires a filter set compatible with Rhodamine (Ex/Em: 550/620nm).
- NO detection requires a filter set compatible with Cyanine 5 (650/670nm).

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

9. Data Analysis

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 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

1. In the presence of NO, the NO Detection Reagent (Red) will demonstrate a red punctuate cytoplasmic staining pattern.

2. 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).

3. Increased levels of oxidative stress give a uniform green cytoplasmic staining in the presence of the Oxidative Stress Detection Reagent.

4. Nitric oxide (NO) positive control samples, induced with NO Inducer (L-Arginine), should exhibit red fluorescence, with punctuate cytoplasmic staining pattern. Keep in mind that fluorescence of the NO Detection Reagent (Red) is not very

bright. Depending on the filters used, the spill-over of the red signal may be visible in the orange channel. However, in this case, the staining pattern will differ significantly from the superoxide generated signal (punctuate cytoplasmic staining versus nuclear staining). Moreover, this staining should disappear upon pretreatment with NO Scavenger (c-PTIO).

5. 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.

6. Cells pre-treated with the ROS Inhibitor (N-acetyl-L-cysteine) should not demonstrate any green or orange fluorescence upon induction.

7. Cells incubated with the NO Scavenger (c-PTIO), post-induced with NO Inducer (L-Arginine) should not demonstrate red fluorescence.

8. Untreated samples should present only low auto-fluorescent background signal in any channel.

10. Troubleshooting

11. 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.

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