

Version 12 Last updated 2 February 2018

# ab113851 DCFDA Cellular ROS Detection Assay Kit

For measurement of reactive oxygen species (ROS) in cells

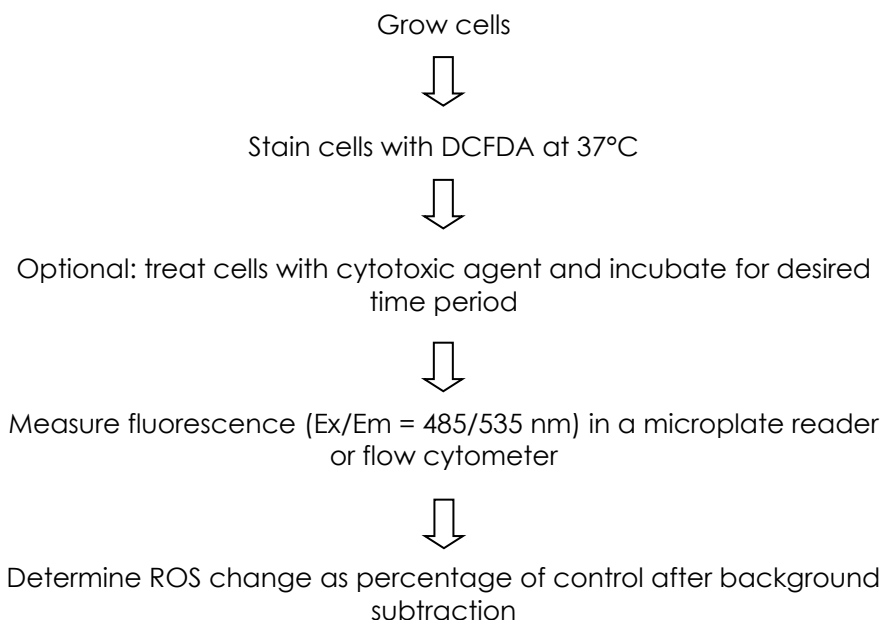
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# 1. Overview

The DCFDA - Cellular Reactive Oxygen Species (ROS) Detection Assay Kit (ab113851) quantitatively measures ROS in suspension or adherent cells using either a microplate assay or flow cytometry. The assay uses the cell permeant reagent 2',7' -dichlorofluorescein diacetate (DCFDA), a fluorogenic dye that measures hydroxyl, peroxy and other ROS activity within the cell. After diffusion in to 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.



## 2. Quick Assay Procedure

**Δ Note:** this procedure is provided as a quick reference for experienced users. Follow the detailed procedure when performing the assay for the first time.

### 2.1 Suspension Cell Microplate Assay

- 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  $\mu\text{M}$  DCFDA in 1X Buffer for 30 min at 37°C
- Wash cells once in 1X Buffer
- Optional: Seed cells at  $1.5 \times 10^5$  / 50  $\mu\text{L}$  / well and overlay 50  $\mu\text{L}$  of 2X treatment; incubate for desired time period
- Read signal at Ex/Em: 485/535 nm
- Determine change as percentage of control after background subtraction

### 2.2 Adherent Cell Microplate Assay

- Harvest  $3\text{-}4 \times 10^6$  cells
- Seed cells at  $2.5 \times 10^4$  cells/well on a 96 well plate
- Allow cells to attach overnight
- Wash cells once in 1X Buffer
- Stain cells with 25  $\mu\text{M}$  DCFDA in 1X Buffer for 45 min at 37°C
- Wash cells once in 1X Buffer
- Optional: Add 100  $\mu\text{L}$ /well of treatment and incubate for desired time period
- Read signal at Ex/Em: 485/535 nm
- Determine change as percentage of control after background subtraction

### 2.3 Flow Cytometry Assay

- Grow  $1.5 \times 10^5$  cells per experimental condition (1 well)
- Collect cells in conical test tube
- Stain cells with 20  $\mu\text{M}$  DCFDA for 30 min at 37°C
- DO NOT wash cells after staining
- Optional: Aliquot cells and treat for desired time period
- Read signal at Ex/Em: 485/535 nm
- Determine change as a percentage of control after background subtraction

### 3. Materials Supplied and Storage

Store kit at 4°C in the dark immediately on receipt and check below for storage for individual components. For longer term storage, keep at -20°C or -80°C. Kit can be stored for 1 year from receipt, if components have not been reconstituted.

Aliquot components in working volumes before storing at the recommended temperature.

Avoid repeated freeze-thaws of reagents.

Item	Quantity	Storage temperature (before prep)	Storage temperature (after prep)
20 mM DCFDA (in DMSO)	35 µL	4°C	4°C
10X Buffer	10 mL	4°C	4°C
55 mM Tert-Butyl Hydrogen Peroxide (TBHP)	50 µL	4°C	4°C

## 4. Materials Required, Not Supplied

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

- Fetal Bovine Serum (FBS) without phenol red
- DMSO (cell culture grade)
- Microplate reader or flow cytometer capable of measuring fluorescence at Ex/Em = 485/535 nm (use similar settings to those used to detect FITC)
- Sterile, tissue culture treated, 96 well plate with clear flat bottom and black sides
- (Optional) Test compounds/diluents of interest
- (Optional) Other ROS inducing control compounds such as doxorubicin, idarubicin, or antimycin
- (Optional) Decane: Solvent used for preparation and stabilization of TBHP

## 5. General guidelines, precautions, and troubleshooting

Please observe safe laboratory practice and consult the safety datasheet.

For general guidelines, precautions, limitations on the use of our assay kits and general assay troubleshooting tips, particularly for first time users, please consult our guide:

[www.abcam.com/assaykitguidelines](http://www.abcam.com/assaykitguidelines)

For typical data produced using the assay, please see the assay kit datasheet on our website.



## 6. Reagent Preparation

Briefly centrifuge small vials at low speed prior to opening.

The sample volumes below are sufficient for 96 x 100  $\mu$ L tests; adjust volumes as needed for the number of strips in your experiment.

### 6.1 10X Buffer

Prepare 1X Buffer by diluting 10X buffer in ddH<sub>2</sub>O: to make 100 mL 1X Buffer, combine 10 mL 10X Buffer with 90 mL ddH<sub>2</sub>O. Mix gently and thoroughly. Label this solution as “**1X Buffer.**”

Equilibrate to 37°C before use. 1X Buffer can be kept frozen or at 4°C for future use.

### 6.2 1X Supplemented Buffer

Prepare 1X Supplemented Buffer by adding 2 mL FBS to 18 mL of 1X Buffer (section 6.1). Make fresh prior to each use and do not store.

### 6.3 DCFDA Solution

Prepare a working DCFDA solution by adding the appropriate volume of 20 mM DCFDA to 1X Buffer. For example, to generate a 20  $\mu$ M final concentration, add 10  $\mu$ L of 20 mM DCFDA solution to 10 mL 1X Buffer.

Long term storage (weeks/months) of diluted DCFDA is not recommended.

The exact concentration of DCFDA required will depend on the cell line being used but a general starting range would be 10 – 50  $\mu$ M. Exact concentrations must be determined by the end user. Typical working concentrations for certain cell lines are shown in the table below:

Sample Type	Recommended Concentration ( $\mu$ M)
Adherent cells (HepG2)	25 $\mu$ M
Suspension cells (HL60, Jurkat)	20 $\mu$ M

#### **6.4 Tert-Butyl Hydrogen Peroxide (TBHP) Solution (Positive Control)**

Prepare a 50 – 250  $\mu\text{M}$  TBHP working solution by diluting 55 mM TBHP stock solution in the 1X Supplemented Buffer (section 6.2). Make fresh each time and do not store for future use (storage may lead to TBHP degradation).

TBHP may also be diluted in complete media with 10% FBS without phenol red.

**Δ Note:** The concentration of TBHP to use will depend on the sensitivity of the cell line. For example, HL60 and Jurkat cells are very sensitive to TBHP whereas HepG2 cells are very insensitive.

**Δ Note:** Optional alternative compounds to induce ROS in cells: HepG2 (cultured in glucose based media): 50  $\mu\text{M}$  antimycin, 50  $\mu\text{M}$  clozapine, 50  $\mu\text{M}$  fluvastatin, 50  $\mu\text{M}$  camptothecin.

HL60 (cultured in glucose based media): doxorubicin, idarubicin, menadione, methyl aminolevulinate, TNHP.

HL60 (cultured in galactose based media – glucose free): idarubicin, menadione.

#### **6.5 Optional: 5 mM Decane**

Dilute Decane (TBHP diluent; not included in the kit) 1,100X in 1X Supplemented Buffer or in complete media with 10% FBS without phenol red.

#### **6.6 Optional: Compounds/Diluents of Interest**

If performing toxicity assays, dilute compounds of interest in 1X Supplemented Buffer to final desired concentration for the experiment. A 96-deep well microplate may be used in this step. Compounds may also be diluted in complete media with 10% FBS without phenol red.

## 7. Assay Procedure

- Equilibrate all materials and prepared reagents to room temperature just prior to use and gently agitate.
- Assay all controls and samples in duplicate.
- Live cells must be used; fixed samples are not compatible
- Include background controls including blank wells (media or buffer only) and untreated or diluent treated stained cells

### 7.1 Suspension Cells Protocol for Microplate Assay

1. Grow suspension cells so that approximately  $1.5 \times 10^5$  cells per well are available on the day of the experiment.
2. Collect cells in a conical tube and wash by centrifugation once in PBS.
3. Stain the cells by resuspending in the diluted DCFDA Solution (section 6.3) at a concentration of  $1 \times 10^6$  cells/mL and incubate at 37°C for 30 minutes in the dark.
4. Wash cells by centrifugation with 1X Buffer maintaining the same concentration of cells.
5. Resuspend cells in 1X Supplemented Buffer or complete media with 10% FBS and no phenol red to a concentration of  $1 \times 10^6$  cells/mL.
6. Seed a dark, clear bottom 96-well microplate with 100,000 stained cells/well and measure fluorescence immediately (see step 8).
7. If performing toxicity assays, overlay each well with previously diluted 2X compounds and treat cells for desired time (1 - 6 hours). Include appropriate controls. DO NOT wash after treatment.
8. Measure plate on a fluorescence plate reader at Ex/Em = 485/535 nm in end point mode in the presence of compounds, media, or buffer.

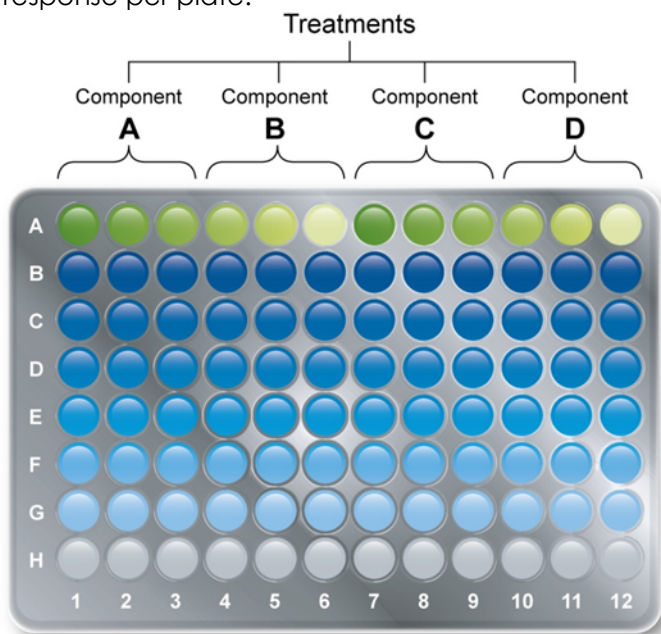
### 7.2 Adherent Cells Protocol for Microplate Assay

1. Grow adherent cells in standard cell culture media so that  $3 \times 10^6 - 4 \times 10^6$  cells are obtained the day before the experiment.
2. Harvest cells and seed a dark, clear bottom 96-well microplate with 25,000 cells per well. Allow cells to adhere overnight.
3. Remove the media and add 100  $\mu$ L/well of 1X Buffer.

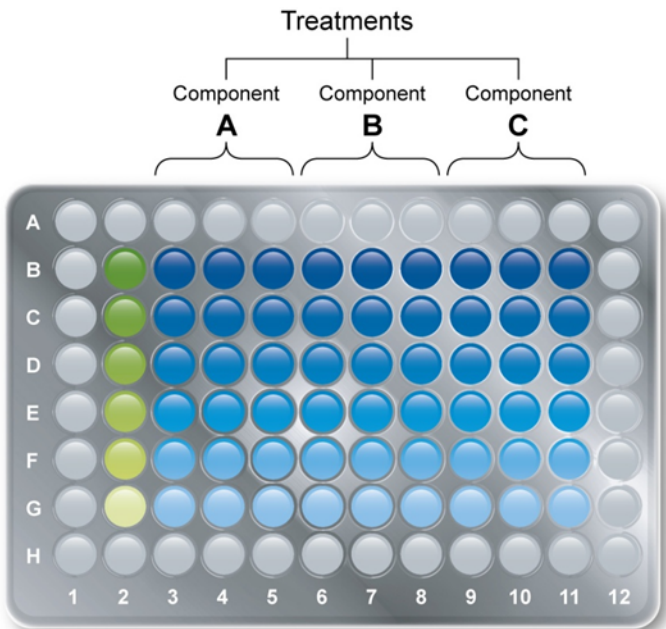
4. Remove 1X Buffer and stain cells by adding 100  $\mu$ L/well of the diluted DCFDA Solution (section 6.3).
5. Incubate cells with the diluted DCFDA Solution for 45 minutes at 37°C in the dark.
6. Remove DCFDA Solution. Add 100  $\mu$ L/well of 1X Buffer or 1X PBS and measure fluorescence immediately (see step 8).
7. If performing toxicity assays, remove 1X Buffer/PBS and add 100  $\mu$ L of previously 1X diluted compound(s) of interest. Treat cells for desired time (1 – 6 hours). Include appropriate controls. DO NOT wash after treatment.
8. Measure plate on a fluorescence plate reader at Ex/Em = 485/535 nm in end point mode in the presence of compounds, media, or buffer.

### 7.3 High Throughput Testing

- This assay may be used for screening pharmacological induction of ROS in any cell line. Depending on microplate template, either 4 or 3 compounds can be tested in triplicate dose response per plate.



**Figure 1: Suggested assay template for screening 4 compounds in dose response.** Row A contains vehicle control to determine maximal signal in the absence of compound. Row H contains non-stained cells to determine background fluorescence.



**Figure 2: Suggested assay template for screening 3 compounds in dose response.** Perimeter wells have been set as background fluorescence wells and column 2 has been set as vehicle titration control. Column 1, column 12, row A and row H contain non-stained cells.

## 7.4 Flow Cytometry Measurement

1. Grow cells (adherent or suspension) 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 accounts for 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 (e.g. trypsinize and quench with media).
3. Stain cells in culture media with  $20 \mu\text{M}$  DCFDA and incubate for 30 minutes at  $37^\circ\text{C}$ . Once the incubation is completed, DO NOT wash the cells.
4. After staining, treat the cells with compound(s) of interest and ensure appropriate controls are included. If using THBP as positive control, optimal signal is obtained after 4 hours of treatment.
5. Gently pipette cells up/down to ensure single cell suspension.
6. Analyze on flow cytometer. Establish forward and side scatter gates to exclude debris and cellular aggregates from analysis.
7. DCF is excited by the 488 nm laser and detected at 535 nm (typically FL1).
8. Ideally 10,000 cells should be analyzed per experimental condition. Cells should not be overly dense during the experiment ( $<1 \times 10^6$  cells/mL).

## 8. Data Analysis

### 8.1 Fluorescent Microplate Measurement

Subtract blank readings from all measurements and determine fold change from assay control (diluent treated cells if performing toxicity studies).

### 8.2 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.

## 9. FAQs / Troubleshooting

I want to treat my cells on a microplate for 24-48 hours. Will DCFDA be stable inside the cells for this long?

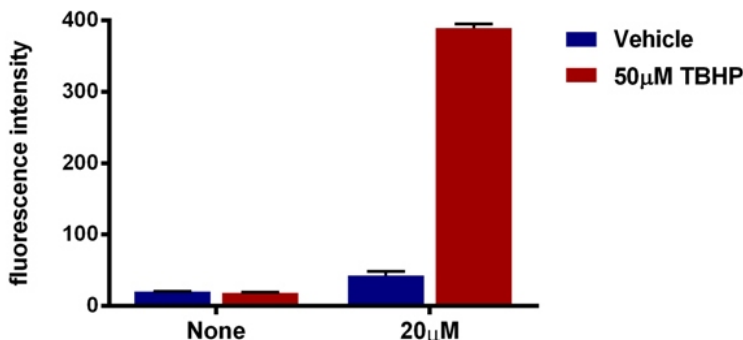
We don't know whether DCFDA is stable for more than 6 hours. This kit is not tested for prolonged treatments. However, in this situation we recommend following the steps below:

- Dilute compounds of interest in complete media without phenol red. Make twice the volume required.
- Treat suspension or adhered cells for the desired period of time. If treating cells for microplate measurements, treat with 100  $\mu$ L per well.
- Include blank wells with no cells but with compound at the same concentration used for treatment.
- Include at least 2 positive control wells, to be reserved for TBHP treatment, containing cells but none of the test compounds.
- 4 hours prior to completion of treatment, dilute TBHP to 10X of the final concentration (500  $\mu$ M) and spike 10X TBHP into the reserved positive control wells by adding 11  $\mu$ L per well.
- 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.
- 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  $\mu$ L of 2X DCFDA dilution per well.
- Incubate DCFDA and compounds for the desired period of time (30 – 45 minutes).
- Transfer the plate to the microplate reader without washing and read end point in the presence of compounds and DCFDA with Ex/Em = 485/535 nm.
- Ratio the relative fluorescence intensity of control and treated wells to the relative fluorescence intensity of the blank wells.

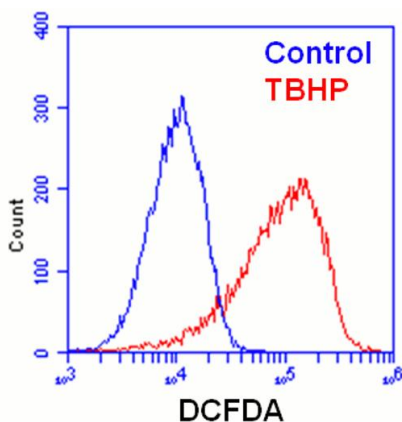


## 10. Typical Data

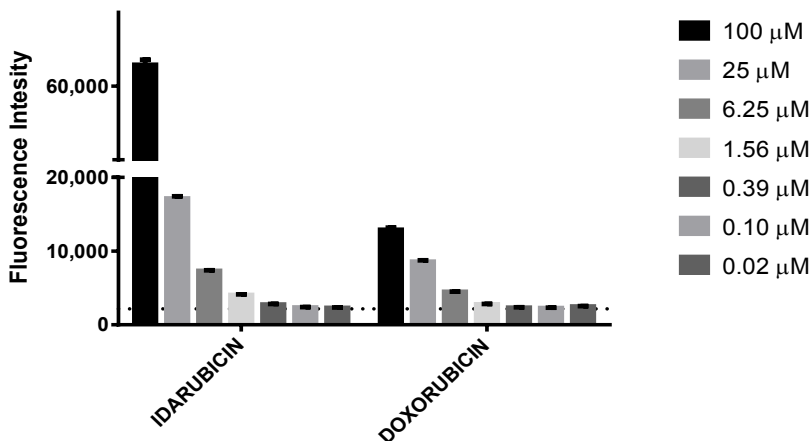
Data provided for demonstration purposes only.



**Figure 3. DCFDA microplate assay result.** Jurkat cells were labeled with DCFDA (20  $\mu$ M) or unlabeled (none) and then cultured an additional 3 hours with or without 50  $\mu$ M tert-butyl hydrogen peroxide (TBHP) according to the protocol. Cells were then analyzed on a fluorescent plate reader (SpectraMax M4 Microplate Reader). Mean  $\pm$  standard deviation is plotted for 4 replicates from each condition. TBHP mimics ROS activity to oxidize DCFDA to fluorescent DCF.



**Figure 4. DCFDA flow cytometry assay result.** Labeled and unlabeled Jurkat cells were treated with 50  $\mu$ M tert-butyl hydrogen peroxide (TBHP) as described in Figure 3 and then analyzed by flow cytometry. There is a 10.1-fold difference between the control and TBHP mean fluorescent intensities.



**Figure 5. 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 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.

## 11. Notes

## Technical Support

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