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The GOX/CAT system: A novel enzymatic method to independently control hydrogen peroxide and hypoxia in cell culture

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APPENDIX A

Protocols generating H_2O_2 with or without hypoxia using the GOX/CAT system

Preface:
Most protocols are adapted to cells grown in 6-well plates. For other culture dishes, the reader is referred to conversion tables 3 and 4.

***Protocol 1*: Exposing cells to steady state concentrations of 1, 5 and 10 µmol/l H_2O_2 for 24 h under normoxic (21% O_2) conditions**

Objective: Long-term exposure of cells to low dose steady state concentrations of H_2O_2.
Equipment
1.CO_2-incubator
2.Cells grown in 6 well plates to 60% confluency
3.DMEM (25 mmol/l glucose) + additives (FCS, antibiotics)
4.Sterile plastic ware (15ml tube, serum pipettes, pipette tips) for cell culture
5.Glucose oxidase (stock solution, Sigma G0543).
6.Catalase (stock solution, Sigma C3155)
7.Tools and reagents to harvest cells at the end of experiment (cell scraper, reagents e.g. Trizol®-Reagent for RNA-isolation, RIPA-buffer for protein-extraction)

Procedure
1. Cells are grown to 60% confluence in a 6-well plate over night.
2. The next day, a H_2O_2 -generating GOX/CAT is prepared according to Table S1. Prepare 2 ml per each well.

H_2O_2 (µmol/l)	GOX dilution	CAT dilution
1	1:100,000	1:66,000
5	1:100,000	1:280,000
10	1:100,000	1:1000,000

3. After mixing allow an equilibration time of ca. 5 min.
4. Then replace the cell culture medium by GOX/CAT-DMEM (2ml/well) and incubate for the desired period (e.g. 24 h) with the cells. No replacement of the GOX/CAT-DMEM is necessary since less than 1.3 mmol/l glucose will be consumed during this time.
5. After 24 h, cells harvested for protein- or RNA-isolation in the appropriate buffers.

Background information for chosen conditions:
According to the enzyme activities of the commercially available stock solutions (see Section 1 of this article) GOX 1:100.000 has an activity of 0.015 µmol/l*s. The cellular catalase activity was assumed to be 0.001 s^{-1}. According to d H_2O_2 /dt= K_{CAT} * [H_2O_2]ss, catalase activity can be obtained. The added external activity is then obtained by subtracting cellular catalase from the calculated value. It should be noted that under certain conditions no external catalase needs to be added since the cellular catalase is high enough. However, GOX should not be incubated in the cell free DMEM for more than a few seconds since H_2O_2 can rapidly accumulate and eventually lead to uncontrolled cytotoxicity or cell death.

Adaptation of experiment to another cell culture dish format:
1. GOX- and CAT dilutions remain the same.
2. Volumes of culture medium for other culture dishes can be obtained from Tables 3 and 4.

Tips and Tricks:
1. Run a test series of different [H_2O_2]ss levels before the real experiment to define the individual H_2O_2-sensitivity of your particular cell type. This can be done in 96-well plates. Microscopic read-out by eye-balling or trypan blue exclusion is sufficient to exclude cytotoxicity or cell death. Usually cytotoxicity manifests after 8-12h of treatment with H_2O_2, allowing a shortened incubation period for the test run within a working day.
2. Even after having determined the [H_2O_2]ss tolerance of the cell line, we usually set up a range of 2-3 [H_2O_2]ss concentrations for each experiment.
3. Before harvesting the cells we check cell morphology by inverse microscopy to exclude cytotoxic effects.

This protocol has been used in the publication of Andriopoulos et al 2007 [18].

***Protocol 2*: Measuring cytotoxicity in a 96-well plate by trypan blue exclusion under various H_2O_2 and hypoxia conditions**

This protocol refers to section 5.1. (biological validation experiment) and to *Fig. 13*.

Objective: To compare the sensitivity of cell lines to H_2O_2.
Equipment:
1. CO_2-incubator
2. Cell line(s) to be tested for H_2O_2-sensitivity. Cells are grown in 96 well plates to 60% confluency over night.
3. DMEM (25 mmol/l glucose) + additives (FCS, antibiotics)
4. Sterile plastic ware (15ml tube, serum pipettes, pipette tips) for cell culture
5. Glucose oxidase (stock solution, Sigma G0543).
6. Catalase (stock solution, Sigma C3155)
7. Trypan blue solution

Procedure
1. Cells are grown in 96-well plates to 60% confluency.
2. Prepare GOX/CAT combinations as shown in *Fig. 13* (upper panel).We recommend using 100 or 200 µl DMEM, GOX-dilutions between 1:10,000 and 1:100,000, and catalase dilution as indicated in each square.
3. Discard old medium and replace by GOX/CAT-DMEM.
4. Remove GOX/CAT-DMEM after 12-24 h and wash cells gently with PBS using a multichannel pipette. Stain the whole 96-well plate with trypan blue solution.
5. Assess trypan blue positive cells semi-quantitatively under an inverted light microscope (e.g. 0, 1+, 2++, 3+++ for increasing rates of cell death).
6. Chose appropriate GOX/CAT combinations for later experiments.

Tips and Tricks:
1. The easiest way to prepare GOX/CAT-DMEM combinations is a type of checkerboard titration: Dilute the catalase by a 1+1 dilution from left to right in an empty 96 well plate (180µl volume; double final concentration). Prepare GOX-dilutions (double final concentrations) in 15ml tubes. Then add 180 µl of GOX-dilutions to 96 well plate, mix gently and equilibrate for 5 min. Transfer GOX/CAT-DMEM to the 96 well plate using a multichannel pipette.
2. Instead of trypan blue exclusion, other cell viability tests can be performed e.g. MTT. However, cells should be carefully washed to remove GOX before incubation with MTT-substrate in order to prevent artifacts.

***Protocols 3*: Exposing cells to enzymatic hypoxia of 2% oxygen in a 6-well plate**

Objective: To study cells under hypoxia.
Equipment:
1. CO_2-incubator
2. Cells grown in 6-well plate
3. DMEM (25 mmol/l glucose) + additives (FCS, antibiotics)
4. Sterile plastic ware (15ml tube, serum pipettes, pipette tips)
5. Glucose oxidase (stock solution, Sigma G0543).
6. Catalase (stock solution, Sigma C3155)
7. Tools and reagents to harvest cells at the end of experiment (cell scraper, reagents e.g. Trizol®-Reagent for RNA-isolation, RIPA-buffer for protein-extraction….)

Procedure
1. Cells are grown in a 6-well plate to 60% confluence over night.
2. Prepare hypoxia medium by diluting glucose oxidase 1:10,000 and catalase 1:1000 in cell culture medium (DMEM 25 mmol/l glucose + additives). The solution is gently shaken. For each well 2.5 ml of hypoxia medium are needed (also see Tables 3 and 4).
3. Exchange cell culture medium to hypoxia medium. Prepare normoxic control by fresh medium alone.
4. Move the cell culture plate carefully to the CO_2-incubator (5% CO_2, room air, 37°C). Be careful not to move the culture dishes during incubation or you will destroy the oxygen-gradient.
5. Harvest cells after desired incubation period for protein- or RNA-isolation in the appropriate reagents.

Tips and Tricks:
1. Do not move the dishes during incubation period or you will destroy the oxygen gradient and re-oxygenate the cells.
2. Choose GOX/CAT combinations depending on the intended incubation period as described in section 4.2.

***Protocol 4*: Exposure of cells to steady state H_2O_2 –concentrations under enzymatic hypoxia of 2% oxygen in a 6-well plate**

Objective: To study cells under conditions of ROS under hypoxia (as occur e.g. in the liver)
Equipment:
1. CO_2-incubator
2. Cells grown in 6-well plate
3. DMEM (25mmol/l glucose) + additives (FCS, antibiotics)
4. Sterile plastic ware (15ml tube, serum pipettes, pipette tips)
5. Glucose oxidase (stock solution, Sigma G0543).

6. Catalase (stock solution, Sigma C3155)
7. Tools and reagents to harvest cells at the end of experiment (cell scraper, reagents e.g. Trizol®-Reagent for RNA-isolation, RIPA-buffer for protein-extraction….)

Procedure:

1. Cells are grown in a 6-well plate to 60% confluence over night.
2. Prepare hypoxia/H_2O_2 medium by diluting glucose oxidase 1:10,000 and catalase (both Sigma, St. Louis, MO) at various concentrations (we suggest 1:1,000 for pure hypoxia, 1:5,000, 1:10,000 and 1:50,000. Most cells will not tolerate a catalase-dilution of 1:100,000 due to H_2O_2-toxicity) in DMEM. Shake the solution gently. For each well 2.5ml of hypoxia/H_2O_2 medium are needed (also see Tables 3 and 4).
3. Change the cell culture medium to hypoxia/H_2O_2 medium. Control cells are treated with fresh medium alone.
4. Incubate cells in CO_2-incubator (5% CO_2, room air, 37°C) for the desired incubation time. Be careful not to move the culture dishes during incubation or you will destroy the oxygen-gradient and increase H_2O_2-toxicity!
5. After the incubation period, cells are harvested for protein- or RNA-isolation in the appropriate medium.

Tips and tricks:

1. Too low confluence of the cells results in increased toxicity of H_2O_2 – be careful to keep cell density constant between experiments.
2. We recommend running in parallel several CAT-dilutions in each experiment to make up for variations in cell density.
3. Always check for cytotoxicity under the microscopes before harvesting.

APPENDIX B

Non-enzymatic ultrasensitive H_2O_2 determination using luminol and NaOCl

B1. Real-time determination of [H_2O_2]ss formation

The ability to measure very low micro and submicromolar H_2O_2 levels in real time by the luminol/hypochlorite system [10, 13] has been instrumental in developing the GOX/CAT system on a quantitative basis. This non-enzymatic chemiluminescence assay is based on the two electron oxidation of luminol (5-amino-2,3-dihydro-1,4-phthalazinedione) by sodium hypochlorite (NaOCl). Luminol is oxidized by NaOCl to diazaquinone in a two-electron oxidation which is further specifically converted by H_2O_2 to an excited aminophthalate via an α-hydroxy-hydroperoxide. The blue chemiluminescence with a maximum wavelength of 431 nm is only emitted in the presence of H_2O_2.

The luminol-hypochlorite system is ideal for monitoring enzyme catalysis involving H_2O_2 as source of product for several reasons: A) This specific two electron oxidation system by NaOCl allows to avoid otherwise unspecific light reactions e.g. via luminol radicals and creates the high specificity for H_2O_2 which is in contrast to many other e.g. peroxidase-based assays. B) Due to the efficient energy transfer of the luminol chemistry, the assay is very sensitive. Nanomolar concentrations can be easily measured and picomolar concentrations have been detected under optimized, special conditions. C) Since the reaction can be performed at pH 7.4 within 400 ms, real-time approach can be established in form of a flow apparatus [13, 54].

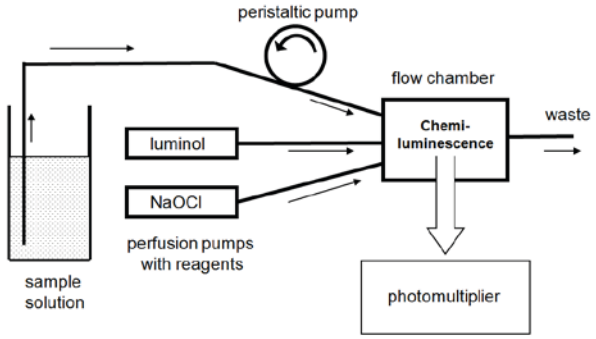
Fig. 4 shows in real time the formation of a H_2O_2 steady state after addition of GOX to a solution containing catalase and glucose. The luminol/hypochlorite assay is not only useful in measuring or confirming the [H_2O_2]ss in cell cultures, but it also allows to measure GOX and catalase activity under the conditions that are actually used in the cell culture (pH 7.4, appropriate, temperature). However, it should be pointed out that, with some limitations, alternative H_2O_2 assays such as described by Dringen et al., [46] or others may be also used or adopted. In addition, others have used oxygen electrodes to indirectly determine enzyme activities [47].

The luminol/hypochlorite assay can be used in two ways, either as a flow system or as an injection system. The systems differ in their preconditions, potentials and limitations. The flow system allows the continuous H_2O_2 determination in a rather large sample volume and is most suited to follow rapid enzyme-dependent changes in H_2O_2 concentration. The injection system permits the end point determination of H_2O_2 in small sample volumes and allows a high sample throughput. Both systems are discussed in the following by presenting typical applications.

***Protocol 5*: Determination of catalase activity [45, 55]**

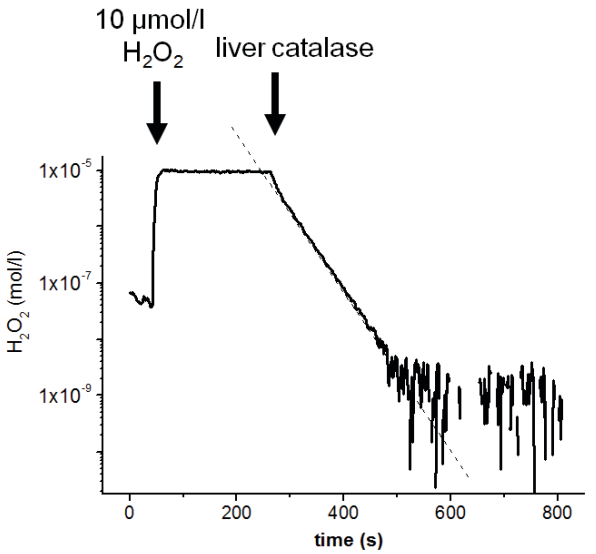
In the flow system, the sample is continuously pumped out from a reaction reservoir and luminol and NaOCl are continuously added to the sample allowing real-time registration of H_2O_2 e.g. to determine fast enzyme kinetics (*Fig. 19*). An advantage is that the sample solution in the reservoir is not in contact with any of the reagents allowing measurements of H_2O_2 independent of the detection system. Another advantage is that conditions can be controlled such as control of temperature or stirring or they can be changed such as adding substrate or inhibitors. Last, several parameters can be determined in parallel (e.g. H_2O_2 and oxygen measurements). A disadvantage of the flow technique is that it requires a large sample volume (up to 100 ml) as the sample is continuously removed. This is usually not limiting since enzyme solutions can be highly diluted due to the sensitivity of the assay. The modus has been successfully employed to measure catalase activity [55], to study H_2O_2 degradation in human erythrocytes [45] and to determine H_2O_2 production in intact rat liver peroxisomes [31,43].

Figure 19. **Scheme of the luminol/hypochlorite assay in the flow modus.**



The system allows interventions during the experiment (e.g. injection of additional compounds, temperature control, magnetic stirring, and measurement of additional parameters such as pH or oxygen levels). The flow system has been decisive for kinetic study of enzymes such as catalase, glutathione peroxidase and oxidases at ultra-low H_2O_2 concentrations in real time.

Figure 20. **Exponential degradation of H_2O_2 by liver catalase as measured in real time.**



The exponential degradation of H_2O_2 by liver catalase is measured in real time using the luminol/hypochlorite assay in a flow modus (see text). The flow system has been calibrated by 10 µmol/l H_2O_2 and background chemiluminescence has been subtracted. H_2O_2 kinetics can be measured down to nanomolar H_2O_2 concentrations. Liver catalase activity can be calculated by linear regression analysis (dotted line) as the exponential rate constant k (see Appendix A).

Equipment (Fig. 19)

- luminometer (any luminometer can be used which allows the installation of a flow cell in front of the photomultiplier e.g. the AutoLumat LB 953 from Berthold EG&G, Wildbad, Germany or the commonly used Fluostar (BMG Labtechnologies GmbH, Offenburg, Germany)). The luminometer should be controlled by a computer equipped with a software for further processing of time/luminescence intensity data.
- perfusion pump for NaOCl and luminol
- peristaltic pump for sample aspiration
- flow cell: A flow cell is required which allows separate and continuous addition of luminol and NaOCl buffered in PBS at pH 7.4 and the continuous addition of the sample. In the authors` laboratory a peristaltic pump is used with a 3 mm polyethylene pipeline to continuously aspirate the sample solution (ca. 4 ml/min). Black 50 ml plastic syringes are loaded with luminol and NaOCl work solutions and both reactions are continuously pumped via the same perfusion pump into the polyethylene pipeline (ca. 12 ml/h).
- graduated cylinder 100 ml for sample solution
- magnetic stirrer to continuously mix sample solution
- temperature control unit (if necessary)

Reagents

- 50 ml 10^{-4} mol/l luminol in 10 mmol/l PBS at pH 7.4 (working solution)
- 50 ml 10^{-4} mol/l NaOCl in tridistilled water (working solution)
- 100 ml 10^{-2} mol/l H_2O_2 in tridistilled water for calibration

Procedure

1. The syringes are loaded with the working solutions of NaOCl and luminol and 50-100 ml PBS is added into the graduated cylinder. All pumps are switched on and the system is allowed to equilibrate for about 5 min. The optimal measuring range is found by adjusting the perfusion pump and calibrated by addition of 10^{-5} mol/l H_2O_2 and catalase (1:100.000 from stock), respectively.
2. An example measurement is now described in more detail demonstrating calibration, data processing and data analysis. In *Fig. 20*, the raw chemiluminescence data of a typical catalase experiment is shown. Initially, H_2O_2 (10 µmol/l final concentration) is added into 100 ml 10 mmol/l PBS.
3. Liver homogenate is further added (arrow). A magnetic stirrer is used to ensure a rapid mixing of the enzyme substrate solution. An exponential decay of the chemiluminescence can be observed that reaches background levels after several minutes. At this time, no changes in luminescence are detectable. The "background luminescence" has several causes and is H_2O_2 dependent (caused by small H_2O_2 impurities of the reagents) or H_2O_2 independent. Factors that contribute to H_2O_2 independent background chemiluminescence are the purity of the NaOCl solution and other side reactions of the luminol/hypochlorite system that also lead to the excited aminophthalate e.g. in association with transition metals in the sample. The concentration of the NaOCl solution clearly defines the extent of the H_2O_2 independent background luminescence and should be as low as possible. Very often, the background luminescence is neglectably small. It can be determined e.g. in the presence of catalase and subtracted from the data. Fig. 20 actually shows the data after subtraction of the background. Such a background subtraction increases sometimes the sensitivity over more

than two orders of magnitude. Indeed, the exponential decay is now observed over a larger range and the detection limit is finally determined by the signal to noise ratio.

4. Catalase activity is finally calculated from the exponential decay of H_2O_2 by linear regression analysis [55].

Comments:

The hypochlorite/luminol technique provides several advantages in comparison to conventional spectrophotometric and titrimetric catalase assays: i) due to the low H_2O_2 concentrations used, molecular oxygen is completely dissolved and not liberated in gaseous form that causes artifacts ii) since maximal extracellular H_2O_2 concentrations are known to reach only micromolar levels, determinations of catalase activity at submicromolar concentrations reflect physiological conditions. iii) repetitive measurements are possible without loss of enzyme activity or cell viability. The assay has been successfully used to study catalase in intact peroxisomes [31,43].

***Protocol 6*. End point determination of H_2O_2 using an injection system**

In this procedure, luminol is premixed with the sample (e.g. culture medium or perfusate sample). At the appropriate time, NaOCl is added and the luminescence intensity is measured immediately within the next 2 seconds. The measurement is fast and only a small sample volume is needed. Theoretically, luminol can be added just before the NaOCl injection to avoid any interferences of sample and detection system. So far, no such interferences have been observed by us, and premixing of luminol with the samples is the usual way for practical reasons. An injection device in measuring position is a requisite condition because the luminescence reaction reaches completion within less than 2 s.

Equipment

- luminometer with injection device in measuring position required (e.g. AutoLumat LB 953 from Berthold EG&G, Wildbad, Germany or Fluostar from BMG Labtechnologies GmbH, Offenburg, Germany). Other injection devices are helpful for complete automatization of the experiment e.g. addition of cell stimulators.
- polystyrene tubes or 96-well microplate for luminometer

Reagents

- stock solution of 10^{-3} mol/l luminol in 10 mmol/l PBS at pH 7. 4 (final concentration of luminol between 10^{-5} – 10^{-4} mol/l)
- stock solution of 10^{-4} mol/l NaOCl in tridistilled water (final concentration of NaOCl between 10^{-6} – 10^{-5} mol/l)
- 10^{-3} mol/l H_2O_2 in tridistilled water for calibration

Procedure

The injector in measuring position is loaded with NaOCl solution and washed. For optimal measuring range, samples with PBS and luminol are loaded containing catalase (e.g. 1:100.000 of stock) and 5 µmol/l H_2O_2. If necessary, the NaOCl concentration needs to be adjusted. In a typical experiment using a final volume of 1000 µl, the injection device adds 50 µl of NaOCl (10^{-6} - 10^{-5} mol/l final concentration) into 950 µl sample with luminol ($5x10^{-5}$ mol/l final concentration). Usually, samples are measured together with an H_2O_2 calibration solution at the beginning and the end of the batch. For a typical microplate luminometer, each well should contain a minimum of 100 µl volume prior to the injection of 20-50 µl NaOCl. A minimum volume is required to allow complete mixture of NaOCl with the luminol solution.

***Protocol 7*: Determination of GOX activity**

Equipment

- microplate luminometer with injection device in measuring position (Fluostar from BMG Labtechnologies GmbH, Offenburg, Germany).
- 96-well microplate for luminometer

Reagents

- stock solution of 10^{-3} mol/l luminol in 10 mM PBS at pH 7. 4 (final concentration of luminol between 10^{-5} – 10^{-4} mol/l)
- stock solution of 10^{-4} mol/l NaOCl in tridistilled water (final concentration of NaOCl between 10^{-6} – 10^{-5} mol/l)
- 10^{-3} mol/l H_2O_2 in tridistilled water for calibration

Procedure

The enzyme rate of GOX can be determined from the accumulation of H_2O_2 over time. For the assay, 100 µl of double concentrated luminol plus 50 µl of four-fold concentrated GOX solution is plated in sequential wells of a 96-well plate. Final GOX concentrations might range from 1:50,000 to 1: 1 million. The enzyme reaction is started by adding 50 µl of four-fold concentrated glucose solution. Final glucose concentration should be between 5 and 25 mmol/l. The addition of glucose can either be done by hand using a multitip pipettor or by injecting it with an automated injector. The first way has the advantage of starting the reactions at the same time in all wells, in which H_2O_2 levels can then sequentially be measured by the injection of 50 µl NaOCl. It has the disadvantage that it will take some time for the measurements to start (injection, closing of door, start of program). The second way has the advantage of being more accurate since the enzyme reaction is started by computer-driven injection of substrate instead of manual injection. However, this method requires a luminometer with two injectors and the possibility to adjust the time interval between the injections of glucose and NaOCl. In either case, K_{GOX} can be determined by the accumulation of H_2O_2 over time, and GOX kinetic diagrams such as Lineweaver Burk plots can be created by plotting initial enzyme rates versus the glucose substrate concentration.

***Protocol 8*. Measurement of H_2O_2 steady state concentration by GOX/CAT**

Equipment and Reagents
- as described in Protocol 7

Procedure
Steady state H_2O_2 levels are determined in a similar way as described above for the determination of K_{GOX}, except that in this case, GOX and CAT will be added to the buffered luminol/glucose solution. Briefly, 100 µl of double concentrated luminol, 25 µl of 8-fold concentrated GOX, and 25 µl of 8-fold concentrated CAT, will be pipetted in multiple wells of a 96-well microtiter plate. The GOX and CAT concentrations are varied to achieve different $[H_2O_2]ss$ concentrations as explained in section 3 and exemplarily shown in Table 1. The reaction will be started by addition of 50 µl of 4-fold concentrated glucose. The establishment and the maintenance of the $[H_2O_2]ss$ can be measured for the next minutes to hours by adding 50 µl of NaOCl to each well at the time of measurement and quantifying the resulting luminescence within the next 2 seconds. Luminescence of given amounts of H_2O_2 in the same solutions (e.g. the same buffer, medium, cells, etc.) but without GOX and CAT can be used for calibration.

1. The injector in measuring position is loaded with NaOCl solution and washed. The GOX/CAT system is started in a typical setting e.g. GOX 1:100.000 and CAT 1: 20.000 that should lead to a H_2O_2 ss of ca. 1 µmol/l H_2O_2 in the presence of 5 mmol/l glucose.
2. H_2O_2 measurements are performed by injecting NaOCl to the GOX/CAT solution mixed with luminol.
3. 5 µmol/l H_2O_2 solutions in the same luminol-PBS buffer are used for calibration.

B2. General comments on troubleshooting, instrumentation and data processing of H_2O_2 determination by luminol-hypochlorite

The luminol/hypochlorite assay is very sensitive and detects H_2O_2 at concentrations as low as 10^{-9} mol/l. Under optimal conditions, even smaller H_2O_2 concentrations can be determined. The assay is linear up to 5-10 µmol/l H_2O_2.

It should be noted that normal aqueous solutions already contain traces of H_2O_2 due to light irradiation mostly by UV light, sometimes as high as up to 10^{-7} mol/l. This is one of the reasons to calibrate the assay with at least 10^{-6} mol/l H_2O_2 or to add catalase to check for H_2O_2 impurities. The samples should be kept in the dark due to the same reason-that is to prevent UV light exposure. Any unspecific chemiluminescence can be detected upon H_2O_2 removal with catalase. The sample volume should be at least 10 times higher than the volume of reagents, because small contamination of reagents with H_2O_2 can significantly decrease the sensitivity of the assay.

NaOCl working solutions should be prepared with tridistilled water to minimize its degradation and they should be freshly prepared and kept in the dark. For each system, the optimal NaOCl concentration should be identified separately. Too high NaOCl concentrations favor unspecific oxidation reactions of luminol. Therefore, NaOCl concentration should be chosen as low as possible (usually 10^{-6}-10^{-5} mol/l). Any compound containing e. g. sulfhydryl- or amino groups will compete with luminol for NaOCl leading to a decrease in sensitivity. In these cases, samples are diluted (e.g. whole blood 1: 100 to 1: 10000) and/or higher concentrations of NaOCl should be used.

pH is critical for the reaction and should be kept stable at a value of 7.4 with any appropriate buffer. No luminescence develops at values below 6.5. The chemiluminescence duration increases with pH above 7.4.

Troubleshooting should include: 1) correct concentrations of reactants 2) proper installation of the flow cell in front of the photomultiplier 3) proper injection of NaOCl in front of the photomultiplier 4) low content of NaOCl-reactive compounds (e.g. solutions of sulfhydryl group-containing proteins should be diluted at least to less than 10^{-6} mol/l) 5) stability of NaOCl solutions. Some injection devices contain metals that may rapidly degrade NaOCl.

The luminol/hypochlorite assay is calibrated with known concentrations of H_2O_2 usually between 10^{-6} – 10^{-5} mol/l taken from stock solutions. Commercial stock solutions of 30% H_2O_2 are stable for many months once kept at 4° C and in the dark. Commercial stock solutions of NaOCl are also stable at 4°C and in the dark. Stock solutions of NaOCl and H_2O_2 can be determined spectrophotometrically at ε_{290} = 350 $M^{-1}cm^{-1}$ at (pH 12) and ε_{230} = 74 $M^{-1}cm^{-1}$, respectively [56,57]. A routine calibration for established conditions requires at least one sample with a known H_2O_2 concentration and a sample without H_2O_2 upon removal by 1 nM catalase. These measurements provide the available measuring range. If necessary, the hypochlorite/luminol assay allows the subtraction of the unspecific luminol-dependent chemiluminescence. It simply needs to be measured prior to NaOCl addition and subtracted from the overall luminescence intensity. The same is valid for any non-H_2O_2-related luminescence which is detected after addition of catalase.

The appropriate instrumentation takes some special considerations. The injection modus requires a luminometer with an injection device in front of the photomultiplier. The flow modus requires a flow cell that is made of inert material to avoid radical reactions in the presence of hypochlorite of H_2O_2. The flow modus should go together with an automated data collection.