Moxi GO – Oxidative Stress Staining using Thermo Fisher's CellROX™ Green ROS Staining Protocol (from Thermo Fisher CellROX™ Green Manual*)

*CellROX® Green Flow Cytometry Assay Kits – User Manual - https://tools.thermofisher.com/content/sfs/manuals/cellrox_green_orange_flow_cyt_assays_man.pdf

Overview

The following protocol is sourced and adapted from the Thermo Fisher CellRox Green Flow Cytometry Kit User Manual. The CellRox family of dyes allows for detection of reactive oxygen species (ROS) in cells. Specifically the dyes are readily oxidized by ROS and convert from a non-fluorescent to fluorescent state upon oxidation. As the dyes are plasma membrane permeant, they can be used to measure oxidative stress in living cells. The CellRox kits include the CellROX Green Reagent, N-acetyl cysteine (an antioxidant, for negative control), and tert-butyl hydroperoxide solution (TBHP, an inducer of ROS for use as a positive control).

Reagents/Components:

- Orflo Moxi GO II 488nm Next Generation Flow Cytometer (Orflo Cat #MXG002)
- CellRox Green Flow Cytometry Assay Kit (ThermoFisher, Cat# C10492)
- MF-S+ Cassettes (Orflo Cat #MXC030)
- Cell media (e.g. RPMI or DMEM)
- *Optional:* PBS (any formulation, e.g. Gibco, Cat #10010023)
- *Optional/Recommended:* Orflo Flow Reagent (Orflo Cat #MXA080)

Protocol:

1. Dilute cells to ~1e5 – 5e5 cells/ml in standard cell media (e.g., RPMI or DMEM). If an adherent cell line is used, ensure that the cells are sub-confluent. **Note: Staining of cells in phosphate buffered saline (PBS) is not recommended.**
2. Induce ROS in cells using the desired method (TBHP).
3. Prepare positive (TBHP) and negative controls (no TBHP). You can prepare the negative control by incubating the cells in the absence of the ROS inducing agent or by incubating the cells with the antioxidant (NAC).
   a. Reconstitute one vial containing 10 mg of NAC (Component C) with 245µL of PBS to make a 250mM solution.
   b. Prepare a 50mM intermediate dilution of TBHP (Component E) by adding 3.22µL of the 70% stock (7.78 M) to 496.8µL of PBS or complete media.
   c. *Negative control:* Prepare a negative control by incubating the cells with NAC before treatment with TBHP. Add NAC to the negative control sample and incubate for 1 hour under normal growth conditions (ex: 37°C, 5% CO2). Although the suggested final concentration of NAC for use is 200–5000µM, the optimal final concentration is cell-dependent and should be determined experimentally for each cell line being tested.
   d. *Positive control:* Create a positive control by adding 100–400µM of TBHP to a sample of cells. Ensure that the same concentration of TBHP is used in both positive and negative controls (Ex: add 4µL of the 50mM intermediate TBHP solution per mL cells for a final concentration of 200µM TBHP).

e. Following the 1hour incubation with NAC, add TBHP to the negative control cells from step 3c.

f. Incubate the samples from step 3d and 3e for 30–60 minutes under normal growth conditions before staining with the CellROX ROS detection reagent.

4. Briefly centrifuge the vial of CellROX reagent (Component A) before opening the vial. Add the CellROX reagent at a final concentration of 500–1000nM to the samples and/or appropriately induced cells, and incubate for 30–60 minutes at 37°C, protected from light.

   a. It is best to prepare an intermediate dilution of the CellROX reagent in DMSO (Component D). Mix the intermediate dilution well by pipetting up and down, and then add the specified amount of the diluted solution to the cells, so that the final concentration of the reagent incubated with the cells is 500–1000nM (Ex: combine 1µL of CellROX reagent with 9µL of DMSO to make a 250µM solution; use 2µL of this intermediate solution to stain 1 mL of the cell suspension for a final concentration of 500nM).

5. *Optional:* Wash the cells once with 3X the stain volume using PBS or other appropriate buffer (ex: wash 1 mL of stained cells with 3 mL of PBS). However, washing is not required following staining with CellROX reagents.

6. *Optional Recommended: Add 20µL Orflo Flow Reagent per ml of cells and inversion mix sample*

7. Read the samples using the Moxi GO II system with the "Open Flow Cytometry" assay. Make sure you select PMT 1 for analysis of CellRox Green Dye.