

MAXWELL SENSORS INC.

Application Notes

LuminMax-C Luminometer

(Attomoles Luminescence Detector)

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1. Background of Chemiluminescence and Bioluminescence

Light-emitting chemical reactions (Chemiluminescence) and light-emitting biological reactions (bioluminescence), which generate signal (light) through chemical reactions, have a diverse range of analytical applications. They are distinct from fluorescence or phosphorescence, which need an external light source to excite the material in order to generate light in other wavelengths. The most common bioluminescence phenomenon is the glowing cold light produced in firefly tails. The generated light is the end product of a chemical reaction, the conversion of the substrate luciferin to oxyluciferin and light, catalyzed by the enzyme luciferase. In this case (see equation), the reaction is driven by the dephosphorylation of adenosine triphosphate (ATP). The light generated by this reaction can be measured directly with a luminometer.

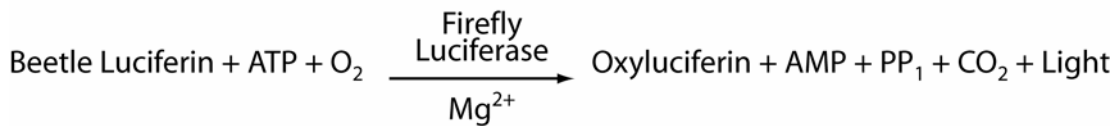
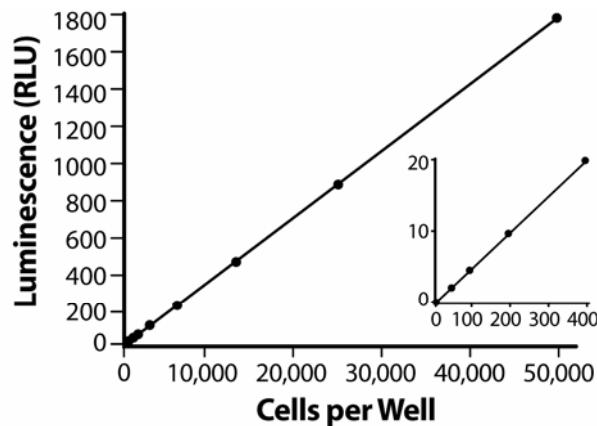


Fig. 1. It shows the luminescence signal as a function of cell (ATP) concentration, RLU (relative light unit).

In order to generate light, all the chemical components in the equation are necessary. However, three chemicals are particularly interest:

1. **Substrate:** Luciferin
2. **Enzyme:** Luciferase
3. **ATP**

The amount of light (measured in photons) produced by the reaction is linearly proportional to the concentration of one of these chemicals, if we keep others in excess. Therefore, we can quantify substrate, enzyme, or ATP by measuring the amount of light generated. For example, **the light intensity is linear to the concentration of ATP, if all others are in excess (Fig. 1)**. The above figure shows that there is a linear relationship between the luminescent signal and the number of cells (ATP in cell) from 0 to 50,000 cells. This experiment has been used in cell viability tests, restaurant sanitary tests, bacterial decontamination tests, and others. Chemiluminescence is also used for detecting **superoxide (O_2^-)** and **NADPH oxidase** in cell culture. The O_2^- in the presence of substrate (luminol) and peroxidase generates light for quantitative measurement.

2. The Basic of Chemiluminescence Detection

In many **protein and nucleic acid experiments**, because no cell is involved, ATP is not required. The two important chemicals are **enzymes** and **substrates**. If we put enzymes and substrates together, they generate light. Enzymes can catalyze the substrates, which leads to the breakdown of the substrate and produces light (see Equation & Fig. 2). Therefore, **the intensity of the light is linear to the concentration of enzyme**. There are many enzymes and substrates commercially available. For example:

Enzymes: Luciferase
Alkaline phosphatase (AP)
Horseradish peroxidase (HRP)
Galactosidase, etc.

Substrates: Luciferin
Acridinium ester
Luminol
Isoluminol
Lumi-PPD
Lumigen-PS-atto, Lumigen TMA-6, etc

Substrate + Enzyme + H₂O₂ solution --> Intermediate compound (from substrate)

-> Light + final product

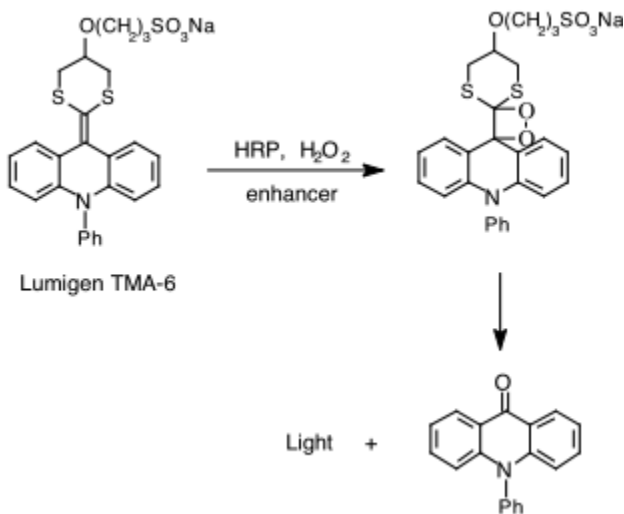


Fig. 2: Lumigen TMA-6 substrate

Many companies provide kits with enzyme, substrate, and solution plus all other necessary chemicals. For example, Promega offers Dual Luciferase Reporter Assay (DLR[™]) and GLO[™], and Stratagene kits for easy cell assay. Pierce, KPL, Lumigen, etc. offers different chemiluminescence substrates.

Different enzyme-substrate pairs generate light with different optical properties (color, response time, longevity (see Fig. 3 & 4), and intensity). Typically,

- the colors are in the visible range (red, green, blue),
- the response times start at a few seconds and last for few hours

Light sticks commonly sold at concerts and amusement parks are based on chemiluminescence. They generate light almost instantaneously and last for 3-6 hours. The common enzymes used are luciferase, alkaline phosphatase (ALP), and horseradish peroxidase (HRP). Companies are continuing to develop new substrates.

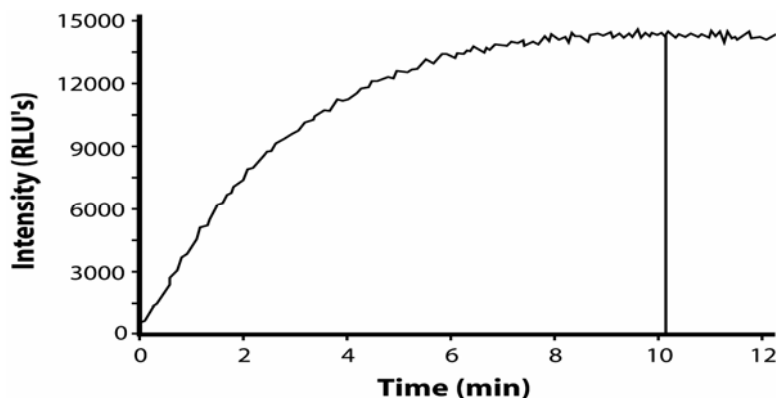


Fig. 3 Kinetic profile of a chemiluminescent substrate - 140 attomoles alkaline phosphatase added to 40 μ l of substrate (Lumi-PPD) solution.

The chemiluminescence substrate in combination with amplification enzymes (ALP or HRP) offers very high detection sensitivity. Figure 4(a) shows an example of the very rapid onset of chemiluminescence: stable peak intensity, reached in seconds, and excellent sensitivity with **less than 10^{-19} moles HRP** (Fig. 4(b)). Sustained luminescence makes it possible to read a signal at any time in order to produce linear calibration curves.

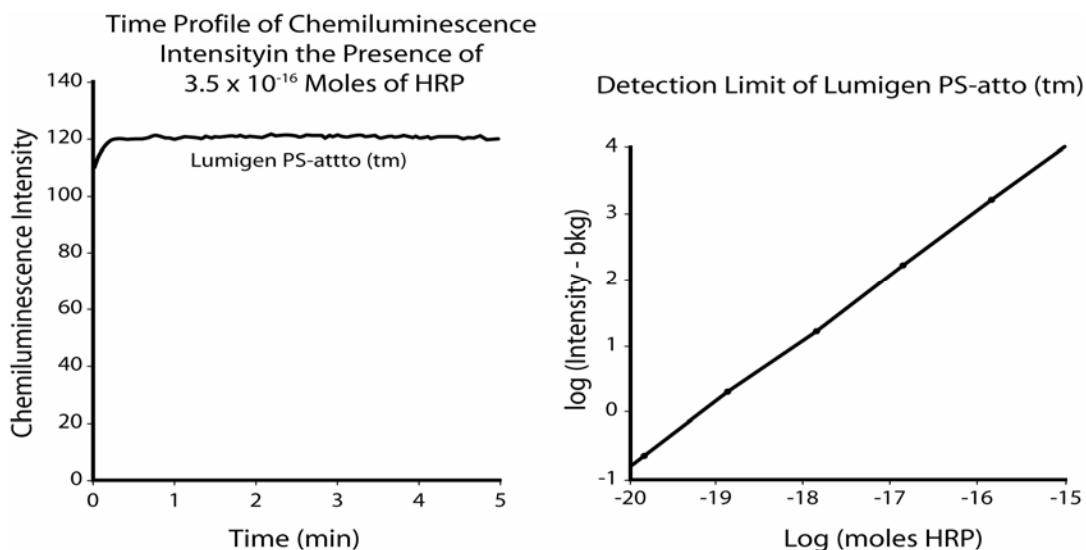


Figure 4. (a) The time profile and (b) detection limit of PS-atto for rapid (< 30 seconds) peroxidase detection. Reaction of the substrate with an HRP label generates sustained high-intensity.

3. Chemiluminescence for Immunoassay & Nucleic Acid Assay

We will now discuss the use of chemiluminescence in immunoassay (protein) and nucleic acid assays.

We know the intensity of the light from chemiluminescence is linearly proportional to the concentration of an enzyme. **Fortunately enzymes such as alkaline phosphatase (ALP), or horseradish peroxidase (HRP), can be coupled (or bound) to protein (antibody, antigen, toxin, bacteria, etc.), or nucleic acid (DNA, RNA, cDNA, etc) molecules by simple reactions.** So once the enzyme is bound to protein or nucleic acid, then **the light intensity is proportional to the amount of protein or nucleic acid.** Based on this method, we can quantify the number of proteins or nucleic acids in a solution.

Immunoassay

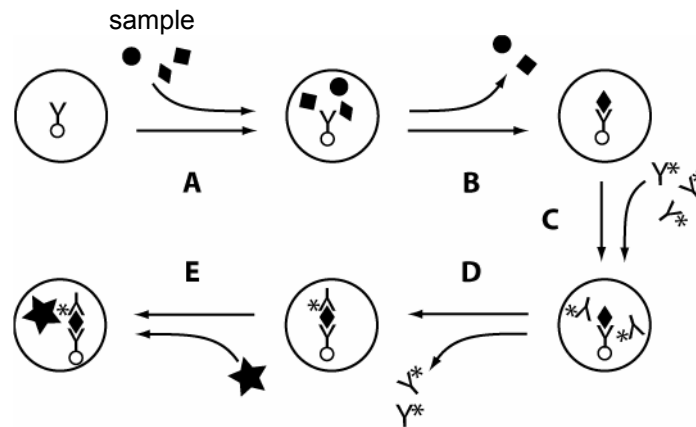


Fig. 5. The schematic shows the common immunoassay sandwiched assay protocol for detecting antigen (antigen is in the sample). Here Y is the antibody, \blacklozenge is the antigen, and * is the enzyme, **Y* enzyme labeled antibody**

For example:

- Coating of capture antibody: Pipette 0.1ml of 1-10ug/ml capture antibody to each well of a multiwell plate. Incubate the plate for 2 hours at 37°C. Remove the coating antibody solution. Wash the plate 3 times with washing buffer.
- Addition of standards, controls and samples: Add 0.1ml of standards, controls and samples into each of the wells. Incubate the plate at room temperature for 1 hour. Remove the standard, control or sample solution. Wash the plate 3 times with washing buffer.
- Addition of detection antibody: Pipette 0.1ml of diluted enzyme (HRP or ALP) labeled detection antibody (Y*) to each well. Incubate the plate at room temperature for 30 minutes. Remove the detection antibody. Wash the plate 3 times with washing buffer to remove unbound detection antibody.
- Addition of Substrate: Add appropriate chemiluminescence substrate solution.
- Count: Immediately read count on the **LuminMax-C Luminometer**.

The microwell will glow only when the sandwich immunocomplex ((e.g. Ab-Ag-Ab*), positive identification) is formed and generates **bright twinkling**

luminescence for highly sensitive detection. The light intensity is proportional to the amount of enzyme (*), and also the amount of antigen (♦).

Reference:

[1]. OS Khalil, TF Zurek, J Tryba, CF Hanna, R Hollar, C Pepe, K Genger, C Brentz, B Murphy, and N Abunimeh
 Abbott prism: a multichannel heterogeneous chemiluminescence immunoassay analyzer
Clin. Chem., Sep 1991; 37: 1540 - 1547.

[2]. D. Robert Dufour, Mageli Talastas, Maria D.A. Fernandez, and Barbara Harris
 Chemiluminescence Assay Improves Specificity of Hepatitis C Antibody Detection
Clin. Chem., Jun 2003; 49: 940 - 944.

Nucleic acid assay

Similarly, nucleic acid assay depends on the pairing of the A, T, G, C sequence. The complex will form if the two nucleic acid chains are complementary to each other. Enzymes can be coupled to one of the DNA chains for detection. Figure 4 illustrates DNA hybridization assays using the target-attached beads. This chemistry is used to attach a 30 nucleotide long target oligo to the beads or coat the nucleotide on the bottom of the microtiter plate. The target nucleotides are subsequently hybridized in solution to a complementary 30-mer oligo **probe, which carried a covalently bound alkaline phosphatase** at its 5' end. By adding the substrate, the enzyme will react with substrate resulting in chemiluminescence.

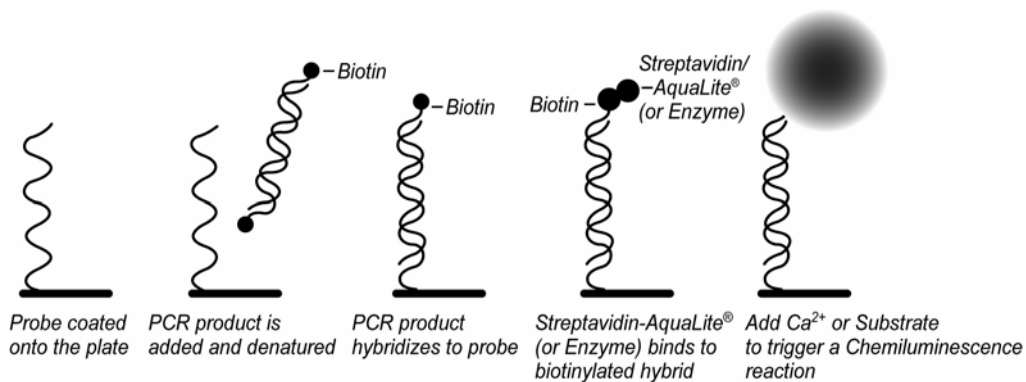


Fig. 6. One of the protocols for nucleic acid detection based on chemiluminescence (Chemicon, Temecula, CA).

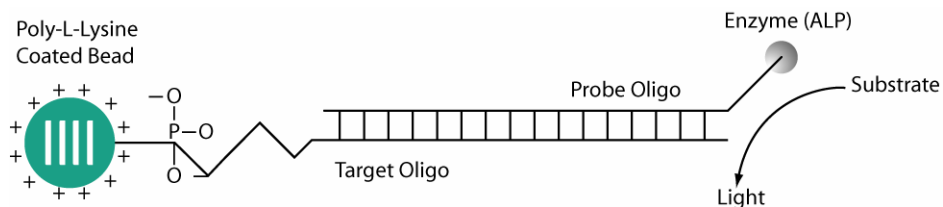


Fig. 7. Polymer bead or magnetic bead-based DNA hybridization assay with a label ALP for chemiluminescence detection. Once the substrate is added, the reaction produces luminescence.

In recent years, the use of luminescence in nucleic acid assays has been expanded from initial qualitative measurements with gel-based systems to solution-based quantitative measurements. LuminMax-C can be used with all commercially available and well-established chemical and biological luminescence reagent systems, suitable for the 96-flat bottom well format. These reagent systems include the QuantiGene[®] Reagent System (Genospectra, Fremont, CA) for quantitating RNA levels in biological samples, the bioluminescent ChemFlash[®] platform (Chemicon, Temecula, CA) for the detection of mRNA expression levels in biological samples and in vitro amplified nucleic acids, and the Dual-Luciferase[®] Reporter Assay System (Promega, Madison, WI) for quantifying gene expression in mammalian cells.

The ChemFlash Platform from Chemicon is described below.

ChemFlash[®] Platform

Amplification reactions are performed using biotinylated primer sets (custom design and synthesis available through Chemicon), and the amplified product is immobilized to the streptavidin-coated capture plate and hybridized to a specific detection probe. Chemicon's XpressPack[®] assays are ideal for the rapid detection of mRNA expression. The 96-well format of these assays offers convenience and increased throughput and is intended to replace more conventional and cumbersome methods of detection, especially Northern blot analysis and RNase protection assays. The primers provided in the XpressPack assays have been specifically designed across splice junctions to exclude genomic DNA amplification and allow for amplification of mRNA targets. The detection probe is designed internal to the primers and is irreversibly coupled to the surface of the plate. The assay utilizes the bioluminescent photoprotein AquaLite[®] (<http://www.chemicon.com/Resource/newsletters/ns9-4.pdf>) (see P.14)

Reference:

[1] Soumet, C. Chemiluminescent and Colorimetric Enzymatic Assays for the Detection of PCR- Amplified Salmonella sp. Products in Microplates *BioTechniques*, 19, No.5, 792-796 (1995).

[2] Fabiana Fini, Giorgio Gallinella, Stefano Girotti, Marialuisa Zerbini, and Monica Musiani Development of a Chemiluminescence Competitive PCR for the Detection and Quantification of Parvovirus B19 DNA Using a Microplate Luminometer *Clin. Chem.*, Sep 1999; 45: 1391 - 1396.

LuminMax-C : Attomoles Luminometer

Detecting Proteins and Nucleic Acids with Ultra-Sensitivity

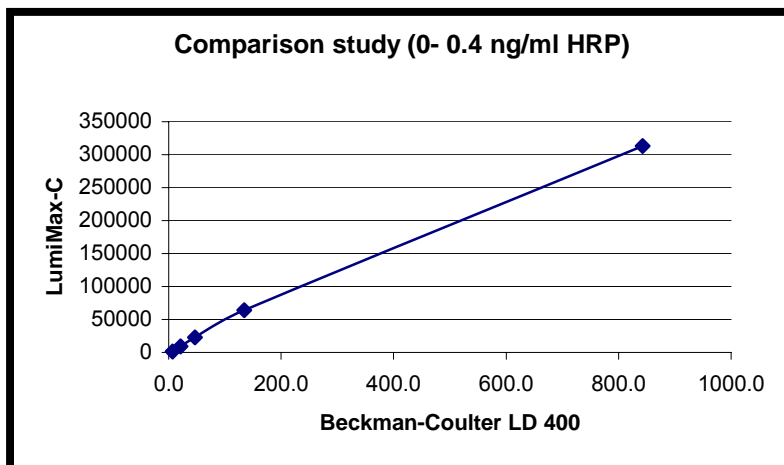
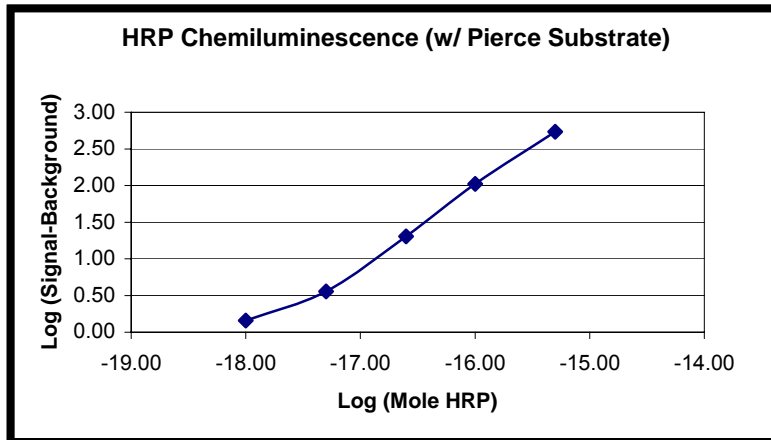
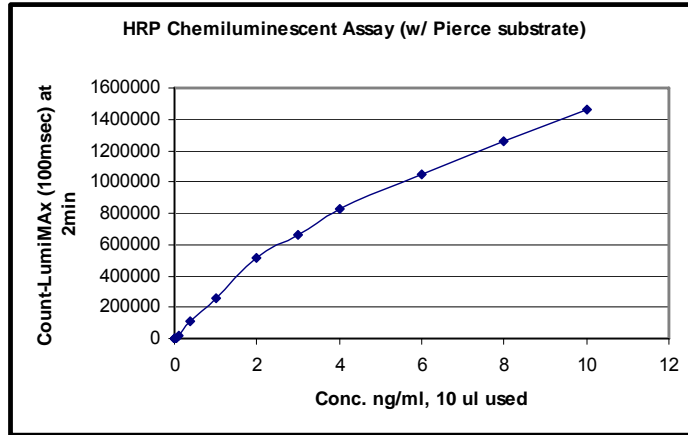
By Maxwell Sensors Inc., <http://www.maxwellsensors.com>

LuminMax-C is a compact model designed for ultra-sensitive chemiluminescence and bioluminescence detection. The system measures luminescence intensity in a 96-well microplate (black, white, or opaque). These microplates have microwells with clear bottoms, allowing the luminescence to be detected from the bottom of the well. Many companies, such as Nunc, Costar, Corning, BD Biosciences, Perkin Elmer, etc., have microplates for luminescence applications. Because LuminMax-C uses a state-of-the-art photoncounting multiplier tube as the detector, the system is extremely light sensitive. LuminMax-C has the ability to accurately count the number of photons generated from a chemiluminescent or bioluminescent experiment, allowing the system to detect very small amounts of analyte, in the range of attomoles, in the samples. The detector is sensitive in all visible ranges.

LuminMax-C combines the latest, low-noise detector and electronic circuitry to provide very low background noise and very high signal levels. Its broad dynamic range covers over 6 orders of magnitude. The superior mechanical design eliminates most of the cross-talk down to $\sim 10^{-5}$ between the adjacent wells. The system is flexible and easy to use. The user can select to scan any one or any set of the 96 wells, at a measuring time of 0.1-10 seconds per well. The system can also be used for kinetic and luminescence time profile studies. After clicking on the "Go" button, the system will automatically and quickly scan all of the reactions in the microwells and display the results.

LuminMax-C utilizes a Windows-based PC or notebook as its microprocessor. The system is interfaced to a computer by a simple plug-in (serial port or USB) connection. A CD, with user-friendly software, is provided for easy installation. The resulting data is displayed as a spreadsheet in Microsoft-Excel or other format. The data, reported as number of optical counts, is displayed as it is collected. The system also offers software with easy click on data processing for routine calibration or operation. The system dimensions are approximately 16" x 11" x 4" and at 20 lb, it is easily portable for sharing between laboratories or researchers.

The following four figures show the LuminMax's sensitivity and comparison studies:



HRP Chemiluminescent Assay (Different Substrates)

