Instructions for Use

For the measurement cellular reactive oxygen species

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

Reactive oxygen species (ROS) assay kit ab113851 uses the cell permeant reagent 2’, 7’-dichlorofluorescein diacetate (DCFDA), a fluorogenic dye that measures hydroxyl, peroxyl and other ROS activity within the cell. After diffusion into the cell, DCFDA is deacetylated by cellular esterases to a non-fluorescent compound, which is later oxidized by ROS into 2’, 7’-dichlorofluorescin (DCF). DCF is a highly fluorescent compound which can be detected by fluorescence spectroscopy with maximum excitation and emission spectra of 495nm and 529nm respectively. Each reactive oxygen species assay kit contains sufficient materials for approximately 300 measurements in microplate format and 70 measurements (35 mL) by flow cytometry.

The two major sources of cellular ROS are complex I (NADH dehydrogenase ubiquinone-ubiquinol reductase) and complex III (ubiquinol cytochrome c reductase), both part of the mitochondrial electron transport chain. These two complexes generate ROS particularly when electron transport is slowed by high mitochondrial membrane potential (Δψm). The major product of ROS in mitochondrial is in the form of superoxide and hydroperoxyl radical. Superoxide generated in complex III occurs in the presence of slow electron transport which allows for the ubisemiquinone anion radical to react with oxygen dissolved in the membrane. The exact source
of superoxide generated by complex I is less known and it is believed to be due to electron leakage from its iron-sulfur clusters.

Low levels (or optimum levels) of ROS play an important role in signaling pathways. However when ROS production increases and overwhelms the cellular antioxidant capacity, it can induce macromolecular damage (by reacting with DNA, proteins and lipids) and disrupt thiol redox circuits. In the first instance, damage can lead to apoptosis or necrosis. Disruption of thiol redox circuits can lead to aberrant cell signaling and dysfunctional redox control.

Limitations:

- FOR RESEARCH USE ONLY. NOT FOR DIAGNOSTIC PROCEDURES.

- Use this kit before expiration date.

- Do not mix or substitute reagents from other lots or sources.

- Any variation in operator, pipetting technique, washing technique, incubation time or temperature, and kit age can cause variation in binding.
2. Assay Summary

**ROS ASSAY ON SUSPENSION CELLS** (e.g. HL60)

**SAMPLE – MICROPLATE ASSAY**
- Grow 1.5x10^6 cells per experimental condition (1 well).
- Collect cells in a conical tube.

**SAMPLE – FLOW ASSAY**
- Grow 1.5x10^6 cells per experimental condition (1 measurement).
- Collect cells in a conical tube.

**STAINING – PLATE ASSAY**
- Wash cells once with PBS.
- Stain cells with 20μM DCFDA in 1X buffer for 30 minutes at 37°C.
- Wash cells once with 1X buffer.

**STAINING – FLOW ASSAY**
- Stain cells with 20μM DCFDA in complete media for 30 minutes at 37°C.
- Do not wash the cells.

**DOSING (OPTIONAL)**
- Seed cells at 1x10^5 cells/50μL/well.
- Overlay 50μL of 2X treatment.
- Incubate for desired period of time.

**DOSING (OPTIONAL)**
- Aliquot cells.
- Treat cells for desired period of time.

**ROS ASSAY ON ADHERENT CELLS** (e.g. HepG2)

**SAMPLE – MICROPLATE ASSAY**
- Harvest 3 – 4 x10^6 cells.
- Seed cells at 2.5x10^4 cells/well on a 96-well plate.
- Allow to attach overnight.

**STAINING**
- Wash cells once with 1X buffer.
- Stain with DCFDA 25μM in 1X buffer for 45 minutes at 37°C.
- Wash cells once with 1X buffer.

**DOSING (OPTIONAL)**
- Add 100μL/well of treatment.
- Treat cells for desired period of time.

**READING AND ANALYSIS**

- Excitation wavelength = 485nm.
- Emission wavelength = 535nm.
- Determine change as percentage from control after background subtraction.
3. Kit Contents

- 20 mM DCFDA (in DMSO) (~1000x) : 0.035 mL
- 10X Buffer : 10 mL
- 55 mM TBHP: 0.05 mL

4. Storage and Handling

Store all components at 4°C in the dark. The kits are stable for at least 6 months from receipt. For longer term storage, keep at -20°C to -80°C in the dark.

5. Additional Materials Required

- Fluorescence plate reader or Flow cytometer. DCF can be detected with similar settings to those used to detect FITC. The suggested excitation/emission wavelengths: 485nm/535nm
- General tissue culture supplies
- PBS
- Fetal bovine serum
- Sterile, tissue culture treated, black 96-well microplates
• 50 – 300μL multichannel pipettor

• Optional:
  • Test compounds of interest
  • Optional ROS inducing control compounds include
tert-butyl hydroperoxide, doxorubicin, idarubicin or
antimycin.

6. Assay Procedure

FLUORESCENCE MICROPLATE MEASUREMENT

A. Suspension cell culture and treatment (example human HL60 cells)

1. Grow HL60 cells in glucose based media so that approximately 1.5X10^7 cells are available on the day of the experiment per plate.

2. Make a **1X buffer solution** as follows: 90mL of sterile deionized water + 10mL of 10X buffer.

3. Make a **1X supplemented buffer** solution as follows: 18mL of 1X buffer solution + 2mL of FBS.

4. If performing toxicity assays, dilute compounds of interest in 1X supplemented buffer to 2X of final desired concentration
for the experiment. A 96-well deep well microplate may be use in this step. Compounds may also be diluted in complete media with 10% FBS with or without phenol red. Include positive (50μM Tert-butyl hydroperoxide) and negative controls (vehicle of choice).

5. Prepare DCFDA mix as follows: 10mL of 1X buffer solution + 10μL of 20mM DCFDA (final concentration 20μM).

6. Collect cells and wash by centrifugation once in PBS.

7. Resuspend cells in 10mL of DCFDA mix and incubate at 37°C for 30 minutes in the dark.

8. Wash cells by centrifugation with 10mL of 1X buffer solution.

9. Resuspend 1x10^7 cells in 5mL of 1X supplemented buffer.

10. Seed a 96-well dark plate as follows: 100,000 stained cells/50μL/well. Include blank wells (with non-stained cells).

11. If performing toxicity assays, add to each well 50μL of previously diluted 2X compounds and treat for desired period of time.
B. Adherent cell culture and treatment (example human HepG2 cells)

1. Grow HepG2 cells in standard media so that $3 \times 10^6$ to $4 \times 10^6$ cells are obtained the day before the experiment.

2. Harvest cells the day before the experiment and seed a dark 96-well microplate with 25,000 cells per well. Allowed to attach overnight.

3. On the day of the experiment, make a **1X buffer solution** as follows: 90mL of sterile deionized water + 10mL of 10X buffer.

4. On the day of the experiment, make a **1X supplemented buffer** solution as follows: 18mL of 1X buffer solution + 2mL of FBS.

5. On the day of the experiment, prepare **DCFDA mix** as follows: 10mL of 1X buffer solution + 12.5μL of 20mM DCFDA (final concentration 25μM).

6. If performing toxicity assays, dilute compounds of interest to the final desired concentration in 1X supplemented buffer solution. A 96-well deep well microplate may be use in this step. **Compounds may also be diluted in complete media with 10% FBS without phenol red.** Include positive (50μM Antimycin) and negative controls (vehicle of choice).
7. Wash the HepG2 cells seeded on the 96-well plate with 100µL/well of PBS once.

8. Add 100µL/well of DCFDA mix and incubate for 45 minutes at 37°C in the dark. Include blank wells (with non-stained cells).

9. Wash the plate once with 1X buffer solution.

10. If performing cytotoxicity assays, add compounds of interest and treat for desired period of time.

C. Reading

1. Set the fluorescent plate reader to perform an endpoint read.

2. Set excitation wavelength at 485nm and emission wavelength at 535nm. Settings for the detection of FITC can be used to detect DCF.
FLOW CYTOMETRY MEASUREMENT

A. Sample preparation and treatment of cells

1. Grow cells (adherent or non adherent) in glucose based media so that on the day of the experiment there are at least \(1.5 \times 10^4\) cells per assayed condition (treatment, dose, time). Include in the calculation enough cells for control signal (control compound, control vehicle and non-stained control cells). This number takes into account any cell loss experienced during processing.

2. Harvest cells and ensure a single cell suspension by (1) gently pipetting up and down suspension cells or (2) by fully detaching adherent cells.

3. Stain cells resuspended in Media with 20µM DCFDA and incubate for 30 minutes at 37°C. Once the incubation is completed, DO NOT wash the cells.

4. After staining, treat the cells with compounds of interest and ensure appropriate controls are included. If Tert-butyl hydroperoxide is chosen as the positive control compound, optimal signal is obtained after 4 hours of treatment.
B. Reading and Analysis

1. Establish forward and side scatter gates to exclude debris and cellular aggregates from analysis.

2. DCF should be excited by the 488nm laser and should be detected at 535nm.

3. Ideally 10,000 cells should be analyzed per experimental condition. Cells should not be overly dense during the experiment (< 1x10^6 cells/mL).
7. Data Analysis and Sample Data

Subtract background (non-stained cells) from all measurements and determine fold change from assay control (vehicle if performing toxicity studies). The data in Figure 1 and 2 below show that the response of Jurkat cells to Tert butyl hydroperoxide after acute treatment (3-4 hours) can be measured by microplate assay or flow cytometry.

i. **Microplate assay:**

![Figure 1. DCFDA microplate assay result.](image)

Labeled (20µM) and unlabeled (none) Jurkat cells were treated with +/- 50µM tert-butyl Hydrogen Peroxide (tbHP) for 3 hours at 37°C. Cells were then washed once with PBS, transferred to a microplate and read on a spectrophotometer. Mean +/- standard deviation is plotted for 3 replicates from each condition. tbHP mimics ROS activity to oxidize DCFDA to fluorescent DCF.
ii. **Flow cytometry:**

![Flow Cytometry Diagram](image)

**Figure 2. DCFDA flow cytometry assay result.** Labeled and unlabeled Jurkat cells were treated with 50µM tert-butyl Hydrogen Peroxide (tbHP) as described in Figure 1 and then analyzed by flow cytometry.

iii. **High Throughput testing:**

This assay may be used for screening pharmacological induction of ROS in any cell line. Depending on microplate template (see Fig. 3) either 3 or 4 compounds may be tested in triplicate dose response per plate.
A.
Figure 3. **Suggested assay templates.** Two assay examples are shown above. The example (A) allows for screening of four compounds in dose response. Row A contains the vehicle control to determine maximal signal in the absence of compound. Row H contains non-stained cells to determine background fluorescence. The example (B) only allows for screening of
three compounds in dose response with perimeter wells as the background fluorescence and column 2 as the vehicle titration control. Column 1, column 12, row A and row H contain non-stained cells.
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