Standard Operating Procedure: Sciensano

PROTOCOL	**Oxidative Stress – H2DCF-DA/MTT Assay in HaCaT cells**
DATE	01/05/2025
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1. Objective and Purpose

This procedure describes the measurement of ROS generation in HaCaT cells using the combined H2DCF-DA and MTT assays used in the laboratory of experimental toxicology. The H2DCF-DA is used to quantify the amount of ROS generated within HaCaT cells, while the MTT is used to normalize this against the number of cells present within the same sample, correcting for changes in growth and cytotoxicity as a result of exposure.

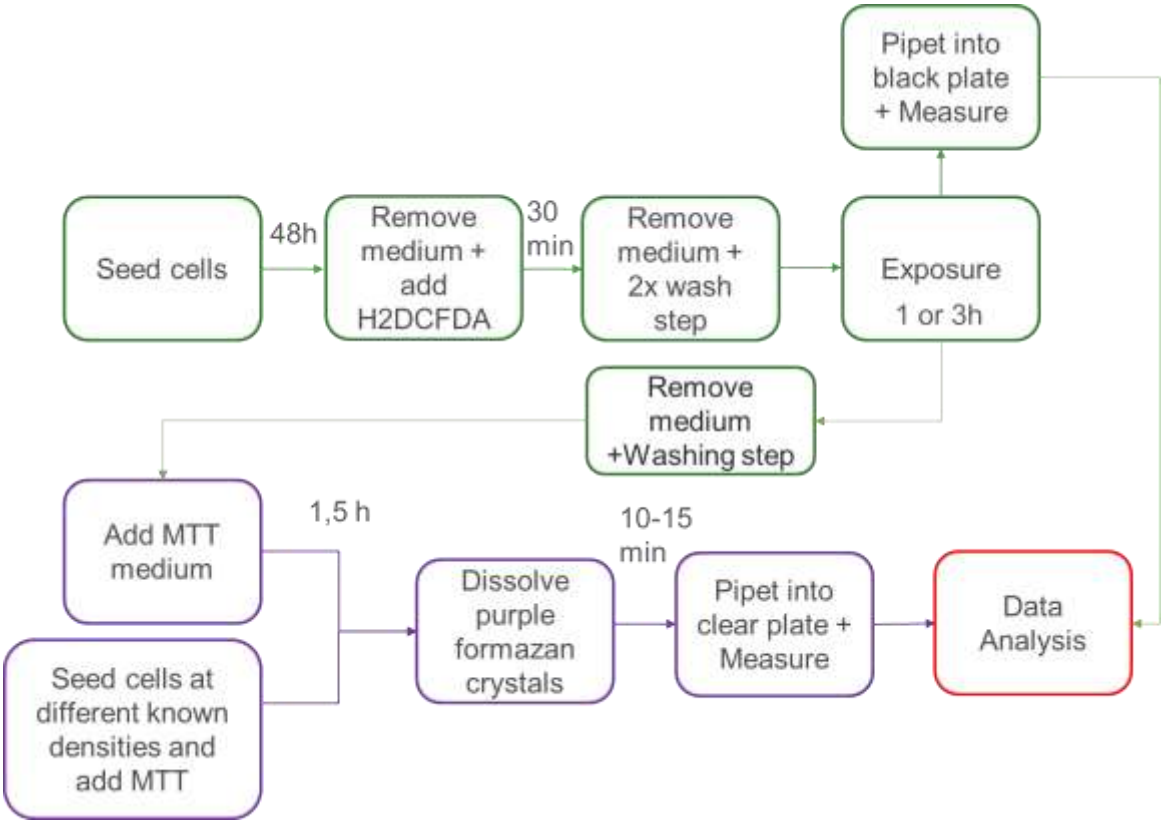


Figure 1: Schematic overview of the combined H2DCF-DA/MTT assay

2. Definitions et abbreviations

DMEM	Dulbecco's Modified Eagle Medium
DMSO	Dimethylsulfoxide
H2DCF-DA	2',7'-dichlorodihydrofluorescein diacetate
DCF	2',7'-dichlorofluorescein
FBS	Foetal Bovine Serum
MTT	3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide

3. Procedure

3.1. Principle

- Cell-permeable dye: H2DCF-DA is a non-fluorescent, cell-permeable compound. It easily diffuses into live cells.

- Intracellular deacetylation: Once inside the cell, cellular esterases remove the acetate groups from H2DCF-DA, converting it into 2′,7′–dichlorodihydrofluorescein (DCFH), which is still non-fluorescent but now trapped inside the cell.
- Oxidation by ROS: DCFH is oxidized by ROS, especially hydrogen peroxide (H_2O_2), hydroxyl radicals (•OH), and peroxynitrite ($ONOO^-$), to form 2′,7′–dichlorofluorescein (DCF).
- Fluorescence emission: DCF is highly fluorescent, and its fluorescence intensity (excitation ~485 nm, emission ~535 nm) is proportional to the amount of ROS present in the cell.

The fluorescent signal measuring ROS generation then gets normalized against the MTT signal, allowing us to calculate an amount of signal vs the amount of cells present within the sample.

- MTT is a yellow tetrazolium salt (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide).
- Uptake and reduction: Viable cells with active mitochondria take up MTT and reduce it using NAD(P)H-dependent oxidoreductase enzymes.
- Formation of formazan: MTT is converted into insoluble purple formazan crystals. This reaction occurs primarily in the mitochondria of metabolically active cells.
- Solubilization: The purple formazan crystals are insoluble in aqueous media, so a solubilizing agent (e.g., DMSO, SDS in acidified isopropanol) is added to dissolve them.
- Quantification: The resulting purple solution is quantified by measuring absorbance at 540–570 nm using a spectrophotometer or plate reader.

More absorbance = more viable/metabolically active cells.

3.2. Security

Protective Clothing: Eye-shield, laboratory coat purple nirtil gloves

All manipulations involving the culturing of the cell cultures should be performed in a biosafety cabinet.

3.3. HaCaT Cell Line

HaCaT is a spontaneously immortalized keratinocyte cell line from the adult human skin of a 62-year-old male. Due to their high capacity to differentiate and proliferate in vitro, these cells are extensively utilized in skin biology and differentiation research.

3.4. Chemicals

Amphotericin B *(Thermo Fisher Scientific)*

Fluorobrite DMEM (Dulbecco's Modified Eagle Medium) *(Thermo Fisher Scientific)*

DMSO (Dimethylsulfoxide) *(Sigma-Aldrich)*

FBS (Foetal Bovine Serum) (Thermo Fisher Scientific)

Gentamycin (Thermo Fisher Scientific)

Glutamax (Thermo Fisher Scientific)

Non Essential Amino Acids (Thermo Fisher Scientific)

PBS pH 7.2 (Thermo Fisher Scientific)

Sodium Pyruvate (Thermo Fisher Scientific)

TrypLE™ (Thermo Fisher Scientific)

Trypsin-EDTA (Thermo Fisher Scientific)

2',7'-dichlorodihydrofluorescein diacetate (Merck)

3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (Merck)

Menadione (Merck)

H2O2 30% (Merck)

3.5. Solutions

3.5.1. Complete Medium

Add to a 500 ml Fluorobrite DMEM bottle:

- 6 ml Gentamycin, Glutamax, Sodium Pyruvate & Non Essential Amino Acids
- 60 ml FBS (2 aliquots)
- 600 µl Amphoterecin B (1 aliquot)
- Label & store at 4°C for maximum 1 month.

3.5.2. H2DCF-DA stock solution

Dissolve the H2DCF-DA powder in DMSO, so that you have an end concentration of 100 mM.

3.5.3. H2DCF-DA loading medium

For an endconcentration of 40 µM, combine

- 49 mL of base fluorobrite DMEM (without additives!)
- 39.2 µL of H2DCF-DA stock solution.

3.5.4. Menadione stock solution

Prepare a Menadione 5 mM solution in DMSO

3.5.5. Menadione exposure medium

Combine, for an end concentration of 5 µM:

- 50 mL base Fluorobrite DMEM medium
- 50 µL Menadione stock solution

3.5.6. H_2O_2 control medium

Combine 20 µl H_2O_2 (30%) with 6 mL base fluorobrite DMEM medium, for a final concentration of 0.1 % H_2O_2

3.5.7. MTT medium

- Weigh 20 mg of 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide
- Dissolve the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide in 4 ml PBS
- Add 36 ml of complete fluorobrite DMEM medium to the solution.

3.6. Experimental procedure for the DCF/MTT assay

3.6.1. Cell seeding

- Seed 2 ml of a cell suspension containing 100 000 cells/ml in a 35 mm cell culture dish (Nunc, NunclonTM Surface or equivalent) in complete fluorobrite DMEM medium. (total of 200 000 cells / dish). Every condition should be performed in triplicate, and include positive control with Menadione and H2O2
- Incubate cells for 24 hours in the incubator (37°C, 5% CO2).

3.6.2. H2DCF-DA Loading

The timing of the loading should be timed, so that the fluorescence will be read out 48 hours after seeding. If exposure will take 3 hours, the loading should be performed 44.5 hours after seeding. For 1 hour exposure, the loading should be performed 46,5 hours after seeding.

- Remove the medium from the cells
- Add 2 ml H2DCF-DA loading medium to the cells
- Incubate cells for 30 minutes in the incubator (37°C, 5% CO2).

3.6.3. Washing step

- Remove the dishes from the incubator and remove medium from the dishes
- Add 2 ml PBS to the dishes
- Remove PBS from the dishes
- Add 2 ml PBS to the dishes
- Remove PBS from the dishes

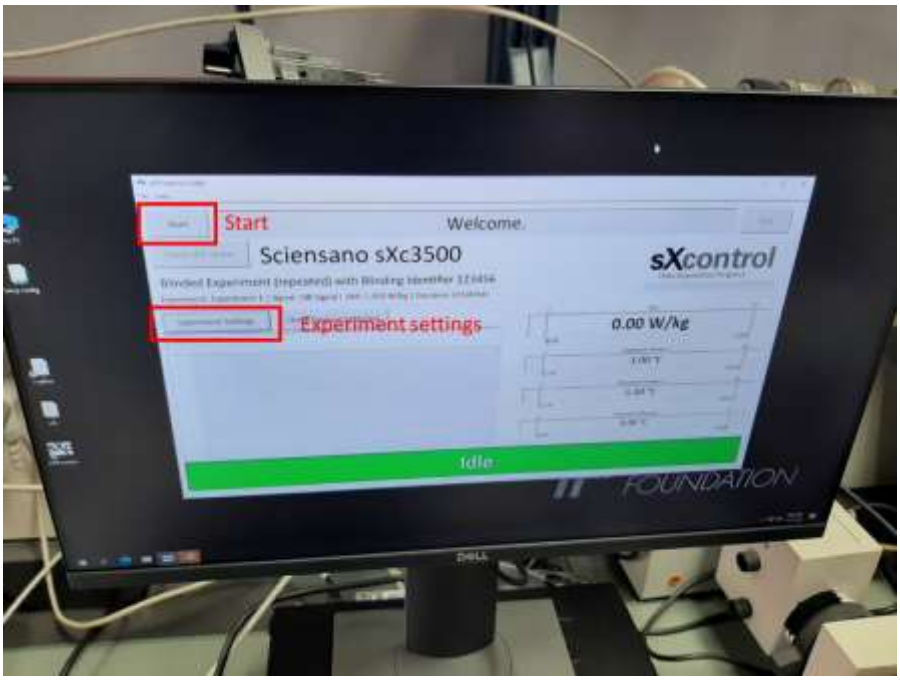
3.6.4. Exposure (RF-EMF)

- Add 3.1 mL cell medium containing the positive control substance (menadione, H2O2) or the solvent (negative control – base fluorobrite DMEM medium).

  Remark:

1. 6 dishes should always be used for the negative control (3 per exposure chamber) and six for the positive control (3 per exposure chamber) to ensure the correct amount of samples to achieve statistical significance. Additionally, an incubator control can be added, including additional negative and positive control samples (both Menadione and H202)

- Place the cells inside the 5G exposure system (inside the sample holder) and incubate the cells for the required time (37°C, 5% CO_2) – either 3 hours or 1 hour, depending on the experiment.



  - For a more in depth description on how to set up the exposure system, see the "sXc 3500 exposure system" guidelines
  - In the "experiment settings" tab, adjustments can be made including SAR, exposure duration and the blinding ID of the experiment
  - Pressing start will enable the 5G EMF to flow inside the exposure chambers and pressing pause will temporarily disable this. It can be enabled again by pressing "start" again.
  - Experiments should always be performed blind, this means that only one of the chambers will be exposed at the same time. This can be decoded by sending the blinding ID to the people at IT'IS (sXc@itis.swiss). This means that the number of samples put inside the two exposure chambers should always be identical, since it is unknown which one of the chambers will be exposed beforehand.

3.6.5. Fluorescence measurement

- After the exposure is finished, take the samples out of the incubator/exposure system.
- Pipet 100 µl into a black 96-well plate. At least 3 wells should be used per sample, in order to account for potential pipetting inconsistencies.

- Measure fluorescence in the Promega GloMax Discover, using excitation at 488 nm and emission at 520 nm.

3.6.6. Washing step

- Remove the DCF medium from the dish and rinse using 2 mL PBS.
- Remove PBS from dishes

3.6.7. Calibration curve cell seeding.

- From a new culture bottle, make a dilution series of cells (600.000 – 300.000 – 150.000 – 75.000 – 37.500 – 0) in an end volume of 1 mL MTT medium.
- Incubate for 2 hours in darkness (covered with alluminum foil).

3.6.8. MTT

- Add 1 mL MTT to the dishes at the same time as doing the calibration curve cell seeding.
- Incubate for 2 hours in darkness (covered with alluminum foil).

3.6.9. Absorbance measurement

- Dissolve the purple crystals in 2 mL DMSO, by putting it on a shaking incubator at 37°C
- Check for complete dissolution of the crystals prior to measurement.
- Pipet 100 µL of of the solution in the dishes into a clear 96-well plate. At least 3 wells should be used per sample, in order to account for potential pipetting inconsistencies.
- Measure absorbance in the Promega GloMax Discover at 560 nm.

4. Data analysis

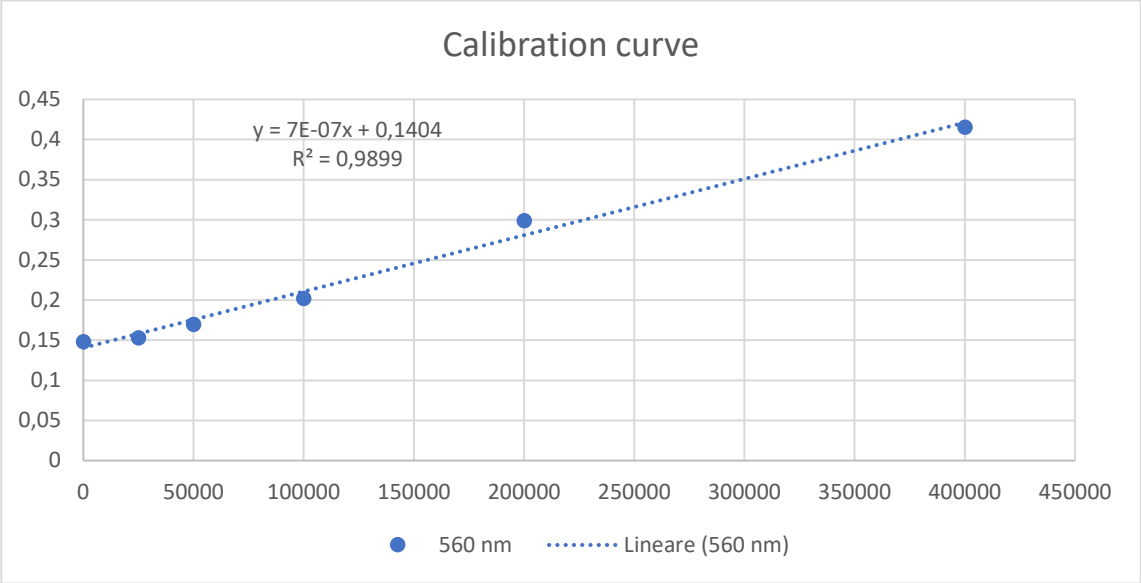
4.1. Fluorescence measurements

Fluorescence measurements calculated against the background (this is an empty well, filled only with the base fluorobrite medium (no cells)

Resulting fluorescence = measured fluorescence – background fluorescence

4.2. MTT calibration curve.

Use the MTT absorbance signal to plot a calibration curve by plotting it against the known number of cells in the sample. Use excel to find the trendline, including the R^2 and the equation.



4.3. MTT signal to cell viability

Use excel to calculate the cell viability:

(% Viability = (Average Absorbance (Treated) – empty well (containing only 1ml MTT medium + 2ml DMSO) / Average Absorbance (Control)) – empty well (containing only 1ml MTT medium + 2ml DMSO) * 100)

4.4. Calculate the amount of cells present in a sample.

Use the equation obtained from the trendline to calculate the amount of cells present in the sample.

If the trendline has the following equation, with

Y = signal

X = amount of cells

$$Y= aX+b$$

This equation can be reformed

$$\frac{Y-b}{a}=X$$

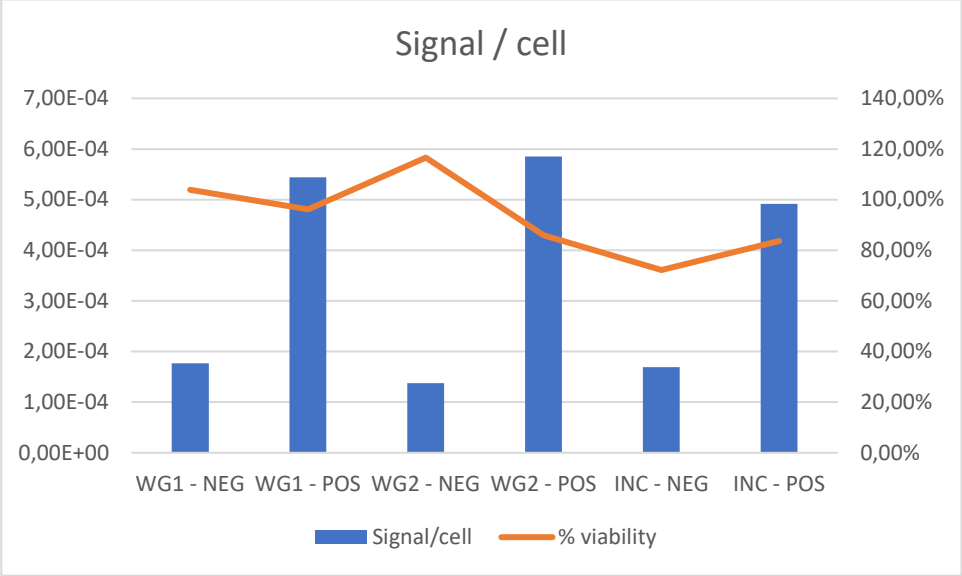
4.5. Calculate signal/ cell

Divide the signal obtained in 4.1. by the amount of cells obtained in 4.4. to obtain the signal/cell

Reported value should be:

Fluorescent signal/cell, averaged over the 3 technical replicate samples.

Ideally, this should be reported on the same graph as the cell viability.


Signal / cell
7,00E-04
6,00E-04
5,00E-04
4,00E-04
3,00E-04
2,00E-04
1,00E-04
0,00E+00
140,00%
120,00%
100,00%
80,00%
60,00%
40,00%
20,00%
0,00%
WG1 - NEG
WG1 - POS
WG2 - NEG
WG2 - POS
INC - NEG
INC - POS
Signal/cell
% viability