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Amplex Red Assay for ROS measurement

Superoxide is the first oxygen radical that is formed in mitochondria. It contains one extra electron. Enzymes such as xanthine oxidase can add one more electron to form hydrogen peroxide. Mitochondrial sodium dismutase converts superoxide to hydrogen peroxide which diffuses to cytosole.

Superoxide and its protonated form, hydroxyl radical, are present in equilibrium in an aqueous solution:

$$O_2^- + H_2O \rightleftharpoons HO_2 + OH^-,$$

although superoxide predominates at physiological pH.

ROS play important signaling role in cell homeostasis. However, under excess production, the ROS initiate oxidative stress and cell destructive processes observed in different pathological conditions. Therefore, the control of generation of ROS is of great importance for cell normal being.

Superoxide is a short living radical which is hard to measure accurately. Therefore, most of the evaluations of ROS production are based rather on measurement of the end product H_2O_2.

ROS production is measured fluorimetrically as a release of H_2O_2 from mitochondria during oxidation of respiratory substrates. We optimized the 96-well plate procedure protocol to use mitochondria in a concentration of 0.2 mg/ml per sample. The master buffer contains the respiration buffer MiR05 supplemented with **5 µM Amplex Red** (non-fluorescent form) and **7 U/ml horseradish peroxidase** (HRP). The mitochondria are added just prior to measurements. The resulting fluorescence signal of resorufin (Em 590 nm), the fluorescent product of Amplex Red, was acquired for 30 min at 37°C on a BioTek Synergy 4 microplate reader (Winooski, VT) with shaking. The signal was calibrated with H_2O_2 prepared freshly for each experiment.

MiP05 - Mitochondria storage and respiration buffer
(http://wiki.oroboros.at/images/d/d9/MiPNet14.13_Medium-MiR06.pdf):

110 mM sucrose,

60 mM K-lactobionate,

20 mM HEPES,

10 mM KH_2PO_4,

3 mM $MgCl_2x6H_2O$,

0.5 mM EGTA,

20 mM taurine,

0.1% fatty acid free BSA

pH 7.2

Master buffer:

MiR05: 18.9 ml

Amplex Red (from 10mM stock): 9.6 µl

HRP (from 450U/ml stock): 298.65 µl

The Amplex Red (Life technology, # A12222) 10 mM stock solution is prepared in DMSO, pre-aliquoted by 10 µl in Eppendorf's and stored at -20°C protected from light.

HRP, 450U/ml stock prepared from 5kU (Sigma P2088).

Procedure:

1. Prepare the Master Buffer.
2. Aliquot the Master Buffer into 20 eppendorfs by 785 µl (This is assuming that the total reaction volume will be 800 µl after addition of mitochondria and modulators).
3. Add modulators as indicated:
   - Glutamate 4 µl (from 2 M stock) – 10 mM
   - Malate 4 µl (from 500 mM stock) - 2 mM
   - Pyruvate 4 µl (from 2 M stock) - 10 mM
   - Succinate 8 µl (from 1 M stock) - 10 mM
   - Palmitoyl-L-Carnitine 1 µl (from 10 mM stock) – 10 µM
   - Rotenon 1.6 µl (from 0.5 mg/ml stock) – 1 µg/ml
   - Myxothiazol 0.64 µl (from 5mM stock) – 4 µM
   - H_2O_2 2 µl (from 10 mM stock) – 25 µM (the higher concentrations of H_2O_2 result in signal decline due to oxidation of the reaction product resorufin to non-fluorescent resazurin)
   - CCCP 0.1 µl (from 5 mM stock) – 0.6 µM

The mitochondria are added right before the measurement. Each sample is vortexed and aliquoted by 200 µl/well in triplicates.