

ab113851 DCFDA / H2DCFDA - Cellular ROS Assay Kit

For measurement of reactive oxygen species (ROS) in cells
For research use only - not intended for diagnostic use.

For overview, typical data and additional information please visit:

www.abcam.com/ab113851

DCF is a highly fluorescent compound with maximum excitation/emission spectra at 495/529 nm. We recommend measuring at 485/535 nm to reduce background noise from excitation and emission crosstalk.

Storage and Stability: Aliquot and store kit at -20°C in the dark immediately on receipt. Refer to list of materials supplied for storage conditions of individual components.

Materials Supplied

Item	Quantity	Storage temp
20 mM DCFDA (in DMSO)	35 µL	-20°C
10X Buffer	10 mL	-20°C
55 mM TBHP	50 µL	-20°C

Materials Required, Not Supplied

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

- Fetal Bovine Serum (FBS)
- DMSO (cell culture grade)
- Microplate reader or flow cytometer or fluorescence microscope 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

Reagent Preparation

Briefly centrifuge small vials at low speed prior to opening. The sample volumes below are sufficient for 96 x 100 µL tests; adjust volumes as needed for the number of strips in your experiment.

10X Buffer. Prepare 1X Buffer by diluting 10X buffer in ddH₂O: to make 100 mL 1X Buffer, combine 10 mL 10X Buffer with 90 mL ddH₂O. 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.

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

DCFDA Solution. Prepare a working DCFDA solution by diluting 20 mM DCFDA in 1X Buffer: to make a 20 µM final concentration, add 10 µL of 20 mM DCFDA solution to 10 mL 1X Buffer. DCFDA may also be diluted in media without phenol red. Use freshly prepared DCFDA solution; long term storage 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 µM.

TBHP Solution (Positive Control). Prepare a 50 – 250 µM TBHP working solution by diluting 55 mM TBHP stock solution in the 1X Supplemented Buffer. 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.

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.

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.

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 in the absence and presence of treatment: media/buffer without cells (microplate only) and cells without DCFDA.
- For treatment longer than 6 hours see FAQ/Troubleshooting.

1.1 Suspension Cells Protocol for Microplate Assay

1. Grow suspension cells so that approximately 1.5×10^5 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 at a concentration of 1×10^6 cells/mL and incubate at 37°C for 30 minutes in the dark.
4. Wash cells by centrifugation with 1X Buffer maintaining the same cell concentration.
5. Resuspend cells in 1X Buffer, 1X Supplemented Buffer or complete media with 10% FBS without phenol red to 1×10^6 cells/mL (Buffer choice dependent on cell line culture requirements).
6. Seed a dark, clear bottom 96-well microplate with 100,000 stained cells/well.
7. Optional: Overlay each well with previously diluted 2X compounds and incubate for desired time (1 - 6 hours). If using TBHP as positive control, optimal signal is obtained after 4h. Include appropriate controls. DO NOT wash after treatment.
8. Measure plate immediately on a fluorescence plate reader at Ex/Em = 485/535 nm in end point mode in the presence of compounds, media, or buffer.

1.2 Adherent Cells Protocol for Microplate Assay

1. Grow adherent cells in standard cell culture media so that 3×10^6 - 4×10^6 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 μ L/well of 1X Buffer.
4. Remove 1X Buffer and stain cells by adding 100 μ L/well of the diluted DCFDA Solution.
5. Incubate cells with the diluted DCFDA Solution for 45 minutes at 37°C in the dark.
6. Remove DCFDA Solution. Add 100 μ L/well in 1X Buffer, 1X Supplemented Buffer or complete media with 10% FBS without phenol red (Buffer choice dependent on cell line culture requirements).
7. Optional: Remove buffer and add 100 μ L of previously 1X diluted compound(s) of interest. Treat cells for desired time (1-6 hours). If using TBHP as positive control, optimal signal is obtained after 4h. Include appropriate controls. DO NOT wash after treatment.
8. Measure plate immediately on a fluorescence plate reader at Ex/Em = 485/535 nm in end point mode in the presence of compounds, media, or buffer.

1.3 Flow Cytometry Measurement

1. Grow cells (adherent or suspension) in standard cell culture media so that on the day of the experiment there are at least 1.5×10^4 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 μ M DCFDA and incubate for 30 minutes at 37°C.
4. Optional: wash cells with 1X Buffer after incubation (excess DCFDA does not significantly affect signal).
5. Treat the cells with compound(s) of interest and ensure appropriate controls are included. If using TBHP as positive control, optimal signal is obtained after 4 hours of treatment.
6. Gently pipette cells up/down to ensure single cell suspension.
7. Analyze on flow cytometer immediately. Establish forward and side scatter gates to exclude debris and cellular aggregates from analysis.
8. DCF is excited by the 488 nm laser and detected at 535 nm (typically FL1).
9. 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).

1.4 Fluorescent microscopy

1. Grow adherent cells in standard cell culture media to an appropriate density on a suitable substrate for live cell imaging (chamber slide, 96 well plate, etc depending on available microscopy setup). Avoid phenol red (background fluorescence).
2. Wash cells 1 or 2 times with 1X Buffer.
3. Stain cells by adding diluted DCFDA Solution.
4. Incubate cells with the diluted DCFDA Solution for 45 minutes at 37°C in the dark.
5. Wash cells 1 or 2 times with 1X Buffer.

6. Perform live cell microscopy with filter set appropriate for fluorescein (FITC). Maintain low light conditions to avoid photobleaching and photo oxidation of DCF. --
--Note that fluorescence will increase due to light exposure and field of view brighten.

Data Analysis

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

2.2 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.

2.3 Fluorescent Microscopy Measurement. Visually score cells for brightness and compare between control and samples or use image analysis methods to compare signal between on digital photographs of cells.

FAQ/ 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:

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 time. For microplate measurements, treat with 100 μ L 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 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 (e.g. 500 μ M for 50 μ M final) and add to the reserved positive control wells (11 μ L to 100 μ L wells).
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. For microplate measurements, overlay 100 μ L of 2X DCFDA dilution per well.
8. Incubate DCFDA and compounds for 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 Ex/Em = 485/535 nm.

If high signal is observed in cell culture media and DCFDA only control, We advise to switch to serum free medium or medium with heat inactivated fbs for the measurement

Technical Support

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