

Technique

Chemiluminescence technique for measuring reactive oxygen species



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Abstract

Accurate assessment of reactive oxygen species (ROS) concentrations may help in the diagnosis of infertility. The chemiluminescence technique, which uses a luminometer to measure ROS, is a common method of assessment. A better understanding of the chemiluminescence technique will allow its proper application in reproductive medicine. A wide range of luminometers are available in the market, and laboratories should select the instruments that suit their individual needs.

Keywords: chemiluminescence, infertility, luminometer, oxidative stress, reactive oxygen species, reproduction

Oxidative stress (OS) occurs when concentrations of reactive oxygen species (ROS) overwhelm the body's antioxidant defence system. Recently, OS has become a real concern in the practice of reproductive medicine because ROS are potentially toxic to spermatozoa, oocytes and embryos. It is important to evaluate the OS status in the male and female reproductive tract, especially because the results have diagnostic and prognostic value in the management of infertility (Agarwal and Saleh, 2002; Agarwal *et al.*, 2003; Aitken *et al.*, 2003, 2004; Agarwal and Allamaneni, 2004).

There are many methods for measuring free radical production in cells. Electron spin resonance, cytochrome *c* reduction, ferrous oxidation of xylenol orange and fluorescent probes such as 2',7'-dichlorofluorescein-diacetate (DCFH-DA) and dihydroethidine (HE) have all been used successfully to detect ROS generation (Curtin *et al.*, 2002). All assays are comparable in terms of technical difficulty and time consumption.

Flow cytometry can be used for the detection of ROS with fluorescent probes. Although accurate, the technique involves the use of a flow cytometer, which is a rather expensive instrument. Moreover, no current studies are available on the standardization of the assay in human spermatozoa. Fluorescent probes can also

be assessed using simple microscopy (Henkel *et al.*, 2003), which is a simple inexpensive procedure. However, relatively small numbers of cells (~200 spermatozoa) are assessed in each sample.

Because human gametes generally produce very low concentrations of ROS, the techniques used to detect ROS production and diagnose OS must be sensitive. One of the most commonly used methods for measuring concentrations of ROS and antioxidants is the chemiluminescence assay (Sharma and Agarwal, 1996; Kobayashi *et al.*, 2001). This assay measures the oxidative end products produced by an in-vitro reaction between ROS and certain reagents. The reaction causes light to be emitted, which is measured with a luminometer. The results can be affected by many variables. Thus, analysts should understand how their luminometer works and the key experimental variables that affect luminescence measurements.

A variety of luminometers can be used to measure the light intensity resulting from the chemiluminescence reaction. Although all luminometers utilize photomultiplier tubes to detect photons, they differ in the processing of signal input. Two different processing designs are presently found in luminometers. Photon counting luminometers count individual

photons, whereas direct current luminometers measure electric current that is maintained by, and is proportional to, the photon flux passing through the photomultiplier tube. The results are expressed as either relative light units (RLU), counted photons per minute (cpm) or mV/s.

The many models of luminometers that are available differ in price, design and features (Stanley, 1999). When comparing models, check their coefficient of variation and the lower limit of detection. The coefficient of variation is the percent change over a set of readings. It provides information on whether the measurements are repeatable and whether all other variables of the assay are kept constant. The lower limit of detection is the minimum sample quantity that generates an instrument response above the blank noise.

Three types of luminometers are commercially available. Single/double tube luminometers are inexpensive and can measure only one or two samples at a given time. These are suitable for small research laboratories. Multiple tube luminometers are more expensive because they can measure multiple samples at one time. These are suitable for centres that are engaged in regular research work on chemiluminescence. Plate luminometers can analyse multiple samples on a single plate. Each plate is disposable and costs approximately \$5. However, the entire plate must be disposed of, even when measuring luminescence for a single sample. These luminometers are therefore more suitable for commercial entities and core research laboratories.

New luminometers are being designed to lower their cost while improving their reliability and ease of use (Porakishvili *et al.*, 2000). **Table 1** contains information on selected luminometers that are available. The Centre for Advanced Research in Human Reproduction currently uses the LB 953 luminometer (Berthold Technologies, Bad-Wildbad, Germany), which is a photon-counting instrument that covers a spectral range from 390 to 620 nm. The automatic sample changer can analyse up to 180 samples, which allows single or

repeated runs of the same samples. On the other hand, the Flash'n Glow LB 955 model, which is manufactured by the same company, consists of a flexible carrier chain for 30 samples.

Multiple factors affect chemiluminescent reactions (Berthold *et al.*, 2000). These include the concentration of reactants, sample volume, reagent injection, temperature control, and background luminescence. The person who operates these instruments should be familiar with these factors, which will enable him/her to obtain consistently accurate results.

The concentration of the reactants (sample and probe) affects the amount of luminescence that is emitted. When the reagent concentration is fixed, luminescence will only vary according to the concentration of the ROS. It is also important to maintain a constant volume of the sample and reagent. More importantly, the volumes and concentrations used should be kept consistent between measurements. Most luminometers offer an optional feature that automatically adds the reagents to the analysed samples. However, for the sake of cost and simplicity, many laboratories prefer to manually add the reagents. In the latter case, strict quality control measures must be followed.

Quality control characteristics of the chemiluminescence assay for measuring ROS in human semen have recently been described, using luminol as a reagent, and the effect of sperm concentration and time of measurement on ROS concentrations has been reported. ROS measurement is both accurate and reliable when the sperm concentration is greater than $1 \times 10^6/\text{ml}$ and the samples are analysed within the first hour after specimen collection (Kobayashi *et al.*, 2001).

The type of reagent(s) used for ROS measurement is critical. Using luminol as a probe has many advantages: (i) luminol is a sensitive chemiluminescent probe that reacts with a variety of free radicals, including superoxide anion,

Table 1. Some commercially available luminometer models, price and manufacturers. ATP = adenosine 5'-triphosphate.

Model	Type	Sensitivity and dynamic range	Price (US \$)	Manufacturer
TD 20/20 ^a	Single tube	0.1 fg luciferase, >5 orders ^b	5250	Turner Biosystems Inc., Sunnyvale, CA, USA
FB-12 ^a	Single tube	1000 molecules of luciferase, 6 orders	5350	Zyflux Corporation, Oak Ridge, TN, USA
Traithler	Single tube	1–10 pg ATP, 7 orders	6000	Bioscan, Washington, D.C., USA
Zyflux FB15 ^a	Single tube	1000 molecules of luciferase, >6 orders	7450	Bio-World, Dublin, OH, USA
Optocomp-2 ^a	Multiple tube	0.1 pg ATP, 6 orders	14,160	MGM Instruments, Inc., Hamden, CT, USA
Autolumat LB 953 ^a	Multiple tube	5 amol of ATP, 6 orders	18,000	Berthold Technologies, Oak Ridge, TN, USA
MicroLumi XS ^a	Microplate	0.1 fg luciferase, >6 orders	9000	Harta Instruments, Gaithersburg, MD, USA
Luminoskan ^a	Microplate	<0.5 fmol ATP, 6 orders	20,000	GMI, Inc., Albertville, MN, USA

^aInstrument offers the option of direct data transfer to personal computer.

^bInstrument has an ability to measure in a range of $1-10^6$ counts without saturation. Beyond this range the instrument has a linear response to the signal input.

hydroxyl radical, and hydrogen peroxide; (ii) luminol measures both intracellular and extracellular free radicals; (iii) the reaction is fast with a very short half-life, suggesting that the time between the addition of the probe and the measurement is very rapid. However, luminol is unable to differentiate between different types of ROS and between intra- and extracellular ROS. Other probes include lucigenin, which yields a chemiluminescence that is more specific for extracellular superoxide anion (Aitken *et al.*, 1992; McKinney *et al.*, 1996).

Chemiluminescence generates a homogeneous population of molecules in the excited state, so the emitted light intensity varies as a function of time (as reaction materials are consumed). Thus, there are two types of systems for measuring the concentration of the substance under analysis. The first system measures the chemiluminescence intensity at a predetermined time after the reaction has started. The second system performs time integration by measuring the luminescence signal within a predetermined interval. The second approach is usually preferred because it is not as prone to variability in time during mixing of the reagents.

The background luminescence is only an approximate estimate of the constant light level, as various factors such as impurities in the reagents and surrounding equipment can affect the results. This is the reason why blank (no sample and reagent) and control aliquots should be assessed along with the test sample in chemiluminescence measurements. The blank reading provides the background luminescence of the instrument whereas the control value (with reagent, without sample in the test tube) shows the background luminescence plus any luminescence caused by the presence of impurities in the reagents. With pure reagents, the control reading is expected to equal the blank values. The control reading should be subtracted from the test reading to calculate the true reading of the sample.

In summary, accurate assessment of OS concentrations in secretions from the reproductive tract (semen, follicular and peritoneal fluid, culture media) may help diagnose OS-induced infertility. Laboratories should select the instrument that suits their individual needs. In addition, the analyst should understand the key experimental variables that can affect luminescence measurements and the importance of standardizing them. Strict quality control measures will help users obtain reliable results.

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Received 9 July 2004; refereed 16 July 2004; accepted 21 July 2004.