

Determination of H₂O₂ Generation by pHPA Assay

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[Abstract] The production of reactive oxygen species, including H₂O₂, is a process that can be used in signaling, cell death, or immune response. To quantify oxidative stress in cells, a fluorescence technique has been modified from a previously described method to measure H₂O₂ release from cells (Panus *et al.*, 1993; Murthy *et al.*, 2010; Larson-Casey *et al.*, 2016; Larson-Casey *et al.*, 2014; He *et al.*, 2011). This assay takes advantage of H₂O₂-mediated oxidation of horseradish peroxidase (HRP) to Complex I, which, in turn, oxidizes p-hydroxyphenylacetic acid (pHPA) to a stable, fluorescent pHPA dimer (2,2'-dihydroxy-biphenyl-5,5' diacetate [(pHPA)₂]). The H₂O₂-dependent HRP-mediated oxidation of pHPA is a sensitive and specific assay for quantifying H₂O₂ release from cells. This assay can measure H₂O₂ release from whole cells, mitochondria, or the NADPH oxidase.

[Background] H₂O₂ generation primarily results from dismutation of superoxide anion (O₂⁻), which occurs at a rapid rate (10⁵-10⁶ M⁻¹ s⁻¹) non-enzymatically. Unlike O₂⁻, H₂O₂ can traverse membranes easily, so it is able to oxidize multiple molecules. ROS can be toxic to cells by oxidizing proteins, lipids, and nucleic acids and are associated with many human diseases. This protocol allows for detection of H₂O₂ release from NADPH oxidase or mitochondria in various cell types.

Materials and Reagents

1. Nunc™ 96-well black bottom plate (Thermo Fisher Scientific, Thermo Scientific™, catalog number: 137101)
2. Eppendorf tubes
3. 15 and 50 ml conical tubes
4. Glucose (Sigma-Aldrich, catalog number: G7528)
5. HEPES (1 M) (Thermo Fisher Scientific, Gibco™, catalog number:15630-080)
6. Sodium bicarbonate (NaHCO₃) (Thermo Fisher Scientific, Fisher Scientific, catalog number: S233-500)
7. 4-hydroxyphenylacetic acid (pHPA) (Sigma-Aldrich, catalog number: H50004)
8. HRP (Sigma-Aldrich, catalog number: P8125)
9. Ca²⁺/Mg²⁺/phenol red-free HBSS (Thermo Fisher Scientific, Gibco™, catalog number: 14175-095)

10. α -Ketoglutaric acid (Sigma-Aldrich, catalog number: K2000)
11. Hydrogen peroxide solution (H₂O₂) (30%, w/w) (Sigma-Aldrich, catalog number: H1009)
12. Tris
13. EDTA
14. Sucrose
15. Protease inhibitor tablets (Sigma-Aldrich, catalog number: 11836170001)
16. Phosphatase inhibitors (EMD Millipore, catalog number: 524625)
17. Antimycin A (optional)
18. MitoTempo (optional)
19. pHPA buffer (see Recipes)
20. 1 mM H₂O₂ working stock solution (see Recipes)
21. Mitochondrial buffer (see Recipes)

Equipment

1. Kontes pellet pestle motor
2. Centrifuge
3. Plate reader (Molecular Devices, model: M2 SpectraMax)

Procedure

1. Prepare pHPA buffer (see Recipes)
2. Prepare H₂O₂ standard curve (see Figure 1)

Note: Prepared in pHPA buffer prior to the assay (The buffer cannot be stored for subsequent measurements, make fresh at time of use).

- a. 8-12 standards in duplicate or triplicate.
- b. Load standards into 96-well plate.

Highest concentration of H₂O₂: 2 μ M (2 μ l of 1 mM H₂O₂ working stock solution/ml pHPA buffer)

Serial dilution: 50%

#wells/standard: 2 or 3

μ l/well: 200

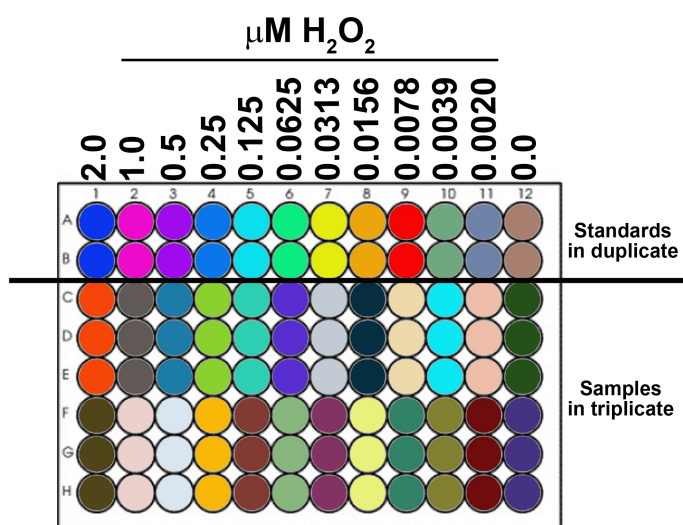


Figure 1. Schematic of 96-well plate with 12 standards (in duplicate) and samples (in triplicate)

3. Prepare samples
 - a. Sample isolation
 - i. Samples can be whole cells, freshly isolated mitochondria or membrane fractions from various cell types (*i.e.*, macrophages, fibroblasts, bronchoalveolar lavage cells, *etc.*).
 - ii. Mitochondrial isolation:
 - 1) Lyse 10 million macrophages in (100 μ l) mitochondrial buffer (see Recipes). Lysis is performed using Kontes Pellet Pestle Motor for 30 sec to homogenize each sample.
 - 2) Centrifuge at 2,000 $\times g$ for 10 min at 4 $^{\circ}$ C, save supernatant at 4 $^{\circ}$ C.
 - 3) Pellet is lysed and centrifuged [repeat steps 1) and 2)].
 - 4) The two supernatants are pooled and centrifuged at 12,000 $\times g$ for 15 min at 4 $^{\circ}$ C.
 - 5) The pellet is resuspended in (50 μ l) mitochondrial buffer without sucrose [same as in 1) but do not add sucrose].
 - b. Each sample should be run in duplicate or triplicate.
 - c. Load samples into 96-well plate:
 - i. Use 5-20 μ g protein or 50,000-100,000 cells per well
 - ii. Suspend sample in pHPA buffer, mix well
 - iii. Load 200 μ l/well in duplicate
 - d. Positive controls may be added (*i.e.*, treat cells with 100 μ M antimycin A).
 - e. Negative controls may be added (*i.e.*, treat cells with 10 μ M MitoTempo).
4. Run assay in plate reader
 - a. Pre-warm plate reader to 37 $^{\circ}$ C.
 - b. Insert plate.
 - c. Run kinetic assay, typically for 4 h with a read every 5-10 min.

- d. Use an excitation wavelength of 320 nm and emission of 400 nm.

Data analysis

1. Use initial readings of standards to generate a standard curve.
2. Determine the H₂O₂ concentration in samples by applying a linear regression to the standard curve and extrapolate.
3. Normalize samples to protein or cell number. Samples can be lysed in protein sample buffer to determine cellular protein levels, or alternatively when using live cells, the number of cells can be used to normalize data.
4. Data can be expressed as a rate over a set time (Figure 2A) or by showing a kinetic curve (Figure 2B); also see Larson-Casey *et al.*, 2016.

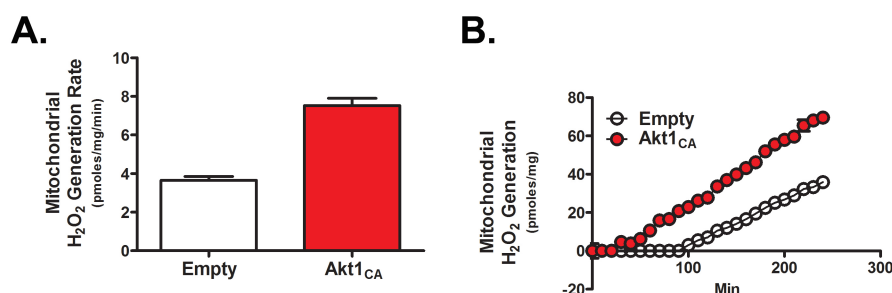


Figure 2. Mitochondrial H₂O₂ generation in macrophages expressing empty or constitutively active Akt1 (Akt1_{CA}) expression vectors

Notes

1. This assay is typically performed on freshly isolated mitochondrial (see Recipes for protocol) or membrane fractions.
2. Prepare all working solutions on day of use and do not reuse.

Recipes

1. pHPA buffer
50 ml total volume made up in Ca²⁺/Mg²⁺/phenol red-free HBSS
Make up buffer in 50 ml conical tube and protect from light

Reagent	Stock	Final concentration	Aliquot for making 50ml pHPA buffer
Glucose	1.39 M (250 mg/ml), (213.85x)	6.5 mM	233.81 µl
HEPES	1 M (1,000x)	6 mM	50 µl
NaHCO ₃	0.89 M (7.5 g/100 ml), (148.33x)	6 mM	337.1 µl
pHPA	(MW:152.1)	1.6 mM	0.012168 g
HRP	-	95 µg/ml	0.00475 g
αKG	-	2.5 mM	0.023 g
HBSS	-	-	49.38 ml

2. 1 mM H₂O₂ working stock solution

1.13 µl 30% (w/w) H₂O₂ stock solution/10 ml H₂O

Note: H₂O₂ MW = 84.01, 30% (w/w) (H₂O₂ stock solution) equivalent to 8.82 M.

3. Mitochondrial buffer

10 mM Tris, pH 7.8

0.2 mM EDTA

320 mM sucrose

1 protease inhibitor tablet

Phosphatase inhibitors diluted 1:100

Acknowledgments

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