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A method to determine reactive oxygen species production in intestinal and liver cell cultures using the 2′,7′-dichlorodihydrofluorescein diacetate assay

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ABSTRACT

Exposure to xenobiotics can increase the production of reactive oxygen species (ROS). When detoxification organs such as the intestines and liver cannot neutralise these xenobiotics, it can induce oxidative stress and cause damage to tissues. Therefore, cell-based bioassays that indicate intracellular ROS production are a useful screening tool to evaluate the effect of these chemicals. Although flow cytometry is commonly used to measure ROS in cells, many research laboratories in the Global South do not always have access to such specialised instrumentation. Therefore, we describe a sensitive but low-cost method that can easily be used to determine ROS production *in vitro*. This method employs the fluorogenic dye, 2′,7′-dichlorodihydrofluorescein diacetate (H_2DCF-DA), which emits fluorescence after being oxidised to a fluorescent derivative. Since the H_2DCF-DA bioassay indicates non-specific ROS production it can be used as a marker of overall oxidative stress. This method was validated by exposing human duodenum epithelial adenocarcinoma (HuTu-80) and rat liver epithelial hepatoma (H4IIE-*luc*) cells to agricultural soil samples.

- Production of ROS can be determined *in vitro* in intestinal and liver cells.
- This method is inexpensive and can be easily performed in standard laboratories.
- The method provides a tool for the high-throughput screening of environmental samples.

Specifications table

Subject area:	Agricultural and Biological Sciences
More specific subject area:	Mammalian cell cultures; environmental samples; pure compounds
Name of your method:	H_2DCF-DA bioassay for cell cultures
Name and reference of original method:	H. Wang, J.A. Joseph. Quantifying cellular oxidative stress by dichlorofluorescein assay using microplate reader, Free Radic. Biol. Med. 27(5–6) (1999) 612–616. doi: 10.1016/S0891–5849(99)00,107–0
Resource availability:	Not applicable

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Method details

General background

Reactive oxygen species (ROS) originate from partial reduction of oxygen and are endogenously produced as by-products of aerobic metabolic processes [1]. Small concentrations of ROS maintain essential physiological functions in organisms [2]. However, exposure to pollutants can increase intracellular production of ROS and cause oxidative stress [3]. To prevent oxidative damage, xenobiotics and resulting ROS are detoxified in the intestines and liver. In those tissues parent compounds and their metabolites are absorbed in the gastrointestinal tract (GIT), which represents the most common exposure route for xenobiotics [4]. After absorption in the GIT, the liver is the main organ responsible for the metabolism of xenobiotics including phase I and II biotransformation reactions which increase the production of ROS [5]. Therefore, intestinal and liver cells are especially susceptible to chemical injury when exposed to pollutants.

Intracellular ROS, including hydrogen peroxide (H_2O_2), can be determined using fluorescent probes—small molecule sensors that react to specific stimuli. An example of such a probe is 2′,7′-dichlorodihydrofluorescein diacetate (H_2DCF-DA). During the H_2DCF-DA assay, the membrane-permeable fluorogenic dye diffuses through the plasma membrane of cells and is hydrolysed to non-fluorescent 2′,7′-dichlorodihydrofluorescein (DCFH) by intracellular enzymes where it remains trapped [6,7]. Under oxidative stress, DCFH reacts with intracellular ROS resulting in the oxidation of DCFH to its highly fluorescent fluorescein derivative, 2′,7′-dichlorofluorescein (DCF) [8]. The emitted DCF fluorescence is proportional to the amount of ROS produced intracellularly. The ability of the fluorogenic dye to diffuse into cells makes it highly suitable for cell-based bioassays [7]. Based on this, the authors provide a simple and cost-effective method that can be used to indicate whether ROS production has been induced in mammalian cell cultures without the use of costly commercial kits. The original method evaluated ROS production in cells derived from the rat adrenal medulla [9]. However, since detoxification organs are susceptible to chemical injury, we describe an H_2DCF-DA bioassay adapted for determining ROS production in intestinal and liver cell lines.

Materials and methods

Equipment

- Cell culture CO_2 incubator that is suitable for tissue culture.
- Centrifuge that can reach 1 000 g.
- pH meter.
- Microplate reader that can measure fluorescence at different excitation and emission wavelengths in 96-well microplates.

Consumables

- 24-well, clear, flat-bottom cell culture microplates.
- 3 mL Plastic Pasteur pipette (non-sterile).
- 2 mL Microcentrifuge tube.
- 96-well, black, flat-bottom, cell culture microplate.

Chemicals and reagents

- Dulbecco's phosphate-buffered saline (DPBS).
- Trypsin-ethylenediaminetetraacetic acid (EDTA) (100X).
- Hydrogen peroxide (H_2O_2) (30%).
- 2′,7′-Dichlorodihydrofluorescein diacetate (H_2DCF-DA).

Working solutions

- DPBS (pH 7.4).
- Trypsin-EDTA (diluted in PBS; 1:9, *v/v*).
- H_2O_2 (350 ng/mL, prepared in DPBS). **Note:** This solution should be prepared fresh before use.
- H_2DCF-DA (10 μM, prepared in DPBS). **Note:** This solution should be prepared and used in the dark since the fluorogenic dye is light-sensitive. The solution can be stored in the dark at -80 °C for up to two years.

Cells

- HuTu-80 (HTB-40™), human duodenum epithelial adenocarcinoma (American Type Culture Collection, Virginia, United States of America).
- H4IIE-*luc*, rat liver epithelial hepatoma (gifted from the University of Saskatchewan, Canada).

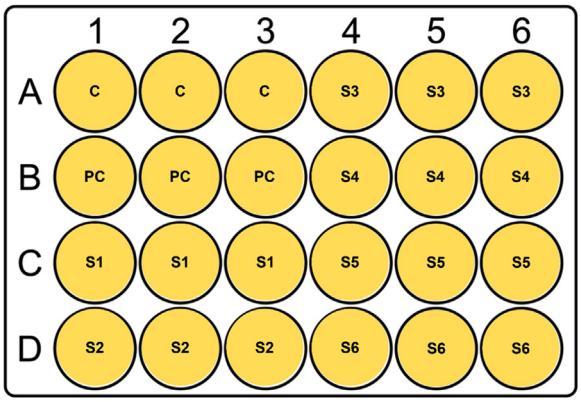

Fig. 1. The 24-well microplate layout for the determination of reactive oxygen species production. C: Control (i.e., untreated cells); PC: Positive control (hydrogen peroxide stimulated cells); S1–S6: Sample 1–6.

Methods

Intracellular ROS

1. Seed 1 mL of cells into a 24-well, clear, flat-bottom microplate. The seeding density will depend on the specific cell line used.
2. Incubate the cells at 37°C in humidified air supplemented with 5% CO_2 for 24 h.
3. Following the attachment of cells, expose the cells to the samples in triplicate for 24 h (Fig. 1). **Note:** Although the different methods of sample preparation are outside of the scope of this paper, it is worth mentioning that the cells can be exposed to samples either via directly dosing into the cell culture medium or by replacing the initial nutrient medium with a medium containing the samples.
4. After adherent growth and exposure to samples (±24 h), the positive control (PC) cells are stimulated with H_2O_2 for 45 min, by directly dosing H_2O_2 (350 ng/mL) into the PC wells (Fig. 1). **Note:** The H_2O_2 concentration required to induce ROS production in the PC cells may vary between different cell lines and it is recommended that this concentration be optimised for individual cell lines before assessing sample responses. Based on prior optimisation experiments, 3.5 ng/mL and 14.2 ng/mL H_2O_2 were found to induce ROS production in the HuTu-80 and H4IIE-*luc* cells, respectively. Consequently, a dosing volume of 10 µL (HuTu-80) and 41 µL (H4IIE-*luc*) of 350 ng/mL H_2O_2 were used for the respective cell lines.
5. After exposure to samples and stimulation with H_2O_2 (PC), remove the nutrient media of the cells by inversion of the microplate.
6. Wash the cells three times with 500 µL DPBS. **Note:** From here on the assay is performed in the dark since H_2DCF-DA is light-sensitive.
7. Add 200 µL H_2DCF-DA (10 µM) to all wells and incubate at 37°C and 5% CO_2 for 30 min.
8. Remove the H_2DCF-DA from the wells by inversion of the microplate.
9. Wash the cells three times with 500 µL DPBS.
10. Add 150 µL trypsin-EDTA to the cells and incubate at 37°C in 5% CO_2 for 3 min (cell-specific).
11. Add 500 µL DPBS to stop trypsin activity.
12. Harvest the cells and transfer the cell suspension of individual wells to a 2 mL microcentrifuge tube using a plastic Pasteur pipette. **Note:** The microcentrifuge tubes should be labelled in such a way that it corresponds to the well number of the 24-well microplate.
13. Centrifuge the cell suspension at 1 000 g for 4 min at room temperature (± 25°C).
14. Discard the supernatant (be careful not to discard the cell pellet).
15. Resuspend the cell pellet in 800 µL DPBS.
16. Transfer 200 µL of the cell suspension to a black 96-well, flat-bottom, cell culture microplate in triplicate.
17. Measure fluorescence (relative fluorescence units, RFUs) at excitation and emission wavelengths of 480 nm and 535 nm, respectively, using a SpectraMax® iD3 multi-mode microplate reader (Molecular Devices, LCC, Lasec SA (Pty) Ltd, South Africa).
18. The RFUs of sample responses are compared to the control. **Note:** This method can only be used for the indication of ROS production and not the quantification thereof.

Method validation

To validate the H_2DCF-DA bioassay for intestinal and liver cell cultures, human duodenum epithelial adenocarcinoma (HuTu-80) and rat liver epithelial hepatoma (H4IIE-*luc*) cells were exposed to polar chemical mixtures extracted from agricultural soil samples. Briefly, cells were seeded (80 000 cells/mL) in 24-well microplates, followed by incubation at 37°C in a humidified atmosphere with

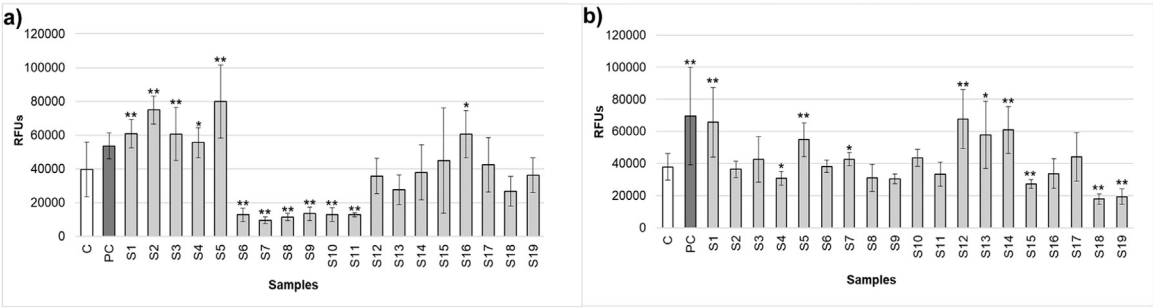

Fig. 2. ROS production in a) HuTu-80 and b) H4IIE-*luc* cells. Data are expressed as mean ± standard deviation ($n = 9$). The Mann-Whitney *U* test was used to determine statistically significant ($^{*}p \leq 0.05$ and $^{**}p \leq 0.01$) responses compared to the control (C). PC: Positive control (i.e., hydrogen peroxide stimulated cells); RFUs: Relative fluorescence units; S1–S19: ample 1–19.

Table 1
Validation parameters for the positive control cells of the adapted H_2DCF-DA bioassay.

	x̄	σ	SE
HuTu-80	53 617	7 562	2 521
H4IIE-*luc*	69 628	30 366	10 122

σ: Standard deviation; x̄: Mean; H_2DCF-DA: 2′,7′-Dichlorodihydrofluorescein diacetate; SE: Standard error.

5% CO_2 and 95% air for 24 h. After attachment, the cells were exposed to the samples (83 mg soil equivalents/mL) for 24 h by replacing the initial nutrient media with an exposure media. First, soil extracts were obtained by adding 20 mL of deionised water to 10 g of soil and shaking the mixture for 1 h (150 rpm) on a mechanical shaker. This was followed by centrifugation at 3 000 g for 20 min. The process was repeated twice and the supernatants were pooled. The soil extracts were used to prepare nutrient media for the cells (i.e., exposure media). Positive control cells were stimulated with H_2O_2. The H_2O_2 concentrations were 3.5 ng/mL and 14.2 ng/mL for the HuTu-80 and H4IIE-*luc* cells, respectively. Control (i.e., untreated) cells were also included. After adding the fluorogenic probe (H_2DCF-DA), fluorescence was measured in black 96-well microplates. The data show statistically significant ($p \leq 0.05$ and $p \leq 0.01$) ROS responses between the control (i.e., untreated) and cells exposed to the soil samples (Fig. 2). Standard deviation and standard error were, respectively, used as reliable statistics to report the precision and accuracy of the method (Table 1). The findings show that the method can be used to indicate ROS production as a marker of oxidative stress in cell lines derived from detoxification organs.

Ethics statements

No human or animal subjects were used during this study. Based on the review by the Faculty of Natural and Agricultural Sciences Ethics Committee (FNASREC) of the North-West University (NWU), South Africa, this study was cleared as having no ethical risk (Ethics number: NWU-01,690–20-A9).

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

CRediT authorship contribution statement

Ilzé Engelbrecht: Conceptualization, Methodology, Formal analysis, Investigation, Writing – original draft, Writing – review & editing, Visualization, Project administration. **Suranie Horn:** Conceptualization, Resources, Writing – review & editing, Visualization, Supervision, Funding acquisition. **John P. Giesy:** Methodology, Writing – review & editing. **Rialet Pieters:** Conceptualization, Resources, Writing – review & editing, Visualization, Supervision, Funding acquisition.

Data availability

Data will be made available on request.

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