Published in final edited form as:

Bio Protoc. 2016 November 20; 6(22): . doi:10.21769/BioProtoc.2010.

Determination of H₂O₂ Generation by pHPA Assay

Jennifer L. Larson-Casey¹ and A. Brent Carter^{1,2,*}

¹Department of Medicine, Division of Pulmonary, Allergy, and Critical Care Medicine, University of Alabama at Birmingham, Birmingham, AL, USA 35294

²Birmingham Veterans Administration Medical Center, Birmingham, AL, USA 35294

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 (1–5). 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 Scientific, NuncTM, catalog number: [137101](#))
2. Eppendorf tubes
3. 15 and 50 ml conical tubes
4. Glucose (Sigma Aldrich, catalog number: [G7528](#))
5. HEPES (1M) (Thermo Fisher Scientific, Gibco[®], catalog number: [15630-080](#))
6. NaHCO₃ (Fisher Scientific, catalog number: [S233-500](#))

*For correspondence: bcarter1@uab.edu.

7. pHPA (Sigma Aldrich, catalog number: [H50004](#))
8. HRP (Sigma Aldrich, catalog number: [P8125](#))
9. $\text{Ca}^{2+}/\text{Mg}^{2+}$ /phenol red-free HBSS (Thermo Fisher Scientific, Gibco®, catalog number: [14175-095](#))
10. α -Ketoglutaric acid (Sigma Aldrich, catalog number: [K2000](#))
11. H_2O_2 (Sigma Aldrich, catalog number: [H1009](#))
12. pHPA Buffer (see Recipes)
13. 30% (w/w) H_2O_2 stock solution (see Recipes)
14. 1 mM H_2O_2 working stock solution (see Recipes)

Equipment

1. Plate Reader: M2 SpectraMax, Molecular Devices
 - a. <https://www.moleculardevices.com/systems/microplate-readers/multi-mode-readers/spectramax-m-series-multi-mode-microplate-readers>

Procedure

1. Prepare pHPA buffer (see Recipes)
2. Prepare H_2O_2 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 H_2O_2 : 2 μM (2 μl of 1 mM H_2O_2 working stock solution/ml pHPA buffer)

Serial Dilution: 50%

#wells/standard: 2 or 3

μl /well: 200
3. Prepare samples

Samples can be whole cells, freshly isolated mitochondria or membrane fractions from various cell types (i.e. macrophages, fibroblasts, bronchoalveolar lavage cells, etc.)

See recipes for mitochondrial isolation protocol.

 - a. Each samples should be run in duplicate or triplicate
 - b. Load samples into 96-well plate:

Glucose:	Final	6.5 mM in H ₂ O
	Stock	1.39 M (250 mg/ml)
	Dilution	213.85x
	Aliquot	233.81 µl
HEPES	Final	6 mM
	Stock	1 M
	Dilution	1000x
	Aliquot	50 µl
NaHCO ₃	Final	6mM in H ₂ O
	Stock	0.89M (7.5 g/100mL)
	Dilution	148.33x
	Aliquot	337.1 µl
pHPA	Final	1.6 mM
	MW	152.1x
	Weight	0.012168 g
HRP	Final	95 µg/ml
	Weight	0.00475 g
α.KG	Final	2.5 mM
	Weight	0.023 g
HBSS	Volume	49.38 ml

2. 30% (w/w) H₂O₂ stock solution

H₂O₂ MW = 84.01

Equivalent to 8.82 M

3. 1 mM H₂O₂ working stock solution

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

4. Mitochondrial Isolation Protocol:

- a.** Lyse 10 million macrophages in mitochondrial buffer (10 mM Tris, pH 7.8; 0.2 mM EDTA, 320 mM sucrose and protease and phosphatase inhibitors). Lysis is performed using Kontes Pellet Pestle Motor for 30 sec to homogenize each sample.
- b.** Centrifuge at 2000 *g* for 10 min at 4°C, save supernatant at 4°C.
- c.** Pellet is lysed and centrifuged (repeat steps a and b).
- d.** The two supernatants are pooled and centrifuged at 12000 *g* for 15 min at 4°C
- e.** The pellet is resuspended in mitochondrial buffer without sucrose (same as in (a) but do not add sucrose).

Acknowledgments

This work was supported by 2R01ES015981 & VA merit review BX001135. This protocol was originally adapted from Panus *et al.*, 1993.

References

1. Panus PC, Radi R, Chumley PH, Lillard RH, Freeman BA. Detection of H₂O₂ release from vascular endothelial cells. *Free Radic Biol Med.* 1993; 14(2):217–223. [PubMed: 8425723]
2. Murthy S, Ryan A, He C, Mallampalli RK, Carter AB. Rac1-mediated mitochondrial H₂O₂ generation regulates MMP-9 gene expression in macrophages via inhibition of SP-1 and AP-1. *J Biol Chem.* 2010; 285(32):25062–25073. [PubMed: 20529870]
3. Larson-Casey JL, Deshane JS, Ryan AJ, Thannickal VJ, Carter AB. Macrophage Akt1 Kinase-Mediated Mitophagy Modulates Apoptosis Resistance and Pulmonary Fibrosis. *Immunity.* 2016; 44(3):582–596. [PubMed: 26921108]
4. Larson-Casey JL, Murthy S, Ryan AJ, Carter AB. Modulation of the mevalonate pathway by Akt regulates macrophage survival and development of pulmonary fibrosis. *J Biol Chem.* 2014; 289(52):36204–36219. [PubMed: 25378391]
5. He C, Murthy S, McCormick ML, Spitz DR, Ryan AJ, Carter AB. Mitochondrial Cu,Zn-superoxide dismutase mediates pulmonary fibrosis by augmenting H₂O₂ generation. *J Biol Chem.* 2011; 286(17):15597–15607. [PubMed: 21393238]

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

Figure 1. Mitochondrial H₂O₂ generation in macrophages expressing empty or constitutively active Akt1 (Akt1_{CA}) expression vectors.