bio-protocol www.bio-protocol.org/e2010 Vol 6, Iss 22, Nov 20, 2016
DOI:10.21769/BioProtoc.2010

Determination of H_2O_2 Generation by pHPA Assay

Jennifer L. Larson-Casey[1] and A. Brent Carter[1, 2, *]

[1]Department of Medicine, Division of Pulmonary, Allergy, and Critical Care Medicine, University of Alabama at Birmingham, Birmingham, AL, USA; [2]Birmingham Veterans Administration Medical Center, Birmingham, AL, USA
*For correspondence: bcarter1@uab.edu

[Abstract] The production of reactive oxygen species, including H_2O_2, 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 H_2O_2 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 H_2O_2-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 H_2O_2-dependent HRP-mediated oxidation of pHPA is a sensitive and specific assay for quantifying H_2O_2 release from cells. This assay can measure H_2O_2 release from whole cells, mitochondria, or the NADPH oxidase.

[Background] H_2O_2 generation primarily results from dismutation of superoxide anion (O_2^-), which occurs at a rapid rate (10^5-10^6 M^{-1} s^{-1}) non-enzymatically. Unlike O_2^-, H_2O_2 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 H_2O_2 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 ($NaHCO_3$) (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. Ca^{2+}/Mg^{2+}/phenol red-free HBSS (Thermo Fisher Scientific, Gibco™, catalog number: 14175-095)

Vol 6, Iss 22, Nov 20, 2016
DOI:10.21769/BioProtoc.2010

10. α-Ketoglutaric acid (Sigma-Aldrich, catalog number: K2000)
11. Hydrogen peroxide solution (H_2O_2) (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 H_2O_2 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 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

Vol 6, Iss 22, Nov 20, 2016
DOI:10.21769/BioProtoc.2010

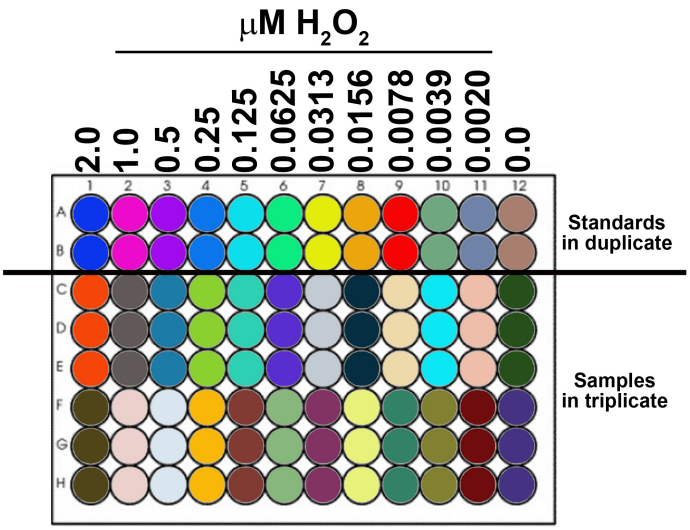

Figure 1. Schematic of 96-well plate with 12 standards (in duplicate) and samples (in triplicate)

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 µl) mitochondrial buffer (see Recipes). Lysis is performed using Kontes Pellet Pestle Motor for 30 sec to homogenize each sample.
         2) Centrifuge at 2,000 *x g* for 10 min at 4 °C, save supernatant at 4 °C.
         3) Pellet is lysed and centrifuged [repeat steps 1) and 2)].
         4) The two supernatants are pooled and centrifuged at 12,000 *x g* for 15 min at 4 °C.
         5) The pellet is resuspended in (50 µl) 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 µg protein or 50,000-100,000 cells per well
      ii. Suspend sample in pHPA buffer, mix well
      iii. Load 200 µl/well in duplicate
   d. Positive controls may be added (*i.e.*, treat cells with 100 µM antimycin A).
   e. Negative controls may be added (*i.e.*, treat cells with 10 µM MitoTempo).
4. Run assay in plate reader
   a. Pre-warm plate reader to 37 °C.
   b. Insert plate.
   c. Run kinetic assay, typically for 4 h with a read every 5-10 min.

Vol 6, Iss 22, Nov 20, 2016
DOI:10.21769/BioProtoc.2010

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 H_2O_2 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.

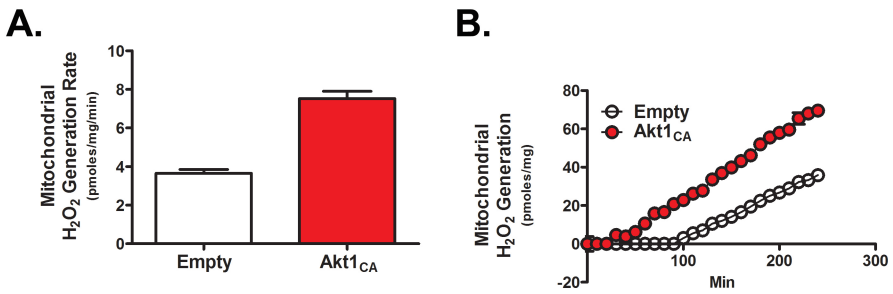

Figure 2. Mitochondrial H_2O_2 generation in macrophages expressing empty or constitutively active Akt1 ($Akt1_{CA}$) expression vectors

Notes

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

Recipes

1. pHPA buffer
   50 ml total volume made up in Ca^{2+}/Mg^{2+}/phenol red-free HBSS
   Make up buffer in 50 ml conical tube and protect from light

Vol 6, Iss 22, Nov 20, 2016
DOI:10.21769/BioProtoc.2010

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 H_2O_2 working stock solution
   1.13 µl 30% (w/w) H_2O_2 stock solution/10 ml H_2O
   Note: H_2O_2 MW = 84.01, 30% (w/w) (H_2O_2 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

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

References

1. He, C., Murthy, S., McCormick, M. L., Spitz, D. R., Ryan, A. J. and Carter, A. B. (2011). Mitochondrial Cu,Zn-superoxide dismutase mediates pulmonary fibrosis by augmenting H_2O_2 generation. *J Biol Chem* 286(17): 15597-15607.
2. Larson-Casey, J. L., Deshane, J. S., Ryan, A. J., Thannickal, V. J. and Carter, A. B. (2016). Macrophage Akt1 kinase-mediated mitophagy modulates apoptosis resistance and pulmonary fibrosis. *Immunity* 44(3): 582-596.
3. Larson-Casey, J. L., Murthy, S., Ryan, A. J. and Carter, A. B. (2014). Modulation of the mevalonate pathway by Akt regulates macrophage survival and development of pulmonary fibrosis. *J Biol Chem* 289(52): 36204-36219.
4. Murthy, S., Ryan, A., He, C., Mallampalli, R. K. and Carter, A. B. (2010). Rac1-mediated mitochondrial H_2O_2 generation regulates *MMP-9* gene expression in macrophages via inhibition of SP-1 and AP-1. *J Biol Chem* 285(32): 25062-25073.

Vol 6, Iss 22, Nov 20, 2016
DOI:10.21769/BioProtoc.2010

5. Panus, P. C., Radi, R., Chumley, P. H., Lillard, R. H. and Freeman, B. A. (1993). Detection of H_2O_2 release from vascular endothelial cells. *Free Radic Biol Med* 14(2): 217-223.