Intracellular Total ROS Activity Assay

Catalog #9144

FOR RESEARCH USE ONLY.
Not for use in diagnostic procedures.

Assess total ROS activity in whole cells using flow cytometry

1. INTRODUCTION

Reactive oxygen species (ROS) are natural byproducts of the normal metabolism of oxygen and play important roles in cell signaling. However, during oxidative stress-related states, ROS levels can increase dramatically. The accumulation of ROS results in significant damage to cellular structures. The role of oxidative stress in cardiovascular disease, diabetes, osteoporosis, stroke, inflammatory diseases, a number of neurodegenerative diseases and cancer, has been well established.

ICT's Intracellular Total ROS Activity Assay provides a good screening option for assessing the potency of oxidative stress inhibitor and activator reagents, and will help to determine how oxidative stress modulates varied intracellular pathways. This kit assesses the overall level of intracellular ROS activity, but does not identify the specific reactive oxygen molecule(s) generated by the oxidative stress event.

The kit provides all the essential reagents and an easy to follow protocol to assess changes in intracellular ROS levels by flow cytometry. The key reagent in the assay is a proprietary dye called Total ROS Green. This dye quickly penetrates membrane structures and accumulates within the cell. In the presence of ROS, the non-fluorescent Total ROS Green dye molecule is oxidized by all various iterations of ROS molecular forms. In the oxidized state, the Total ROS Green dye molecule acquires fluorescence properties that enable its detection by flow cytometry (Ex/Em: 490 nm/520 nm) as an indicator of the relative level of intracellular ROS activity. This assay is not designed to determine which particular cell pathology triggered the increase in intracellular ROS concentration in the treatment cell populations, or which individual species of reactive oxygen are involved in the cell response. Other testing parameters must be analyzed to establish the specific causation process responsible for the increased intracellular ROS activity levels.

ICT's Intracellular Total ROS Activity Assay requires minimal procedural steps and hands-on time to complete. Suspension cells or EDTA disassociated adherent cells are stained for 1 hour to pre-load the cells with Total ROS Green quantitation dye. Cells are then treated experimentally. Since the non-internalized dye probe is non-fluorescent, subsequent wash steps are not required, thus simplifying the assay procedure. Following treatment, cells are ready for analysis by flow cytometry. Each kit will enable the assessment of up to 100 samples (0.5 mL each).

This assay can be adapted for use with a fluorescence microscope or plate reader equipped with FITC/FAM dye-compatible excitation/emission optics. Use and optimization of these alternative fluorescence analysis methods will require further modification of this flow cytometer based protocol.

2. KIT CONTENTS

- 1 vial of Total ROS Green, #6688
- 1 vial of DMSO, 200 µL, #6689
- 1 bottle of Assay Buffer, 10X, 15 mL, #686

3. STORAGE

The entire unopened kit should be stored frozen until the expiration date; however, some components may be stored refrigerated.

- Aliquot and store unused reconstituted Total ROS Green reagent at ≤ -20°C for ≥ 1 month. Avoid repeated freeze/thaw cycles.
- Assay Buffer, 10X may be stored frozen or at ≤ 2-8°C for up to 6 months.
- Protect Total ROS Green reagent from light.

4. SAFETY DATA SHEETS (SDS)

SDS are available at online at www.immunochemistry.com or by calling 1-800-829-3194 or 952-888-8788.

5. RECOMMENDED MATERIALS

- 15 mL polypropylene centrifuge tubes (1/sample)
- FACS tubes
- Cultured cells treated with the experimental conditions ready to be labeled
- Reagents to induce oxidative stress, such as tert-Butyl hydroperoxide (e.g. Sigma catalog #458139) or hydrogen peroxide (e.g. VWR catalog # BDH7690-1).
- Hemocytometer

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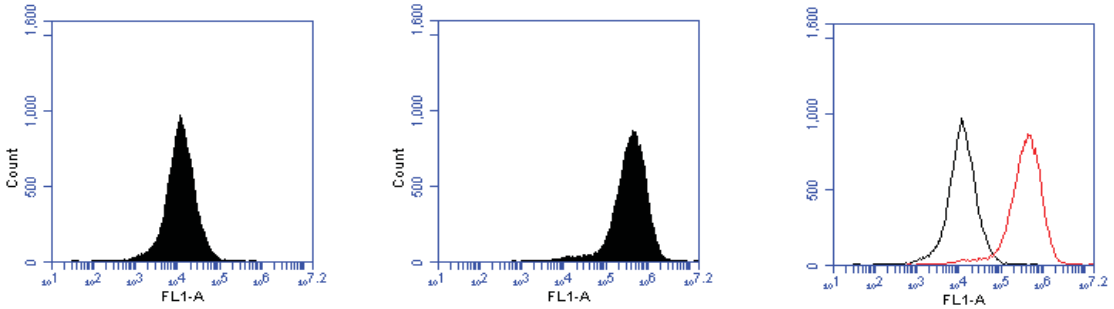
FIGURE 1: ANALYSIS VIA FLOW CYTOMETRY

Jurkat cells were stained with ICT's Intracellular Total ROS Activity Assay (Catalog #9144) for 1 hour, and then treated with a negative control (left histogram) or 100 µM tert-Butyl hydroperoxide, a reactive oxygen-inducing agent used to create the positive control (middle histogram), for 30 minutes at 37°C. Cells were read on the FL1 channel of an Accuri C6 flow cytometer. The median fluorescence intensity (MFI) of stained cells in the negative control was 11,875 in FL1-A (left: Negative), whereas the treated population had a MFI value of 419,067 (middle: Positive), which is an increase of more than 35-fold. The effect of tert-Butyl hydroperoxide on intracellular total ROS activity is easily visible when the samples are overlaid in a single plot (right-most histogram, black: Negative; right, red: Positive). Data courtesy of Dr. Kristi Strandberg, ICT, 224:76.



Negative, Non-induced | Positive, Reactive Oxygen-induced | Overlay

6. DETECTION EQUIPMENT

Total ROS Green can be visualized with a flow cytometer at Ex/Em = 490/520 nm (FL1 channel).

7. PREPARATION OF 500X TOTAL ROS GREEN STOCK

Total ROS Green dye is supplied as a lyophilized powder that must be solubilized with DMSO to create a 500X stock concentrate. Each kit includes 1 vial of Total ROS Green quantitation dye, which provides enough reagent to stain 100 samples (at 0.5 mL per sample) for flow cytometry analysis.

1. Thaw the kit and bring components to room temperature. Protect Total ROS Green from light and use gloves when handling all reagents.
2. Reconstitute each vial of Total ROS Green with 100 µL DMSO (Catalog# 6689, included in kit) to form the 500X stock solution. Mix well. Protect from light. Once reconstituted in DMSO, the stock solution may be aliquoted and stored at ≤-20°C for ≥ 1 month, protected from light. Avoid repeated freeze/thaw cycles.

- **Warning!** Contains Dimethyl sulfoxide (DMSO), which is a combustible liquid, causes skin irritation, and causes serious eye irritation. In case of exposure, immediately flush eyes or skin with water. See SDS for further information.

8. PREPARATION OF 1X ASSAY BUFFER

1. Remove Assay Buffer, 10X from the freezer, and allow to thaw and come to room temperature. A precipitate may have formed during freezing. Mix gently. Allow sufficient time for the precipitate to go back into solution. Do not boil.
2. Dilute 1:10. For example, add 15 mL of 10X Assay Buffer to 135 mL of deionized or distilled water.
3. Filter sterilize 1X Assay Buffer prior to use.
4. Store sterile 1X Assay Buffer for up to 1 month at ≤ 2-8°C.
   - Buffer does not contain presevatives.
   - Avoid contamination. Do not use if buffer appears to be contaminated.
   - Other isotonic buffers such as Hanks Buffered Saline Solution (HBSS) or Phosphate Buffered Saline (PBS) may be used instead of the provided Assay Buffer. Alternate buffers should be evaluated by the end user.

9. STAINING PROTOCOL FOR SUSPENSION CELLS

- **Cells need to be pre-loaded with Total ROS Green dye prior to exposure to experimental conditions.**

Staining/pre-loading with Total ROS Green can be completed in an hour. However, because it is used to label living cells, adequate time must be allotted for the cultivation of cell samples and the experimental treatment or oxidative stress induction process. Allocate a sufficient amount of time for the experimental condition to manifest itself. The optimal exposure time will vary from several minutes to several days depending upon the mechanism involved.

Cell concentrations used for analysis should be 5 x 10^5 to 1 x 10^6 cells/mL prior to labeling. Avoid stressful culture conditions; cell concentrations that are too high or too low can create false positive artifacts and lead to erroneous interpretation of the data.

1. Prepare cells in 0.5 mL 1X Assay Buffer (or an alternative isotonic buffer such as HBSS or PBS) at a cell density of 5 x 10^5 to 1 x 10^6 cells/mL.
2. Transfer cells into 15 mL polypropylene centrifuge tubes or FACS tubes to undergo Total ROS Green staining.
3. When ready to stain cells, dilute the 500X Total ROS Green stock solution 1:10 to prepare a 50X working solution. Dilute 500X stock by adding 900 µL of 1X Assay Buffer to the vial containing 100 µL of 500X stock. This yields 1 mL at 50X.

4. Spike cells with 50X Total ROS Green at a 1:50 v/v ratio directly into samples prepared in 1X Assay Buffer. For example, add 10 µL Total ROS Green to each 490 µL sample. If larger samples are being used, add Total ROS Green at 1:50.
5. Incubate samples for 1 hour at 37°C in a CO_2 incubator. The optimal incubation time may vary with different cell lines. It is recommended that the incubation conditions be optimized within each laboratory to accommodate the varying cell lines being utilized.

10. STAINING PROTOCOL FOR ADHERENT CELLS

- **Cells need to be pre-loaded with Total ROS Green dye prior to exposure to experimental conditions.**

1. Seed adherent cells into culture flasks or plates at a concentration that will provide a monolayer confluency < 80% at the end of the experimental treatment. Different adherent cell lines expand at different growth rates. Each cell line must be evaluated to assess the seeding volume and time to achieve an 80% (or less) monolayer confluency coverage prior to beginning the experiment.
2. Disassociate adherent cells from plate or flask surface:
   a. Remove cell culture media.
   b. Incubate monolayer with sterile 0.5 mM EDTA. Some cell lines may be detachable using only 0.5 mM EDTA. EDTA chelates the Ca^{2+} and Mg^{+} ions that facilitate cell adherence functionality.
   c. As soon as the cell monolayer has detached, pellet the cell suspension by centrifugation at 200 x g.
   d. Wash the cells with fresh cell culture medium.
   e. Pellet cell suspension by centrifugation.
   f. Remove the supernatant and resuspend the cell pellet in 1X Assay Buffer (or other isotonic buffer).
3. When ready to stain cells, dilute the 500X Total ROS Green stock solution 1:10 to prepare a 50X working solution. Dilute 500X stock by adding 900 µL of 1X Assay Buffer to the vial containing 100 µL of 500X stock. This yields 1 mL at 50X.
4. Spike cells with 50X Total ROS Green at a 1:50 v/v ratio directly into samples prepared in 1X Assay Buffer. For example, add 10 µL Total ROS Green to each 490 µL sample. If larger samples are being used, add Total ROS Green at 1:50.
5. Incubate samples for 1 hour at 37°C in a CO_2 incubator. The optimal incubation time may vary with different cell lines. It is recommended that the incubation conditions be optimized within each laboratory to accommodate the varying cell model systems being utilized.

11. EXPERIMENTAL PREPARATION & CONTROLS

1. Stain cells with Total ROS Green according to Section 9 or 10.
2. Create experimental and control cell populations:
   a. Treated experimental population: cells exposed to the experimental condition or treatment.
   b. Negative control: non-treated cells grown in a normal cell culture environment.
   c. Solvent vehicle control: cells grown in a normal cell culture environment spiked with an equivalent concentration of solvent used to dissolve the experimental compound. This control may not be necessary if the solvent used has a low to non-discernible influence on cell viability; in this case, use control 2b instead.
   d. Positive control: cells induced to undergo oxidative stress using a validated treatment method. For example, cells can be treated with 100 µM tert-Butyl hydroperoxide for 30 minutes at 37°C to induce oxidative stress.

If the experimental cell treatment protocol calls for a prolonged incubation period (> 30 minutes), it is recommended that the assay buffer included with the kit be replaced by a more complex cell-friendly buffer media. Dulbecco's phosphate buffered saline containing > 0.1 % bovine serum albumin provides an excellent alternative. Unintentional creation of oxidative stress artifacts due to the environment of the cell suspension buffer should be avoided.

12. ANALYZE DATA

1. Monitor the fluorescence intensity using flow cytometry. Total ROS Green exhibits fluorescence excitation and emission characteristics similar to FITC or FAM fluorophores. Total ROS Green is efficiently excited (490 nm optimal) using standard blue laser optics packages, which are present on most flow cytometers, and it is easily detected using typical FL1 green-emission-channel optics (530/30 nm).
2. Create a FSC-A vs SSC-A dot plot, and then gate on the cells of interest. Plot FL1 emission intensity (X-axis) versus cell number (Y-axis) as a histogram. Compare the median fluorescence intensity (MFI) output generated within the negative control cell population to that observed in the positive control and experimental treatment cell populations. Determine if the solvent vehicle used to deliver the experimental compound (if applicable) had a significant influence on the relative intracellular ROS Activity.
3. In general, the higher the Total ROS Green fluorescence output relative to the fluorescence output of the healthy cell negative control, the greater the degree of ROS activation in those cell treatment groups.

13. REFERENCES

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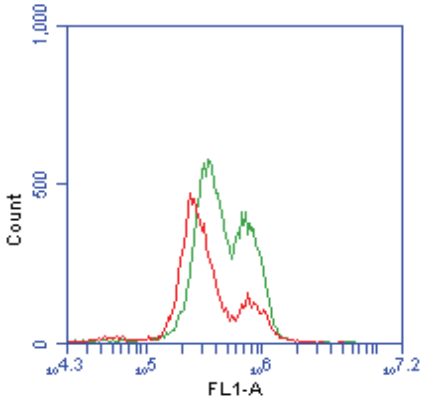
RELATED PRODUCTS:

Reduced glutathione or GSH is the most abundant non-protein thiol in cells. Primarily recognized as a key intracellular source of reducing power for combating the toxic accumulation of free radical byproducts, GSH is also involved with detoxification and removal of exogenous and endogenous toxins and alkylating agents. In its role as a cell signaling agent, GSH is involved in DNA synthesis and cell proliferation regulation. The assessment of GSH levels is a useful indication of intracellular redox potential and a cell's ability to prevent oxidative stress.

To detect intracellular GSH in whole cells by flow cytometry, use ICT's Intracellular GSH Assay (#9137). To quantitatively measure GSH and oxidized glutathione in a variety of samples including cell lysates, use ICT's 96-well-based Glutathione Colorimetric Detection Kit (#9135) or Glutathione Fluorescent Detection Kit (#9133 or #9134).

Visit www.immunochemistry.com for more information.



ABOVE: Jurkat cells were treated with a negative control (green histogram) or staurosporine (red histogram) for 4 hours at 37°C then stained with ICT's Intracellular GSH Assay (#9137).

RIGHT: Glutathione Colorimetric Detection Kit (#9135)

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