bio-protocol

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H_2O_2 Release Assay

Santosh Yadav, Shruthi Sanjitha Sampath, Brian J. Deskin and Victor J. Thannickal*

John W. Deming Department of Medicine, Tulane University School of Medicine, New Orleans, LA 70112, USA
*For correspondence: vthannickal@tulane.edu

Abstract

Reactive oxygen species are ubiquitous in nature, and function as signalling molecules in biological systems; they may also contribute to oxidative stress in several pathobiological disease states. In this report, we describe a simple, reliable, sensitive, and specific assay for the detection and quantitation of hydrogen peroxide (H_2O_2) release by living cells, organoids, or tissues. Furthermore, the low cost of reagents required for this assay makes it inexpensive relative to commercial kits. The high sensitivity and specificity are based on the ability of H_2O_2 to react with heme peroxidases and convert para-substituted phenolic compounds to fluorescent dimers.

Keywords: Hydrogen peroxide, Free radicals, Reactive oxygen species, NADPH oxidases, Oxidative stress, Redox biology, Homovanillic acid

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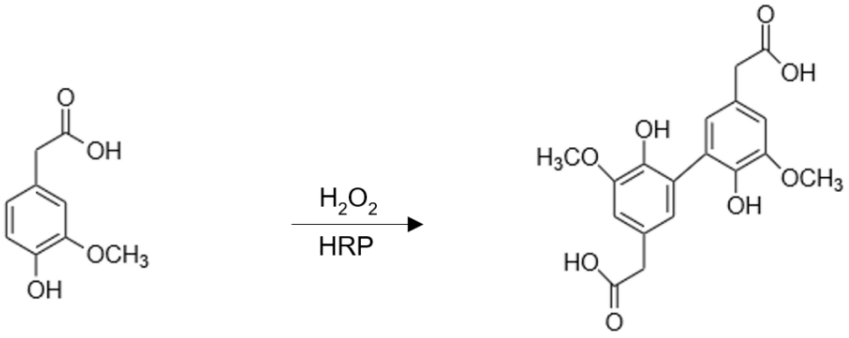
Graphical abstract:



3-methoxy-4-hydroxyphenylacetic acid (HVA)

2,2′-dihydroxy-3,3′-dimethoxydiphenyl-5,5′-diacetic acid (HVA dimer)

Background

Cellular sources of reactive oxygen species (ROS) may derive from a diverse number of enzymatic and non-enzymatic metabolic reactions (Thannickal and Fanburg, 2000). Intracellular organelles such as mitochondria, endoplasmic reticulum, microsomes, and peroxisomes are well known to contain ROS-generating enzymes. Other sources of intracellular ROS include soluble flavoenzymes, such as xanthine oxidase or aldehyde oxidase, and the non-enzymatic autooxidation of small molecules, such as dopamine, flavins, and hydroquinones. The cell is well equipped to combat excess ROS, primarily through the actions of superoxide dismutases, catalase, and glutathione peroxidase. It is now well recognized that ROS induce post-translational modification of proteins that regulate cellular signalling, referred to as “redox signalling” (Thannickal and Fanburg, 2000; Sies and Jones, 2020).

Over the past 25 years, the discovery of the NADPH oxidase (NOX) family of enzymes has further illuminated the field of redox signalling (Lambeth, 2004; Bedard and Krause, 2007). The primary catalytic function of these novel enzymes is the regulated generation of ROS, specifically of superoxide anion ($O_2^{\cdot-}$) and hydrogen peroxide (H_2O_2). H_2O_2 has emerged as a key signalling molecule through its direct effects on susceptible thiols groups in proteins that mediate signal transduction (Veal *et al.*, 2007). The localization of NOX enzymes within biological membranes, including plasma membranes, allows for the detection of NOX activity on the surface of live cell cultures/tissues. However, in the case of H_2O_2, extracellular release may also reflect intracellular generation of H_2O_2 that is capable of diffusing across the plasma membrane, or transported via aquaporin channels (Bienert and Chaumont, 2014).

In this bio-protocol report, we describe a simple, yet reliable, method for the detection and measurement of extracellular H_2O_2 release. Unlike currently available methods for intracellular H_2O_2 detection with fluorescent dyes, this method is specific and sensitive, with detection in the nanomolar range. Furthermore, the steady-state rates of H_2O_2 release can be calculated over a period of minutes to hours in live cells, organoids, or tissues. The low cost of assay reagents and long shelf-life when stored appropriately (see protocol for details), makes it inexpensive relative to commercially available kits. The specificity for H_2O_2 in this assay system is based on the high reactivity of H_2O_2 with transition metal-containing heme peroxides, such as horseradish peroxidase (HRP), at high rate constants, typically in the 10^7–10^8 $M^{-1}s^{-1}$ range (Winterbourn, 2013). The resulting intermediate complex is then able to catalyze the dimerization of a range of substituted phenolic compounds. Here, we have chosen homovanillic acid (3-methoxy-4-hydroxyphenylacetic acid, HVA), which is converted to the highly fluorescent dimer (2,2′-dihydroxy-3,3′-dimethoxydiphenyl-5,5′-diacetic acid) in the presence of H_2O_2 and HRP (Ruch *et al.*, 1983). We have successfully utilized this method to detect the activity of NOX4 (Thannickal and Fanburg, 1995; Waghray *et al.*, 2005; Hecker *et al.*, 2009, 2014; Chanda *et al.*, 2021), which, in contrast to other NOX homologs, has several unique features, including the extracellular generation of H_2O_2 (Martyn *et al.*, 2006).

Materials and Reagents

1. Six round bottom 13 × 100 mm glass tubes (Fisher Scientific, catalog number: 14-961-27)
2. 96-well plate, flat-bottom & black (Corning, catalog number: 3916)
3. 24-well tissue culture-treated plate (Corning, catalog number: 3524)
4. Hank's balanced salt solution (HBSS) (ThermoFisher, catalog number: 14025092)
5. HBSS, no calcium, no magnesium, no phenol red (Gibco, catalog number: 14175095)
6. Homovanillic acid (HVA) (Sigma, catalog number: H1252)
7. Horseradish peroxidase (HRP), Type VI (Sigma, catalog number: P8375)
8. H_2O_2, 30% solution (Sigma, CAS No.: 7722-84-1)
9. IMR-90 (Coriell Institute)
10. Dulbecco's Modified Eagle Medium (DMEM) (Corning, catalog number: MT15013CV)
11. Fetal bovine serum (FBS) (Thermo Fisher, catalog number: A3160402)
12. Penicillin-Streptomycin (Thermo Fisher, catalog number: 15140122)
13. L-Glutamine (Fisher Scientific, catalog number: 25030-081)
14. Trypsin (Fisher Scientific, catalog number: MT25053CI)
15. Transforming growth factor-β1 (TGF-β1) (R&D Systems, catalog number: 101B1)
16. Hemocytometer for cell counting (Fisher Scientific, catalog number: NC1587539)
17. Homovanillic acid (HVA) stock solution, 10 mM (see Recipes)
18. Horseradish peroxidase (HRP), 5,000 U/mL in Ca^{2+} and Mg^{2+} free buffer (see Recipes)
19. H_2O_2, 1 mM (see Recipes)
20. NaOH-EDTA alkalization solution (see Recipes)
21. Assay media (see Recipes)

Equipment

1. Modular multimode microplate reader (Synergy H1, BioTek, model: H1M)
2. High-speed centrifuge (Eppendorf, model: 5430R)
3. Fisher Vortex Genie 2 (Fisher Scientific, catalog number: 02215360)

Software

1. GraphPad Prism 9.1.2 (https://www.graphpad.com)

Procedure

Cell-based assay

Day 1: Seed cells (~50,000 cells/well) in a 24-well tissue culture plate, in complete DMEM media with 10% FBS, 1% Penicillin-Streptomycin, and 2.5 mM L-Glutamine; incubate at 37°C in a humidified incubator with 5% CO_2.
Day 2: Serum-starve cells by reducing FBS concentration to 0.05% overnight (~16 h).
Day 3: In some cases, stimulation with a cytokine, such as activated TGF-β1 (2 ng/mL), may be added to induce enzyme(s) that generate extracellular H_2O_2 for variable durations of time.
Day 3 or 4: Assay for H_2O_2 release (see **Figure 1**).

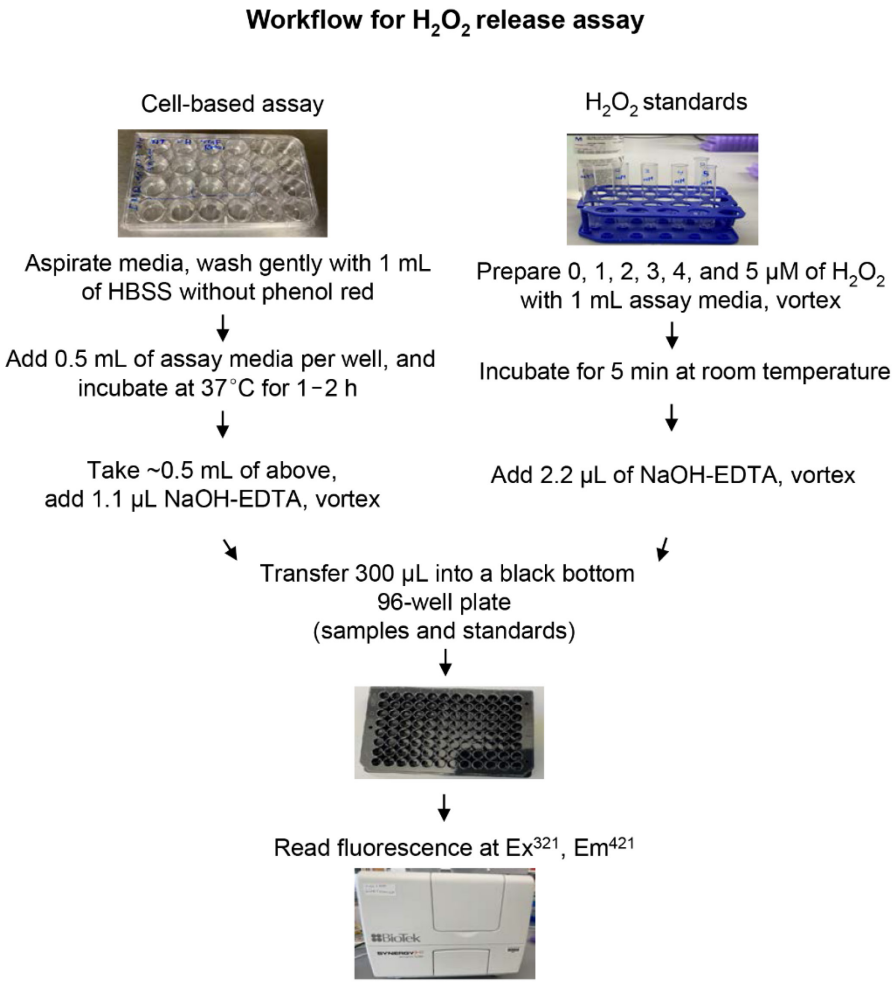


Figure 1. Schematic of workflow to determine rates of H_2O_2 release in a cell or organoid-based assay.

1. Aspirate media from all wells, and wash gently with 1 mL of HBSS without phenol red.
2. Add 0.5 mL of assay media to each well, including three blank wells containing no cells.
3. Incubate at 37°C in the CO_2 incubator for 1–2 h ("t" in minutes, see step #2 under "Data analysis").
4. At the end of the incubation period, transfer the cell-free supernatant to individual 13 × 100 mm glass tubes; add HBSS to the remaining cells for cell counting (to determine the number of cells/well, "n" in millions; see step #2 under "Data analysis").
5. Add 1.1 µL of NaOH-EDTA alkalization solution to the glass tubes containing assay media; vortex, and allow to sit for 5 min.
6. Transfer 300 µL of alkalized assay medium from this into a 96-well black bottom plate; measure fluorescence (relative fluorescence units, RFU) at excitation and emission wavelengths of 321 nm and 421 nm, respectively.

Standard curve

1. Add 1 mL of the same assay medium (used above for cell incubation) in six 13 × 100 mm glass tubes.
2. Standard concentrations of H_2O_2 (0–5 µM) can be made by adding 1:1,000 dilutions to each of the tubes in step #1 above, including a blank (no H_2O_2).
3. The samples are vortexed, and the reaction allowed to proceed at room temperature for 5 min.
4. Add 2.2 µL of NaOH-EDTA solution to each 1 mL tube (in step #2), vortex again and sit for 5 min (together with cell-based samples in step #5 above, under "Cell-based assay").
5. Transfer 300 µL from each standard concentration to a 96-well plate, and read fluorescence as described in step #6 above (under "Cell-based assay").

Data analysis

1. Plot the RFU of the standards using GraphPad Prism (https://www.graphpad.com) to obtain a linear standard curve; determine the slope ("m") (see **Figure 2**).

2. The rate of H_2O_2 release can then be calculated based on the following equation:
   H_2O_2 release rate (in picomoles/min/10^6 cells):

$$\frac{\Delta\text{RFU (experimental sample} - \text{blank sample)}}{\text{m} \times \text{t (min)} \times \text{n } (10^6\text{cells})} \times \text{CF}$$

   where, m = slope of standard curve
   t = duration of cell incubation (in minutes)
   n = number of cells/well (in millions)
   CF = conversion factor (0.5×10^3 to obtain results in *p*moles/min/10^6 cells)
   [µmoles/L (µM) × 0.5 mL (reaction volume) × 10^6 *p*moles/µmoles × 10^{-3} L/mL]

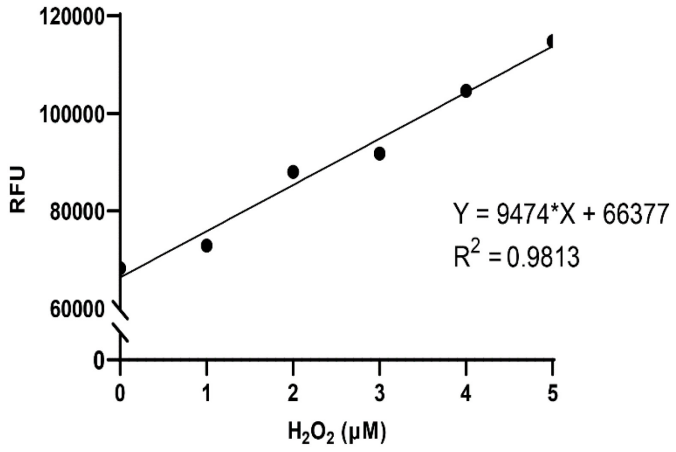


Figure 2. Standard curve for fluorometric assay for H_2O_2.
Known concentrations of H_2O_2 (0–5 µM) are added to the assay media for 5 min of reaction time at room temperature; the pH of the reaction mixture is alkalinized, and fluorescence measured as described in the detailed protocol. In the example above, Y = m*X + c, where slope of the curve m = 9474, slope of the curve, and y intercept c = 66377, y intercept; in our experience, the y intercept represents "background" fluorescence that is similar to the value obtained with blanks (as described in the protocol).

Recipes

1. Homovanillic acid (HVA) stock solution, 10 mM

Reagent Amount	Final concentration
Homovanillic acid 91.1 mg	10 mM
HBSS 50 mL	n/a
Wait until fully dissolved by gentle warming if needed. Make 0.5-mL aliquots in Eppendorf tubes, and store at -80°C.	

2. Horseradish peroxidase (HRP), 5000 U/mL in Ca^{2+} and Mg^{2+} free buffer

Reagent Amount	Final concentration
Horseradish peroxidase 25,000 U	5,000 U/mL
HBSS 5 mL	n/a
Store at -80°C in 50 µL aliquots.	

3. H_2O_2,1 mM

Reagent Amount	Final concentration
H_2O_2, 30% solution 5.67 µL	1 mM
H_2O 50 mL	n/a
Store at 4°C.	

4. NaOH-EDTA alkalization solution

Reagent Amount	Final concentration
NaOH 24 g	
H_2O 45 mL	n/a
Take 45 mL of NaOH solution from above and add glycine 338 mg (0.1 M) and 329 mg of EDTA (25 mM).	
Make 1.5-mL aliquots in Eppendorf tubes and store at room temperature.	

5. Assay media

Reagent Amount	Final concentration
HVA 0.5 mL HVA (10 mM stock)	100 µM
HRP 50 µL HRP (5,000 U/mL stock)	5 U/mL
HBSS with $CaCl_2$ and $MgCl_2$ 50 mL	n/a
Prepare fresh on the day of the assay.	

Acknowledgments

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Competing interests

The authors declare no competing interests in relation to the work described in this report.

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