

## A Highly Sensitive Chemiluminometric Assay for Real-Time Detection of Biological Hydrogen Peroxide Formation

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**ABSTRACT** | Hydrogen peroxide (H<sub>2</sub>O<sub>2</sub>) is a major reactive oxygen species (ROS) produced by various cellular sources, especially mitochondria. At high levels, H<sub>2</sub>O<sub>2</sub> causes oxidative stress, leading to cell injury, whereas at low concentrations, this ROS acts as an important second messenger to participate in cellular redox signaling. Detection and measurement of the levels or rates of production of cellular H<sub>2</sub>O<sub>2</sub> are instrumental in studying the biological effects of this major ROS. While a number of assays have been developed over the past decades for detecting and/or quantifying biological H<sub>2</sub>O<sub>2</sub> formation, none has been shown to be perfect. Perhaps there is no perfect assay for sensitively and accurately quantifying H<sub>2</sub>O<sub>2</sub> as well as other ROS in cells, wherein numerous potential reactants are present to interfere with the reliable measurement of the specific ROS. In this context, each assay has its own advantages and intrinsic limitations. This article describes a highly sensitive assay for real-time detection of H<sub>2</sub>O<sub>2</sub> formation in cultured cells and isolated mitochondria. This assay is based on the luminol/horseradish peroxidase-dependent chemiluminescence that is inhibitable by catalase. The article discusses the usefulness and shortcomings of this chemiluminometric assay in detecting biological H<sub>2</sub>O<sub>2</sub> formation induced by beta-lapachone redox cycling with both cells and isolated mitochondria.

**KEYWORDS** | Beta-Lapachone; Chemiluminescence; Hydrogen peroxide; Luminol; Oxidative stress; Reactive oxygen species; Redox cycling; Redox signaling

**ABBREVIATIONS** | 3-APA, 3-aminophthalate; BSA, bovine serum albumin; CL, chemiluminescence; CPBS, complete phosphate-buffered saline; CPM, counts of photon emission per minute; DMEM, Dulbecco's modified Eagle medium; DMSO, dimethyl sulfoxide; FBS, fetal bovine serum; H<sub>2</sub>O<sub>2</sub>, hydrogen peroxide; HRP, horseradish peroxidase; PBS, phosphate buffered saline; ROS, reactive oxygen species

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## 1. OVERVIEW

Hydrogen peroxide ( $\text{H}_2\text{O}_2$ ) is a non-radical reactive oxygen species (ROS). In contrast to superoxide anion radical,  $\text{H}_2\text{O}_2$  is relatively stable and can readily cross cell membranes via aquaporin-facilitated transmembrane diffusion [1, 2]. Although high levels of  $\text{H}_2\text{O}_2$  cause oxidative stress, leading to cell and tissue injury, at low concentrations and when its formation is tightly controlled,  $\text{H}_2\text{O}_2$  acts as a second messenger, participating in cell signal transduction [3, 4]. Therefore, accurate and sensitive detection and measurement of  $\text{H}_2\text{O}_2$  in biological systems are of critical importance for investigating the physiological and pathophysiological roles of this ROS. Over the past decades, a number of assays have been developed for the detection and/or quantification of biological  $\text{H}_2\text{O}_2$  formation. These methods can be classified into the following four categories: (1) fluorescence-based assays (e.g., the *N*-acetyl-3,7-dihydroxyphenoxazine oxidation assay, also known as the Amplex Red assay, which is available as a kit from various commercial sources) [5]; (2) chemilu-

minescence (CL)-based assays (e.g., the luminol/horseradish peroxidase-dependent CL assay described in this article) [6, 7]; (3) electrode-based assays (e.g., the  $\text{H}_2\text{O}_2$  electrode assay) [8]; and (4) the most recently developed redox protein-based assays [9, 10]. Although numerous assays are available, currently there is no well-established assay for sensitive and specific detection and/or measurement of intracellular  $\text{H}_2\text{O}_2$  production. The method described in this article is primarily for the detection of extracellular  $\text{H}_2\text{O}_2$ . Therefore, there is a strong need for developing assays that allow sensitive and selective detection of intracellular  $\text{H}_2\text{O}_2$ . Nevertheless, when appropriately used in well-defined biological systems, many of the currently available assays, including the one described in this article, could be used to estimate the formation of intracellular  $\text{H}_2\text{O}_2$ .

## 2. METHOD PRINCIPLES

The assay described in this article involves the use of the CL technique. Hence, it is imperative to first in-

introduce the basic chemical principles of CL and chemiluminometry in detecting biological ROS, including  $\text{H}_2\text{O}_2$ .

## 2.1. General Principles of CL and Chemiluminometry

CL is the emission of light with limited release of heat as a result of a chemical reaction. Given reactants A and B, with an excited intermediate  $\text{E}^*$ , the light-emitting reaction can be written as follows:  $[\text{A}] + [\text{B}] \rightarrow [\text{E}^*] \rightarrow [\text{Products}] + \text{light}$ . For example, if [A] is luminol and [B] is  $\text{H}_2\text{O}_2$  in the presence of a suitable catalyst (e.g., myeloperoxidase or horseradish peroxidase), the following reactions occur:  $\text{luminol} + \text{H}_2\text{O}_2 \rightarrow [3\text{-APA}^*] \rightarrow 3\text{-APA} + \text{light}$ , where 3-APA denotes 3-aminophthalate, and 3-APA\* is the excited state. The decay of 3-APA\* to a lower energy level causes photon emission (**Figure 1**). It should be noted that CL differs from fluorescence in that the electronically excited state is derived from the product of a chemical reaction rather than the more typical way of creating electronically excited states, namely, absorption of energy that occurs in the process leading to fluorescence.

By measuring the photon emission using a luminometer, the rate of  $\text{H}_2\text{O}_2$  formation in a biological system can be determined. Because the above CL response is dependent on an exogenously added peroxidase, such as horseradish peroxidase (HRP), which does not cross cell membranes, the luminol/HRP-dependent CL response is indicative of the level of  $\text{H}_2\text{O}_2$  released into the extracellular milieu.

For the detection of a particular ROS, a CL probe (e.g., luminol) along with other required reagents (e.g., HRP) is incubated with the ROS-generating system (e.g., enzymes, cells, tissues), and the light emission resulting from the chemical reactions between the probe and the ROS is measured by a luminometer (e.g., the Berthold LB9505 Multi-Channel Biolumat or LB953 AutoLumat) or a liquid scintillation counter. CL imaging can also be applied to visualize ROS formation in situ in cultured cells or tissues [11, 12].

Similar to fluorescence-based methods, CL assays represent a highly sensitive way of detecting ROS in biological systems. However, many CL probes have limited specificity for different ROS, and this limitation also applies to many other ROS-detecting probes, such as fluorescence probes.

## 2.2. Assay Principle of the Luminol/HRP-Dependent CL

As noted earlier in Section 2.1, the CL probe luminol is oxidized by  $\text{H}_2\text{O}_2$  in the presence of HRP to yield light emission. Hence, luminol-derived CL in the presence of exogenously added HRP can be used to sensitively and selectively detect extracellular  $\text{H}_2\text{O}_2$  [6]. The kinetic mode of modern luminometers allows the real-time detection of the CL responses over time at various temperatures, which may yield important information on the rate of  $\text{H}_2\text{O}_2$  flux in the extracellular milieu. Because  $\text{H}_2\text{O}_2$  like water can readily diffuse across cell membranes via an aquaporin-dependent mechanism [1, 2], intracellularly generated  $\text{H}_2\text{O}_2$  can be instantly released into the extracellular space. Hence, under well-defined conditions, by detecting extracellular  $\text{H}_2\text{O}_2$  release, intracellular  $\text{H}_2\text{O}_2$  formation can be estimated. This is particularly useful for studying chemical redox cycling inside cells to form  $\text{H}_2\text{O}_2$  (**Figure 2**). It is noteworthy that in addition to  $\text{H}_2\text{O}_2$ , other oxidants might also be involved in the reactions leading to luminol/HRP-dependent CL. To determine the specificity of the luminol/HRP-dependent CL for detecting  $\text{H}_2\text{O}_2$  formation, catalase can be added to the reaction mix to determine catalase-inhibitable luminol/HRP-dependent CL.

## 3. MATERIALS AND INSTRUMENTS

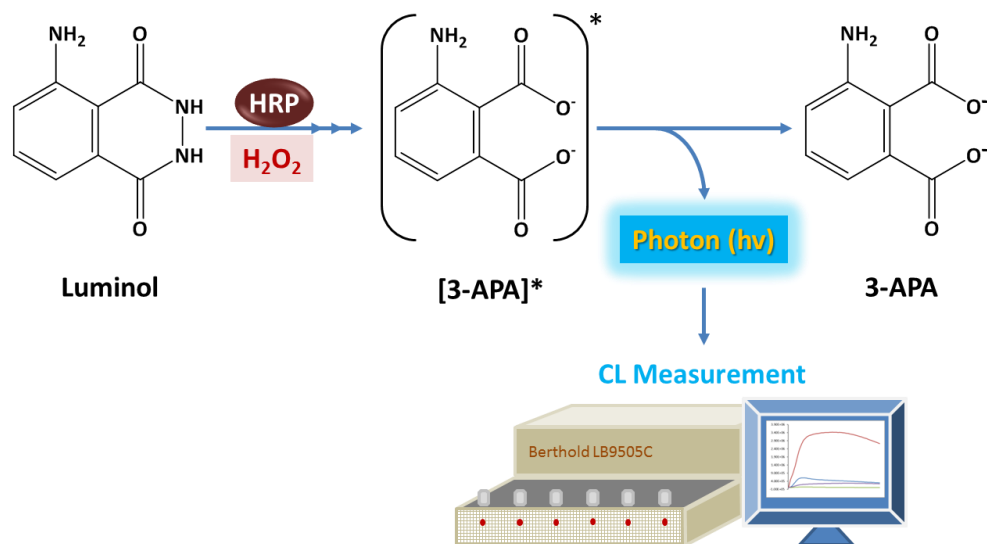
This article describes the catalase-inhibitable luminol/HRP-dependent CL assay for detecting  $\text{H}_2\text{O}_2$  formation by two biological systems: cultured Lewis lung carcinoma (LLC) cells and intact mitochondria isolated from the LLC cells.

### 3.1. Major Materials

The major materials for the catalase-inhibitable luminol/HRP-dependent CL assay are listed alphabetically in **Table 1**.

### 3.2. Major Instruments

- (1) Luminometers: Berthold LB9505 Multi-Channel Biolumat or Berthold LB950 AutoLumat
- (2) Beckman Coulter DU-800 ultraviolet/visible (UV/Vis) spectrophotometer



**FIGURE 1. Schematic illustration of the major chemical reactions leading to luminol-dependent CL.** As shown, in the presence of HRP, luminol reacts with hydrogen peroxide, leading to the eventual formation of the excited state of 3-aminophthalate (3-APA\*), whose decay to a lower energy level results in photon emission, which is measured luminometrically as the chemiluminescence (CL) response.

- (3) Dounce tissue grinder
- (4) Water bath or dry bath
- (5) Cell culture equipment ( $\text{CO}_2$  incubator, tissue culture hood, centrifuge, microscope, etc.)

## 4. PROTOCOLS AND STEPS

This section describes the detailed protocols and steps involved in the use of the catalase-inhibitable luminol/HRP-dependent CL assay to detect  $\text{H}_2\text{O}_2$  generated from cultured LLC cells as well as isolated mitochondria in the presence of beta-lapachone, a potential anticancer compound that undergoes redox cycling to generate superoxide and  $\text{H}_2\text{O}_2$  [13].

### 4.1. Cultured Cells

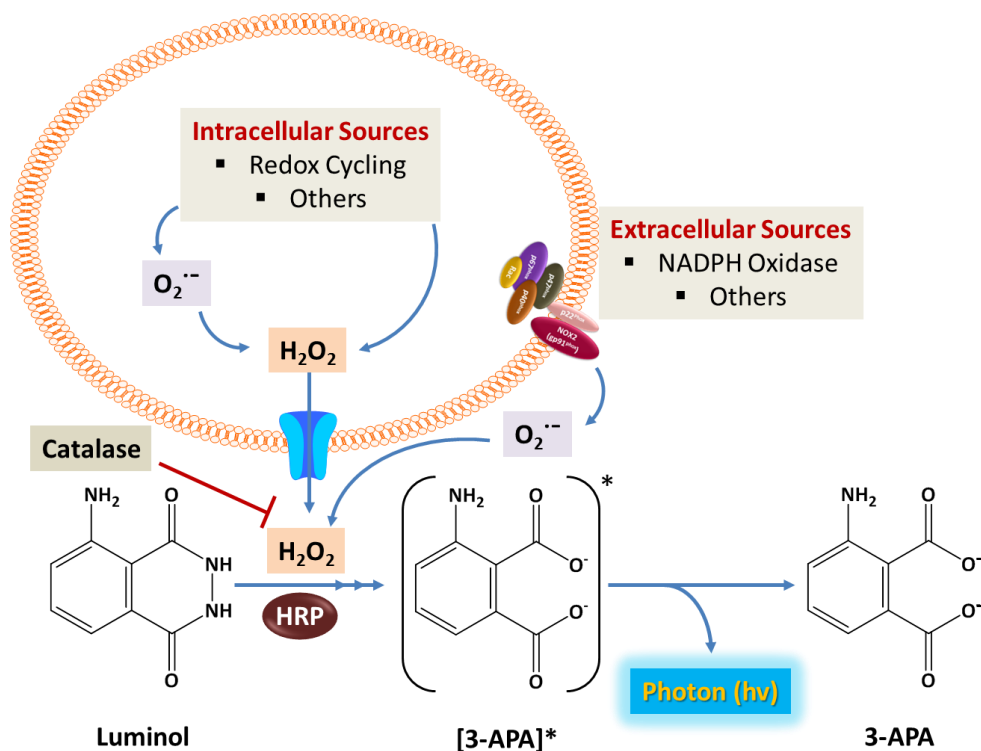
#### 4.1.1. Assay Layout

**Figure 3** illustrates the assay layout for determination of the catalase-inhibitable luminol/HRP-dependent CL resulting from  $\text{H}_2\text{O}_2$  generated by redox cycling of beta-lapachone in LLC cells in CPBS at  $37^\circ\text{C}$  for 30 min. It should be noted that the incu-

bation time can vary from a few seconds to a few hours depending on the experimental design.

#### 4.1.2. Assay Description

The catalase-inhibitable luminol/HRP-dependent CL assay is used to detect extracellular  $\text{H}_2\text{O}_2$  released from cultured LLCs. Briefly, LLC cells are cultured in Dulbecco's modified Eagle medium (DMEM), supplemented with 10% fetal bovine serum (FBS), 100 units/ml of penicillin, and 100  $\mu\text{g}/\text{ml}$  of streptomycin in 150  $\text{cm}^2$  tissue culture flasks at  $37^\circ\text{C}$  in a humidified atmosphere of 5%  $\text{CO}_2$ . The cells are fed every 2–3 days, and subcultured once they reached 80–90% confluence. For the CL measurement, LLC cells are harvested from the cultures and washed once in CPBS. As many as  $1 \times 10^6$  cells are suspended in 1 ml CPBS in a CL tube containing 1  $\mu\text{M}$  beta-lapachone, 10  $\mu\text{g}/\text{ml}$  of HRP, and 10  $\mu\text{M}$  luminol in the presence or absence of 500 units/ml of catalase, and the CL tube is immediately transferred to a Berthold LB9505 luminometer for recording the CL response at  $37^\circ\text{C}$  for 30 min. The results are expressed as the real-time photon emission curve (CL response) and the integrated CL response (the area



**FIGURE 2. Detection of extracellular hydrogen peroxide by the luminol/HRP-dependent CL in cells.** As depicted, H<sub>2</sub>O<sub>2</sub> in the extracellular milieu may be either generated by membrane-associated enzymes such as NADPH oxidases or derived from intracellular sources (e.g., chemical redox cycling). In this context, intracellularly generated H<sub>2</sub>O<sub>2</sub> can diffuse out of cells via an aquaporin-facilitated mechanism. Inclusion of exogenous catalase in the assay, i.e., detection of catalase-inhibitable luminol/HRP-dependent CL, makes it possible to specifically determine the flux of H<sub>2</sub>O<sub>2</sub> in the extracellular milieu.

under the curve, representing the total counts of photon emission over the 30 min of incubation time).

#### 4.1.3. Preparation of Reagents

- (1) Luminol solution (2 mM in PBS): 7.96 mg luminol sodium (molecular mass = 199.14) dissolved in 2 ml PBS (aliquot into microfuge tubes and store at -20°C).
- (2) HRP solution (2 mg/ml in PBS): 4 mg HRP dissolved in 2 ml PBS (aliquot into microfuge tubes and store at -20°C).
- (3) Beta-Lapachone solution (1 mM in dimethyl sulfoxide): first prepare 10 mM beta-lapachone (molecular mass = 242.27) (add 2.06 ml dimethyl sulfoxide to a vial containing 5 mg beta-lapachone), and then dilute this 10 mM beta-

lapachone solution in dimethyl sulfoxide to yield a 1 mM solution.

- (4) Catalase solution (100,000 units/ml in PBS): 50 mg catalase (2,300 units/mg) dissolved in 1.15 ml PBS (aliquot into microfuge tubes and store at -20°C).

#### 4.1.4. Steps

- (1) Aliquot  $1 \times 10^6$  cells into each of the 4 microfuge tubes followed by centrifugation to pellet the cells and discard of the medium. Keep the cell pellets on ice.
- (2) Add 5 µl of 2 mM luminol to each of the 4 CL tubes.
- (3) Add 5 µl HRP (2 mg/ml) to the CL tubes 1, 2, and 4, and 5 µl PBS to the CL tube 3.

**Table 1. Major materials for the catalase-inhibitable luminol/HRP-dependent CL assay**

Major Material	Vendor and Catalog Number	Note
Bio-Rad protein assay dye reagent concentrate	Bio-Rad; 5000006	For measurement of mitochondrial protein
Catalase	Sigma-Aldrich; C9322	Source: bovine liver; 2,300 units/mg
Complete phosphate-buffered saline (CPBS)	Investigator-prepared	Components: 8.1 mM Na <sub>2</sub> HPO <sub>4</sub> , 1.47 mM KH <sub>2</sub> PO <sub>4</sub> , 138 mM NaCl, 2.67 mM KCl, 0.5 mM MgCl <sub>2</sub> , 0.7 mM CaCl <sub>2</sub> , and 0.1% glucose, pH 7.4
Horseradish peroxidase (HRP)	Sigma-Aldrich; P8250	Type II; source: horseradish roots ( <i>Amoracia rusticana</i> ); molecular mass: ~44 kDa
Beta-Lapachone	Sigma-Aldrich; L2037	5 mg size
Lewis lung carcinoma (LLC) cells	ATCC; CRL-1642	LLC cells are derived from Lewis lung carcinoma in C57BL/6 mice.
Luminol (sodium salt)	Sigma-Aldrich; A4685	Luminol sodium salt is soluble in water (50 mg/ml), yielding a clear solution.
Mitochondrial isolation buffer	Investigator-prepared	Components: 250 mM sucrose, 10 mM Hepes, 1 mM EGTA, and 0.5% bovine serum albumin, pH 7.4
Mitochondrial respiration buffer	Investigator-prepared	Components: 70 mM sucrose, 220 mM mannitol, 2 mM Hepes, 2.5 mM KH <sub>2</sub> PO <sub>4</sub> , 2.5 mM MgCl <sub>2</sub> , 0.5 mM EDTA, and 0.1% bovine serum albumin, pH 7.4
Phosphate-buffered saline (PBS)	Investigator-prepared	Components: 8.1 mM Na <sub>2</sub> HPO <sub>4</sub> , 1.47 mM KH <sub>2</sub> PO <sub>4</sub> , 138 mM NaCl, and 2.67 mM KCl, pH 7.4
Succinate	Sigma-Aldrich; S2378	Sodium succinate dibasic hexahydrate

- (4) Add 5  $\mu$ l catalase (100,000 units/ml) to the CL tube 4, and 5  $\mu$ l PBS to the CL tubes 1, 2, and 3.
- (5) Add 1  $\mu$ l of 1 mM beta-lapachone to the CL tubes 2, 3, and 4, and 1  $\mu$ l dimethyl sulfoxide (DMSO) to the CL tube 1.
- (6) Add 984  $\mu$ l of 37°C-prewarmed air-saturated CPBS to the microfuge tube 1, as indicated in Step (1) above, that contains  $1 \times 10^6$  cells to resuspend the cells. Transfer the entire cell suspension to the CL tube 1 immediately followed by mixing of the sample and recording of the CL response at 37°C for 30 min. Do the same for the remaining 3 samples. The background CL levels of the instrument are measured with the CL tubes containing all reactants/components but cells. The background CL levels are subtracted from the CL responses of the tubes containing cells so that the reported CL responses in the figures are due to cellular activity.

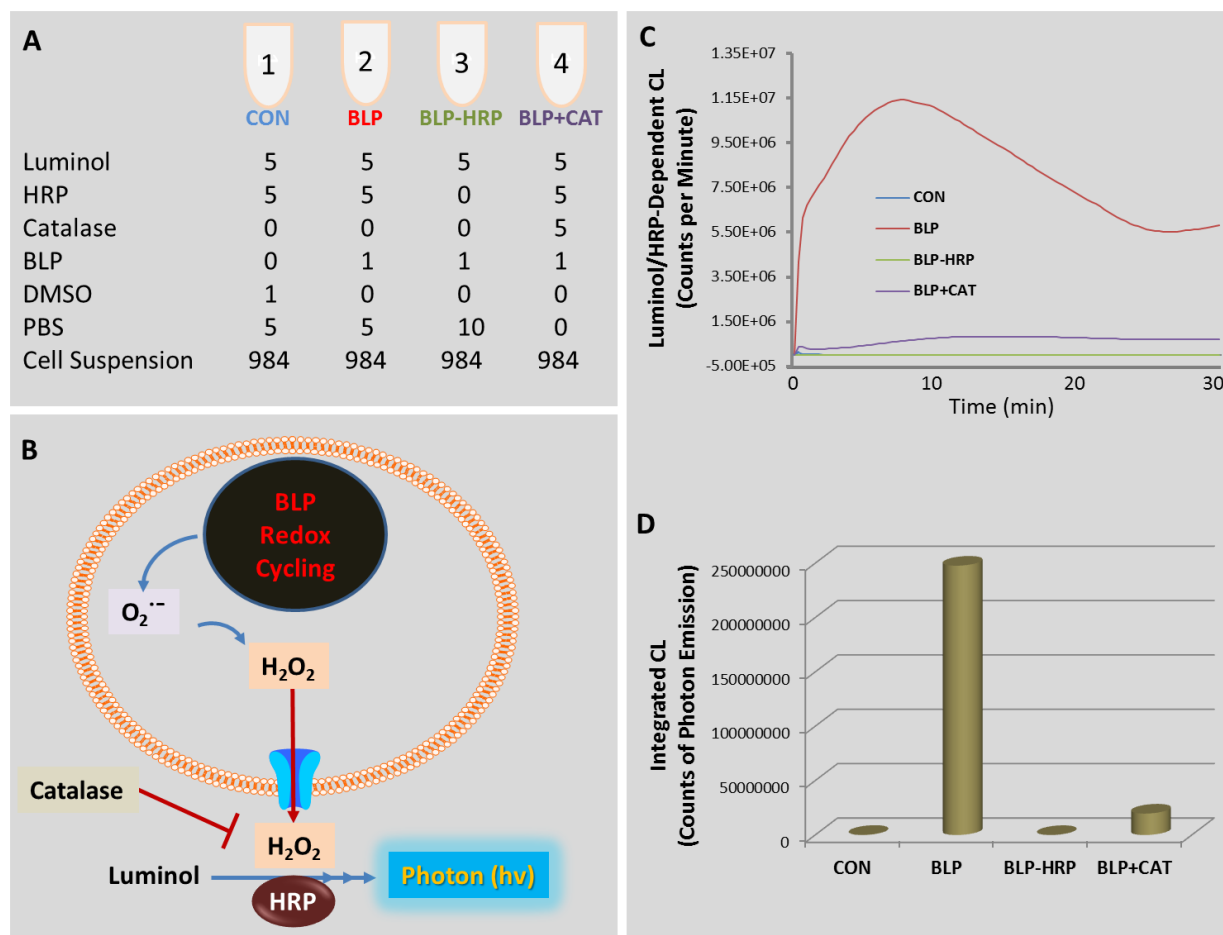
#### 4.1.5. Calculations

The Berthold LB9505 multi-channel luminometer enables real-time measurement of the CL responses from each of the 4 samples (CL tubes) which are reported as the CL response curves (see **Figure 3C**) with the unit of the Y and X axis being counts of photon emission per minute (CPM) and minute, respectively. The luminometer automatically calculates the area under the curve for each of the CL response curves from the 4 samples. This is designated as the integrated CL (see **Figure 3D**) with the unit being total counts of photon emission over the 30 min of incubation time per  $1 \times 10^6$  cells.

#### 4.1.6. Other Considerations

The Berthold LB9505 multi-channel luminometer can measure up to 6 samples simultaneously, whereas the newer model of Berthold LB953 luminometer

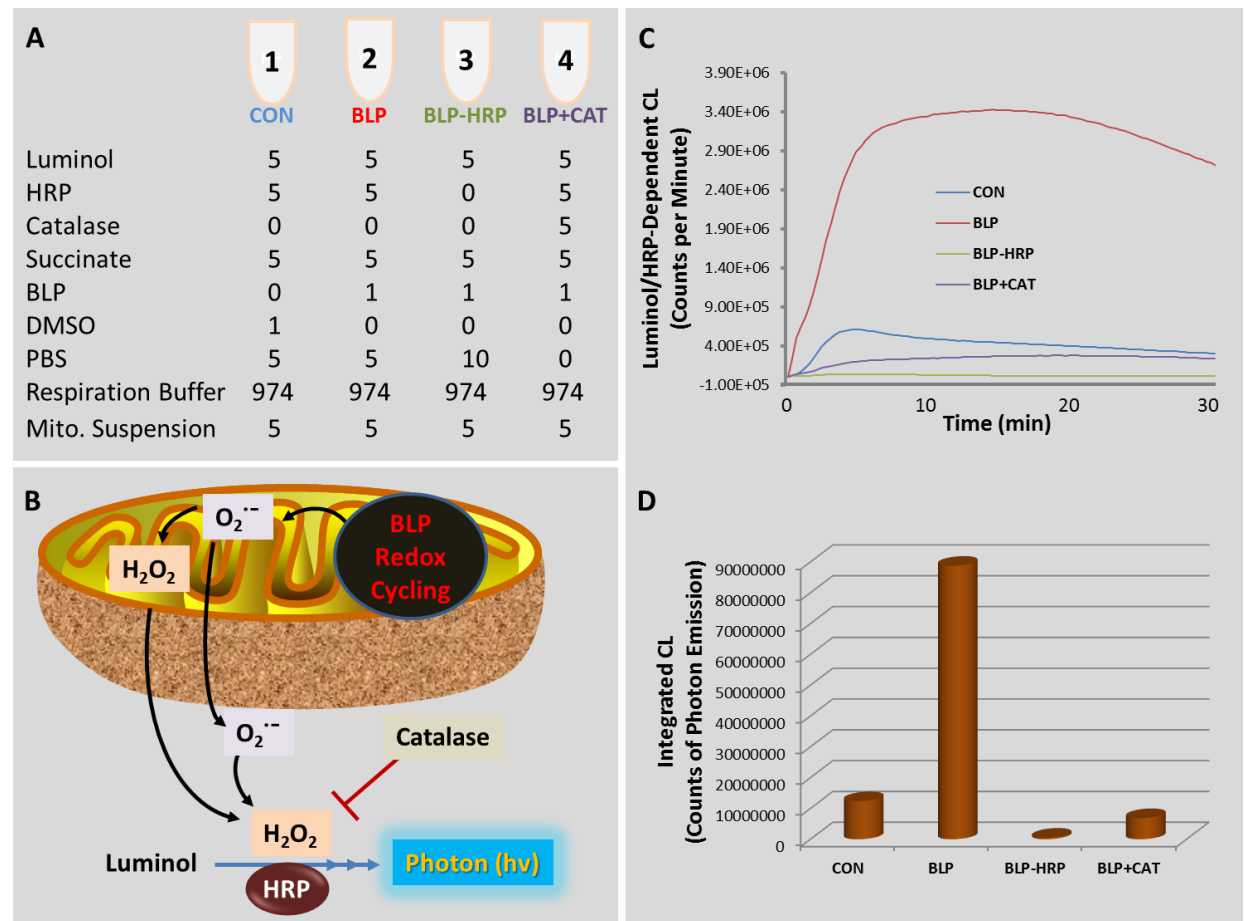




**FIGURE 3. Assay layout for the catalase-inhibitable luminol/HRP-dependent CL responses in LLC cells treated with beta-lapachone.** Redox cycling of beta-lapachone (BLP) in LLC cells generates superoxide which undergoes spontaneous or superoxide dismutase-catalyzed dismutation to form  $H_2O_2$ . The  $H_2O_2$  formed can readily diffuse out of cells and react with luminol in the presence of HRP, resulting in CL responses. Addition of catalase (CAT) inhibits the CL response by >92%, indicating that the assay under the present experimental conditions selectively detects extracellular  $H_2O_2$ . Notably, the CL response is completely abolished in the absence of added HRP, indicating that the CL response is dependent on the presence of HRP, thus confirming that the CL response occurs in the extracellular milieu. The unit in panel A is  $\mu$ l.

with auto-injectors can measure much more samples simultaneously under its kinetic mode. However, our laboratories have consistently noticed that the Berthold LB9505 luminometer is probably one of the most sensitive luminometers for detecting CL responses from biological systems, including tissues, intact cells, and isolated mitochondria, as well as other subcellular components. Both the Berthold LB9505 and LB953 luminometers are equipped with

a temperature control unit, allowing the measurement of the CL responses at various temperatures including  $37^\circ\text{C}$ . Although this article describes the use of the Berthold LB9505 luminometer in detecting biological  $H_2O_2$  formation, the protocols can be easily adopted to detect biological  $H_2O_2$  formation with other commercially available luminometers if the detection sensitivity is not a major concern. As many of the commercially available luminometers are not



**FIGURE 4.** Assay layout for the catalase-inhibitable luminol/HRP-dependent CL responses in isolated mitochondria treated with beta-lapachone. Redox cycling of beta-lapachone (BLP) in succinate-driven mitochondria generates superoxide which undergoes spontaneous or superoxide dismutase-catalyzed dismutation to form  $H_2O_2$ . The  $H_2O_2$  formed can readily diffuse out of mitochondria and react with luminol in the presence of HRP, resulting in CL responses. Superoxide may also exit mitochondria and then undergo spontaneous dismutation, contributing to the extramitochondrial  $H_2O_2$  formation. Addition of catalase (CAT) inhibits the CL response by >92%, indicating that the assay under the present experimental conditions selectively detects the formation of extramitochondrial  $H_2O_2$ . The CL response is completely abolished in the absence of added HRP, indicating that the CL response is dependent on the presence of HRP, confirming that the CL response occurs in the extramitochondrial milieu. The unit in panel A is  $\mu$ l. Mito. denotes mitochondrial.

equipped with the kinetic and temperature control units, to detect the time- and temperature-dependent CL responses, the sample CL tubes can be incubated at various temperatures in a water bath or dry bath and then transferred to the luminometer for the measurement of the CL responses at different time intervals to obtain a time-dependent response.

## 4.2. Isolated Mitochondria

### 4.2.1. Assay Layout

**Figure 4** illustrates the assay layout for determination of the catalase-inhibitable luminol/HRP-dependent CL responses resulting from  $H_2O_2$  gener-



ated by redox cycling of beta-lapachone in isolated mitochondria at 37°C for 30 min. It should be noted that the incubation time can vary from a few seconds to a few hours depending on the experimental requirement and the luminometer used.

#### 4.2.2. Assay Description

The catalase-inhibitable luminol/HRP-dependent CL assay is used to detect H<sub>2</sub>O<sub>2</sub> released from intact mitochondria isolated from LLC cells. Briefly, LLC cells are cultured in DMEM supplemented with 10% FBS, 100 units/ml of penicillin, and 100 µg/ml of streptomycin in 150 cm<sup>2</sup> tissue culture flasks at 37°C in a humidified atmosphere of 5% CO<sub>2</sub>. The cells are fed every 2–3 days, and subcultured once they reach 80–90% confluence. Mitochondria are isolated from the freshly harvested LLC cells according to the method described before [14]. Briefly, LLC cells (~3 × 10<sup>7</sup> cells) are harvested and washed once with PBS. The cell pellet is resuspended in 6 ml of sucrose buffer (250 mM sucrose, 10 mM Hepes, 1 mM EGTA, and 0.5% bovine serum albumin, pH 7.4) and homogenized with a Dounce tissue grinder (40 strokes with the tight pestle) on ice. The homogenate is centrifuged at 1,500 × g for 10 min at 4°C. The supernatant is collected and centrifuged at 10,000 × g for 10 min at 4°C. The resulting mitochondrial pellet is washed twice with the sucrose buffer and then resuspended in bovine serum albumin (BSA)-free sucrose buffer (250 mM sucrose, 10 mM Hepes, and 1 mM EGTA, pH 7.4). The mitochondrial protein is measured with Bio-Rad protein assay dye based on the method of Bradford [15] with BSA as the standard. The mitochondrial suspension is diluted to 1 mg/ml in the above BSA-free sucrose buffer and kept on ice for experiments within 3 hours. For the CL measurement, 5 µl of mitochondrial suspension (giving a final concentration of 0.05 mg mitochondrial protein/ml) is added to a CL tube containing the various reactants (250 µM succinate, 1 µM beta-lapachone, 10 µg/ml of HRP, and 10 µM luminol in the presence or absence of 500 units/ml of catalase) in the respiration buffer (in a final volume of 1 ml; 70 mM sucrose, 220 mM mannitol, 2 mM Hepes, 2.5 mM KH<sub>2</sub>PO<sub>4</sub>, 2.5 mM MgCl<sub>2</sub>, 0.5 mM EDTA, and 0.1% BSA, pH 7.4), and the CL tube is immediately transferred to the Berthold LB9505 luminometer for recording the CL responses at 37°C for 30 min. The results are expressed as the real-time photon emis-

sion (CL response curve) and the integrated CL response (the area under the curve, representing the total counts of photon emission over the 30 min of incubation time).

#### 4.2.3. Preparation of Reagents

- (1) Luminol solution (2 mM in PBS): see Section 4.1.3 for preparation.
- (2) HRP solution (2 mg/ml in PBS): see Section 4.1.3 for preparation.
- (3) Beta-Lapachone solution (1 mM in dimethyl sulfoxide): see Section 4.1.3 for preparation.
- (4) Catalase solution (100,000 units/ml in PBS): see Section 4.1.3 for preparation.
- (5) Succinate solution (0.5 M in deionized water): 1.35 g sodium succinate dibasic hexahydrate (molecular mass = 270.14) dissolved in 10 ml deionized water (aliquot into microfuge tubes and store at –20°C).

#### 4.2.4. Steps

- (1) Add 5 µl of 0.5 M succinate to each of the 4 CL tubes.
- (2) Add 5 µl of 2 mM luminol to each of the 4 CL tubes.
- (3) Add 5 µl of HRP (2 mg/ml) to the CL tubes 1, 2, and 4, and 5 µl PBS to the CL tube 3.
- (4) Add 5 µl catalase (100,000 units/ml) to the CL tube 4, and 5 µl PBS to the CL tubes 1, 2, and 3.
- (5) Add 1 µl of 1 mM beta-lapachone to the CL tubes 2, 3, and 4, and 1 µl dimethyl sulfoxide (DMSO) to the CL tube 1.
- (6) Add 974 µl of 37°C-prewarmed air-saturated mitochondrial respiration buffer to the CL tube 1 followed by the addition of 5 µl of the mitochondrial suspension (1 mg/ml). Upon mixing, the CL tube is transferred to the LB9505 luminometer for recording the CL response at 37°C for 30 min. Do the same for the remaining 3 samples. It is worth mentioning that the background CL levels of the instrument are measured with the CL tubes containing all reactants/components but mitochondria. The background CL levels are subtracted from the CL responses of the tubes containing mitochondria so that the reported data of CL responses in the figures are due to mitochondrial activity.

#### 4.2.5. Calculations

The Berthold LB9505 multi-channel luminometer enables real-time measurement of the CL responses from each of the 4 samples (CL tubes) which are reported as the CL response curves (see **Figure 4C**) with the unit of the Y and X axis being CPM and minute, respectively. The luminometer automatically calculates the area under the curve for each of the CL response curves from the 4 samples. This is designated as the integrated CL (see **Figure 4D**) with the unit being total counts of photon emission over the 30 min of incubation time per 0.05 mg mitochondria.

#### 4.2.6. Other Considerations

In addition to those mentioned in Section 4.1.6, the protocols described in this article can be easily adopted to study the effects of other mitochondrial substrates, such as pyruvate/malate, which provide NADH, thus feeding the electrons through the mitochondrial complex I. It is noteworthy that in addition to using this assay to study chemical redox cycling in mitochondria, the assay can also be employed to detect  $\text{H}_2\text{O}_2$  formation by isolated mitochondria under various conditions. In this context, mitochondria can be isolated from cells or tissues under certain pathophysiological conditions to determine the altered formation of mitochondrial  $\text{H}_2\text{O}_2$ . Notably, mitochondria are the major source of cellular  $\text{H}_2\text{O}_2$  under diverse physiological and pathophysiological conditions and mitochondria-derived  $\text{H}_2\text{O}_2$  plays an important role in biology and medicine [16–18].

## 5. DISCUSSION OF ADVANTAGES AND LIMITATIONS

### 5.1. Advantages

As with other CL-based techniques, the biggest advantage of the luminol/HRP-dependent CL assay is its high sensitivity for detecting biological  $\text{H}_2\text{O}_2$  formation. With the kinetic mode of the Berthold LB9505 (as well as the Berthold LB953) luminometer, the relative rate of formation of  $\text{H}_2\text{O}_2$  in a biological system can be sensitively detected in a real-time manner. Examining the shapes of the CL response curves may also yield important insight into the dynamics of  $\text{H}_2\text{O}_2$  formation in a biological system un-

der a particular physiological or pathophysiological condition.

### 5.2. Limitations

The luminol/HRP-dependent CL assay for detecting biological  $\text{H}_2\text{O}_2$  formation is not without limitations. As noted earlier, this assay only detects the rate of  $\text{H}_2\text{O}_2$  flux in the extracellular or extramitochondrial milieu. Because intracellularly formed  $\text{H}_2\text{O}_2$  can readily penetrate the biomembranes to enter the extracellular/extramitochondrial milieu, detection of the rate of extracellular/extramitochondrial  $\text{H}_2\text{O}_2$  flux can, however, be used to reflect the dynamics of  $\text{H}_2\text{O}_2$  formation from the intracellular sources. Another shortcoming of the assay is related to its limited ability to directly quantify the absolute amounts of  $\text{H}_2\text{O}_2$  formed in a biological system. To circumvent this limitation, a concurrently run experiment using known amounts of  $\text{H}_2\text{O}_2$  may be considered to obtain a standard curve (i.e., a linear relationship between the integrated luminol/HRP-dependent CL and the amounts of exogenously supplemented  $\text{H}_2\text{O}_2$ ). The exogenously supplemented  $\text{H}_2\text{O}_2$  may be in the form of either a  $\text{H}_2\text{O}_2$  solution of known concentration or preferentially a known rate of  $\text{H}_2\text{O}_2$  formation by a standard enzymatic system, such as the glucose/glucose oxidase. Nevertheless, it should be borne in mind that due to the complexity of reactions involved in detecting  $\text{H}_2\text{O}_2$  as well as other ROS in biological systems, the reliability of such a standard curve for quantifying biological  $\text{H}_2\text{O}_2$  should be rigorously tested and validated.

## 6. CONCLUSION

As with many other assays and techniques for detecting biological  $\text{H}_2\text{O}_2$  as well as other members of the ROS family, the luminol/HRP-dependent CL assay has its own advantages and limitations. The biggest advantage of this assay is its high sensitivity and real-time detection of  $\text{H}_2\text{O}_2$  formation. On the other hand, a major limitation of the assay is its inability to directly detect intracellular  $\text{H}_2\text{O}_2$  formation. In addition, the assay does not allow quantification of  $\text{H}_2\text{O}_2$  in biological systems unless a concurrently run standard curve is employed. Nevertheless, as mentioned earlier, if appropriately used under controlled conditions along with the results being correctly in-

terpreted, detection of  $\text{H}_2\text{O}_2$  by the luminol/HRP-dependent CL assay can yield important information on the real-time formation of this ROS in various biological systems under different physiological and pathophysiological conditions. In fact, detecting the changes in the relative rate of  $\text{H}_2\text{O}_2$  formation, rather than the absolute amounts of  $\text{H}_2\text{O}_2$  formed, under different conditions, is frequently the goal of the experiments towards understanding the role of this important ROS in biology and medicine.

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