Instructions for Use

For the measurement cellular reactive oxygen species

This product is for research use only and is not intended for diagnostic use.
# Table of Contents

## INTRODUCTION
1. BACKGROUND 2
2. SUSPENSION CELL ASSAY SUMMARY – MICROPLATE 3
3. ADHERENT CELL ASSAY SUMMARY – MICROPLATE 4
4. SUSPENSION CELL ASSAY SUMMARY – FLOW 5

## GENERAL INFORMATION
5. PRECAUTIONS 6
6. STORAGE AND STABILITY 6
7. MATERIALS SUPPLIED 6
8. MATERIALS REQUIRED, NOT SUPPLIED 7
9. LIMITATIONS 7
10. TECHNICAL HINTS 8

## ASSAY PREPARATION
11. REAGENT PREPARATION 9

## ASSAY PROCEDURE
12. ASSAY PROCEDURE 11

## DATA ANALYSIS
13. CALCULATIONS 14
14. TYPICAL DATA 14

## RESOURCES
15. FREQUENTLY ASKED QUESTIONS 19
16. NOTES 22
INTRODUCTION

1. BACKGROUND

Abcam’s DCFDA - Cellular Reactive Oxygen Species Detection Assay uses the cell permeant reagent 2′,7′ -dichlorofluorescin 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′ –dichlorofluorescein (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.
2. **SUSPENSION CELL ASSAY SUMMARY – MICROPLATE**

Grow $1.5 \times 10^5$ cells per experimental condition (1 well)

Collect cells in conical test tube

Wash cells once in 1X Buffer

Stain cells with 20 µM DCFDA in 1X Buffer for 30 minutes at 37 ºC

Wash cells once in 1X Buffer

Optional Steps: Seed cells at $1.5 \times 10^5$/50 µL/well

Overlay 50 µL of 2X treatment

Incubate for desired period of time

Read signal at Ex485 nm/Em535 nm

Determine change as percentage of control after background subtraction
3. ADHERENT CELL ASSAY SUMMARY – MICROPLATE

Harvest 3-4x10^6 cells

Seed cells at 2.5x10^4 cells/well on a 96 well plate

Allow cells to attach overnight

Wash cells once in 1X Buffer

Stain cells with 25 µM DCFDA in 1X Buffer for 45 minutes at 37 ºC

Wash cells once in 1X Buffer

Optional Steps: Add 100 µL/well of treatment

Incubate for desired period of time

Read signal at Ex485 nm/Em535 nm

Determine change as percentage of control after background subtraction.
4. **SUSPENSION CELL ASSAY SUMMARY – FLOW**

- Grow $1.5 \times 10^5$ cells per experimental condition (1 well)
- Collect cells in conical test tube
- Stain cells with 20 µM DCFDA in 1X Buffer for 30 minutes at 37 ºC
- DO NOT wash cells after staining
- Optional Steps: Aliquot cells
  - Treat cells for desired period of time
- Read signal at Ex485 nm/Em535 nm
- Determine change as percentage of control after background subtraction
5. **PRECAUTIONS**

Please read these instructions carefully prior to beginning the assay.

All kit components have been formulated and quality control tested to function successfully as a kit. Modifications to the kit components or procedures may result in loss of performance.

6. **STORAGE AND STABILITY**

Store kit at 4°C immediately upon receipt.

For longer term storage, keep at -20°C to -80°C in the dark.

Refer to list of materials supplied for storage conditions of individual components. Observe the storage conditions for individual prepared components in section 11.

7. **MATERIALS SUPPLIED**

<table>
<thead>
<tr>
<th>Item</th>
<th>Amount</th>
<th>Storage Condition (Before Preparation)</th>
</tr>
</thead>
<tbody>
<tr>
<td>20 mM DCFDA (in DMSO)</td>
<td>35 µL</td>
<td>4°C</td>
</tr>
<tr>
<td>10X Buffer</td>
<td>10 mL</td>
<td>4°C</td>
</tr>
<tr>
<td>55 mM Tert-Butyl Hydrogen Peroxide (TBHP)</td>
<td>50 µL</td>
<td>4°C</td>
</tr>
</tbody>
</table>
8. **MATERIALS REQUIRED, NOT SUPPLIED**

These materials are not included in the kit, but will be required to successfully utilize this assay:

- Fluorescence plate reader or Flow cytometer. DCF can be detected with similar settings to those used to detect FITC. The suggested excitation/emission wavelengths: 485 nm/535 nm
- General tissue culture supplies
- PBS (1.4 mM KH2PO4, 8 mM Na2HPO4, 140 mM NaCl, 2.7 mM KCl, pH 7.4)
- Fetal Bovine Serum (FBS)
- Cell culture grade DMSO
- Sterile, tissue culture treated, clear bottom, dark sided 96-well microplates.
- Multichannel pipette (50 – 300 μL)
- Optional:
  - Test compounds/diluents of interest
  - Other ROS inducing control compounds such as doxorubicin, idarubicin or antimycin
  - Decane: Solvent used for the preparation and stabilization of TBHP

9. **LIMITATIONS**

- Assay kit intended for research use only. Not for use in diagnostic procedures
- Do not use kit or components if it has exceeded the expiration date on the kit labels
- Do not mix or substitute reagents or materials from other kit lots or vendors. Kits are QC tested as a set of components and performance cannot be guaranteed if utilized separately or substituted
• Any variation in operator, pipetting technique, washing technique, incubation time or temperature, and kit age can cause variation in binding

10. TECHNICAL HINTS

• Clear bottom, dark sided microplates are recommended with this assay. Clear sided microplates have not been tested with this kit

• It is highly recommended to always include a positive control sample (treated with TBHP for 2 - 4 hours) as this assay does not detect very low levels of ROS found in normal, healthy cells

• If working with compounds to test their ability to induce ROS in cell culture, it is recommended to stain first the cells and then carry out acute treatments (1 – 6 hours), followed by reading of ROS in the presence of compound/experimental conditions to prevent removal of ROS species during wash steps

• If longer treatments are required, refer to FAQ section

• Settings for the detection of FITC can be used to detect DCF

• The magnitude of the fluorescent signal generated in these assays is dependent on the type of fluorescent plate reader used and on the settings of the flow cytometer. The relevant measurement is the ratio of fluorescent signal between control and treated samples within an experiment

• This kit is sold based on number of tests. A ‘test’ simply refers to a single assay well. The number of wells that contain sample, control or standard will vary by product. Review the protocol completely to confirm this kit meets your requirements. Please contact our Technical Support staff with any questions
11. REAGENT PREPARATION

Equilibrate all reagents to room temperature (18-25°C) prior to use.

The sample volumes below are sufficient for 96 100 μL tests; adjust volumes as needed for the number of strips in your experiment.

11.1 1X Buffer

Prepare 1X Buffer by adding 10 mL 10X Buffer to 90 mL deionized water. Mix gently and thoroughly.

11.2 1X Supplemented Buffer

Prepare 1X Supplemented Buffer by adding 18 mL 1X Buffer to 2 mL FBS.

11.3 Working DCFDA Solution

Prepare a working DCFDA solution by adding the appropriate volume of 20 mM DCFDA to 1X Buffer. As an example, to generate a 20 μM final concentration solution of DCFDA, add 10 μL of 20 mM DCFDA solution to 10 mL 1X Buffer.

The exact concentration of DCFDA required will depend on the cell line being used but a general starting range would be 10 - 50 μM. Exact concentrations have to be determined on an individual basis by the end user.

Typical working concentrations for certain cell lines are shown in the table below:

<table>
<thead>
<tr>
<th>Typical working concentrations</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Sample Type</strong></td>
</tr>
<tr>
<td>HepG2</td>
</tr>
<tr>
<td>HL60</td>
</tr>
<tr>
<td>Jurkat</td>
</tr>
</tbody>
</table>
11.4 **50 μM TBHP (Positive control)**
Dilute 55 mM Tert-Butyl Hydrogen Peroxide (TBHP) 1,100X in the supplemented buffer. Prepare only what will be used in the assay as storage of this solution may lead to degradation of TBHP. TBHP may also be diluted in complete media with 10% FBS without phenol red.

11.5 **Optional: 5 mM Decane**
Dilute Decane (TBHP diluent - not provided with the kit) in either supplemented buffer or complete media with 10% FBS without phenol red 1,100X.

11.6 **Optional: Compounds/Diluents of interest**
If performing toxicity assays, dilute compounds of interest in 1X supplemented buffer to final desired concentration for the experiment (see section 11.1.7). 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.
12. ASSAY PROCEDURE

- Equilibrate all materials and prepared reagents to 37°C prior to use.
- It is recommended to assay all standards, controls and samples in duplicate.

12.1 Fluorescent Microplate Measurement (Suspension Cells)

12.1.1 Grow suspension cells so that approximately $1.5 \times 10^5$ cells per well are available on the day of the experiment.

12.1.2 Collect cells in a conical tube and wash by centrifugation once in PBS.

12.1.3 Stain the cells by resuspending in the diluted DCFDA Solution at a concentration of $1 \times 10^6$/mL and incubate at 37°C for 30 minutes in the dark.

12.1.4 Wash cells by centrifugation with 1X Buffer maintaining the same concentration of cells.

12.1.5 Resuspend cells in 1X Supplemented Buffer or complete media with 10% FBS and no phenol red to a concentration of $1 \times 10^6$/mL.

12.1.6 Seed a dark, clear bottom 96-well microplate with 100,000 stained cells/well and read immediately (see 12.1.8).

12.1.7 If performing toxicity assays, overlay each well with previously diluted 2X compounds and treat for desired period of time (1 – 6 hours). Include background wells (untreated or diluent treated stained cells) as well as blank wells (media or buffer only).

12.1.8 Read plate end point in the presence of compounds, media or buffer on a fluorescent plate reader with excitation wavelength at 485 nm and emission wavelength at 535 nm.
12.2 **Fluorescent Microplate Measurement (Adherent Cells)**

12.2.1 Grow adherent cells in standard media so that 3\times10^6 to 4\times10^6 cells are obtained the day before the experiment.

12.2.2 Harvest cells and seed a dark, clear bottom 96-well microplate with 25,000 cells per well. Allow to adhere overnight.

12.2.3 Remove the media and add 100 μL/well of 1X Buffer.

12.2.4 Remove 1X Buffer and stain cells by adding 100 μL/well of the diluted DCFDA Solution.

12.2.5 Incubate cells with the diluted DCFDA Solution for 45 minutes at 37°C in the dark.

12.2.6 Remove DCFDA Solution; add 100 μL/well of 1X Buffer or 1X PBS and read immediately (see 12.2.8).

12.2.7 If performing cytotoxicity assays remove 1X Buffer or 1X PBS and add 100 μL of previously diluted 1X compounds of interest. Treat for desired period of time (1–6 hours). Include background wells (untreated or diluent treated stained cells) as well as blank wells (media or buffer only).

12.2.8 Read plate end point in the presence of compounds, media or buffer on a fluorescent plate reader with excitation wavelength at 485 nm and emission wavelength at 535 nm.
12.3 Flow cytometry Measurement

12.3.1 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.

12.3.2 Harvest cells and ensure a single cell suspension by (1) gently pipetting up and down suspension cells or (2) by fully detaching adherent cells (e.g. trypsinize and quench with media).

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

12.3.4 After staining, treat the cells with compound(s) 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.

12.3.5 Gently pipette cells up/down to ensure single cell suspension.

12.3.6 Analyze on flow cytometer. Establish forward and side scatter gates to exclude debris and cellular aggregates from analysis.

12.3.7 DCF should be excited by the 488 nm laser and should be detected at 535 nm (typically FL1).

12.3.8 Ideally 10,000 cells should be analyzed per experimental condition. Cells should not be overly dense during the experiment \(<1 \times 10^6\text{ cells/mL}\).
13. **CALCULATIONS**

- **Fluorescent Microplate Measurement**
  Subtract blank readings from all measurements and determine fold change from assay control (diluents treated cells if performing toxicity studies).

- **Flow cytometry Measurement**
  Exclude debris and isolate cell population of interest with gating. Using mean fluorescent intensity, determine fold change between control and treated samples.

14. **TYPICAL DATA**

Data provided for **demonstration purposes only**. The data in Figures 1 and 2 below show that the response of Jurkat cells to tert-butyl hydrogen peroxide after acute treatment (3-4 hours) can be measured by microplate assay or flow cytometry.

**Figure 1.** DCFDA microplate assay result. Jurkat cells were labeled with DCFDA (20 µM) or unlabeled (none) and then cultured an additional 3 hours with or without 50 µM tert-butyl hydrogen peroxide (TBHP) according to the protocol. Cells were then analyzed on a fluorescent plate reader (SpectraMax M4 Microplate reader). Mean +/- standard deviation is plotted for 4 replicates from each condition. TBHP mimics ROS activity to oxidize DCFDA to fluorescent DCF.
Figure 2. DCFDA flow cytometry assay result. Labeled and unlabeled Jurkat cells were treated with 50 µM tert-butyl hydrogen peroxide (TBHP) as described in Figure 1 and then analyzed by flow cytometry. There is a 10.1-fold difference between the control and TBHP mean fluorescent intensities.
Figure 3. Acute Effect of anthracyclines on ROS production in HL60 cells. Labeled HL60 cells were treated with idarubicin or doxorubicin for 4 hours at multiple doses according to section 10 of the protocol. At the end of the treatment cells were read end point in a fluorescent plate reader (Perking Elmer-Wallac 1420 Victor 2 Multilabel plate reader). Mean +/- standard deviation is plotted for 3 replicates from each condition. The dotted line represents the mean of 24 replicates of HL60 cells treated with 0.5% DMSO.
High Throughput testing
This assay may be used for screening pharmacological induction of ROS in any cell line. Depending on microplate template (Figure 4) either 3 or 4 compounds may be tested in triplicate dose response per plate.

A.
Figure 4. 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.
15. **FREQUENTLY ASKED QUESTIONS**

**Q.** I want to treat my cells on a microplate for 24 – 48 hours. Will DCFDA be stable inside the cells for this period of time?

**A.** We don’t know whether DCFDA is stable for more than 6 hours. This kit has not been tested with prolonged treatments. However, in this situation we recommend to follow the steps below:

1. Dilute compounds of interest in complete media without phenol red. Make twice the volume required.
2. Treat suspension or adhered cells for the desired period of time. If treating cells for microplate measurements, treat with 100 µL per well.
3. Include blank wells with no cells but with compound at the same concentration used for treatment.
4. Include at least 2 positive control wells, to be reserved for TBHP treatment, containing cells but none of the test compounds.
5. 4 hours prior to completion of treatment, dilute TBHP to 10X of the final concentration (500 µM) and spike 10X TBHP into the reserved positive control wells by adding 11 µL per well.
6. 1 hour prior to completion of the treatment, dilute DCFDA at 2X of the final concentration desired in the same media used for treatment (containing experimental compounds) and warm to 37°C.
7. 30 – 45 minutes prior to completion of the treatment, overlay 2X DCFDA dilution on top of the treated cells. If treating cells for microplate measurements, overlay 100 µL of 2X DCFDA dilution per well.
8. Incubate DCFDA and compounds for the desired period of time (30 – 45 minutes).
9. Transfer the plate to the microplate reader without washing and read end point in the presence of compounds and DCFDA.
with excitation wavelength at 485 nm and emission wavelength at 535 nm.

10. Ratio the relative fluorescence intensity of control and treated wells to the relative fluorescence intensity of the blank wells.

Q. Should cells be washed after incubation with the cytotoxicity compound?
A. Cells should NOT be washed after treatment with the TBHP or other compounds of interest.

Q. Will this kit work with fixed cells?
A. Cells must be alive to work with this kit, therefore fixation is not recommended.

Q. How long is DCFDA stable for in cells?
A. We do not know how long DCFDA is stable in cells. We do know that it is stable for 4 hours and have tested DCFDA with treatments up to this point. However as we have not tested beyond this we do not know whether the signal is detectable beyond this point.

Q. Is it possible to dilute the reagents and then freeze them for another time?
A. The 1X Buffer can be frozen or kept at 4°C for use on a different experiment. It is recommended that the customer aliquot the DCFDA into as many experiments as they will run. It is probably only practical to aliquot in 8 – 10 uL volumes to prevent loss of reagent by aliquoting in too small a volume. We cannot ensure the stability long term (weeks/months) of the DCFDA once it is diluted in the dilution buffer. Enough is given for a 96 well plate, but there is no need to run all 96 data points at once. The TBHP will not be stable if diluted long term. There is no need to freeze TBHP, simply keep in the fridge and use as necessary. This is very stable if kept as it is supplied.
Q. Can FBS be included in the buffer whilst incubating the DCFDA?
A. It should work, but we have not tested it. Under the conditions stated in the current protocol, we have not found an increase in background signal.

Q. Why is TBHP being used in this kit instead of H$_2$O$_2$?
A. Either TBHP or H$_2$O$_2$ would work as positive controls.

Q. Can the culture media contain phenol red?
A. The microplate assay should be performed without phenol red both in suspension and adherent cells. If this is not possible we suggest to include all the relevant negative controls (including media + DCFDA with no cells) to ensure that the media does not affect DCFDA reading.

Q. How long can treated cells be stored prior to analyzing via flow cytometry?
A. After treatment, cells cannot be stored as the flow cytometry reading of DCFDA must be run on live cells.
16. **NOTES**
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