Oct 16, 2025

Mitochondrial ROS Analysis

Science Advances

DOI
https://dx.doi.org/10.17504/protocols.io.rm7vz9×98gx1/v1

Szu-Chi Liao[1], Ken Nakamura[1]

[1]Gladstone Institutes

Szu-Chi Liao
Gladstone Institutes

OPEN ACCESS

DOI: https://dx.doi.org/10.17504/protocols.io.rm7vz9x98gx1/v1

External link: https://doi.org/10.1126/sciadv.adu0726

Protocol Citation: Szu-Chi Liao, Ken Nakamura 2025. Mitochondrial ROS Analysis. **protocols.io** https://dx.doi.org/10.17504/protocols.io.rm7vz9x98gx1/v1

Manuscript citation:
Liao S, Kano K, Phanse S, Nguyen M, Margolis E, Fu Y, Meng JX, Moutaoufik MT, Chatterton Z, Saccon T, Broderick K, Aoki H, Simms J, Suteja FX, Sei Y, Huang EJ, McAvoy K, Manfredi G, Halliday G, Babu M, Nakamura K CHCHD2 mutant mice link mitochondrial deficits to PD pathophysiology. Science Advances 11(46). doi: 10.1126/sciadv.adu0726

Protocol status: Working
We use this protocol and it's working

Created: August 19, 2025

Last Modified: October 16, 2025

Protocol Integer ID: 225026

Keywords: ASAPCRN, mitochondrial ros analysis this protocol, mitochondrial ros analysis, levels in isolated mitochondria, mitochondria, isolated mitochondria, ro, h2o2

Abstract

This protocol is used to measure ROS (H2O2) levels in isolated mitochondria.

Materials

- Amplex Red Hydrogen Peroxide/Peroxidase Assay Kit (Invitrogen A22188)
- Mitochondrial respiration buffer
- Respiratory substrate solution

Reagent Preparation

1 Prepare mitochondrial respiration buffer (100 mL; 120 mM KCl, 5 mM KH_2PO_4, 10 mM HEPES, 2 mM $MgCl_2$, 1 mM EGTA).
- 894.6 mg KCl
- 68.0 mg KH_2PO_4
- 238.8 mg HEPES
- 19.0 mg $MgCl_2$
- 38.0 mg EGTA
- pH 7.2 (adjusted with KOH)

2 Prepare 10 mM Amplex Red reagent stock solution.

2.1 Warm up Amplex Red reagent (blue cap) and DMSO (green cap) at RT.

2.2 Immediately before use, add 60 µL of DMSO into a vial of Amplex Red (each vial of Amplex Red is sufficient for 100 assays of 100 µL).

3 Prepare 1X reaction buffer by diluting 4 mL of 5X reaction buffer (white cap) with 16 mL of deionized water.

4 Make 10 U/mL horseradish peroxidase (HRP) stock solution.

4.1 Reconstitute a vial of HRP (yellow cap) with 1 mL of 1X reaction buffer.

4.2 After the assay, aliquot unused HRP stock solution into single-use tubes and store at -20.

5 Prepare 20 mM hydrogen peroxide (H_2O_2) working solution.

5.1 Immediately before use, dilute the ~3% (0.88 M) H_2O_2 (red cap) into appropriate volume of 1X reaction
buffer to make a 20 mM working solution; exact concentration is on the label.

6 Prepare respiratory substrate solution (10 mL; 50 mM pyruvate and 25 mM malate).

- 55.0 mg pyruvate
- 33.5 mg malate
- 10 mL of mitochondrial respiration buffer

Measure H_2O_2 level

7 Dilute 30 µg crude mitochondrial preps to 40 µL with mitochondrial respiration buffer.

8 Prepare positive (10, 5, 2, 1 and 0.5 µM H_2O_2) and negative (1X mitochondrial respiration buffer) controls.

9 Load samples (40 µL/well).

10 Prepare a working solution of 100 µM Amplex Red reagent and 0.2 U/mL HRP by mixing the following:
- 50 µL of 10 mM Amplex Red reagent stock solution
- 100 µL of 10 U/mL HRP stock solution
- 4.85 mL of 1X reaction buffer

11 Add 50 µL of the working solution into each well.

12 Record fluorescence emission at 590 nm (excitation at 560 nm) for 8 min.

13 Add 10 µL of respiratory substrate into each well; final concentration is 5 mM pyruvate and 2.5 mM malate.

14 Record fluorescence emission at 590 nm (excitation at 560 nm) for 45 min.