

An Introduction to Chemiluminescence and Bioluminescence Measurements

1. Chemiluminescence and Bioluminescence

Chemi- and bioluminescence measurements have become extremely popular in recent years. They are often used to determine the amount of a specific unknown present in a sample, and in the last decade, have become extremely important in the study of gene expression and gene regulation. Chemiluminescence is the light emitted by a chemical reaction and bioluminescence is a type of chemiluminescence in which the chemical reaction is catalyzed by an enzyme.

Measurement of light from a chemical reaction is highly useful because the concentration of an unknown can be inferred from the rate at which light is emitted. The rate of light output is directly related to the amount of light emitted and accordingly, proportional to the concentration of the luminescent material present. Therefore, light measurement is a relative indicator of the amount of luminescent material present in the sample of interest.

2. Advantages of Luminescence Measurement

Luminometry, the analytical technique used to measure chemi- and bioluminescent reactions, has several advantages over other analytical techniques. Extraordinary sensitivity; a wide dynamic range; inexpensive instrumentation; and the emergence of novel luminescent assays make this technique very popular.

Superior sensitivity and low background distinguish luminometry from other analytical methods. Luminometry is up to 100,000 times more sensitive than absorption spectroscopy and is at least 1,000 times more sensitive than fluorometry. A well-designed luminometer can detect as little as 0.6 picograms of adenosine triphosphate (ATP) or 0.1 femtograms of luciferase, two common luminescent analytes. The ATP/luciferin-luciferase reaction and its lower limit of detection are shown in Figures 1a and 1b.

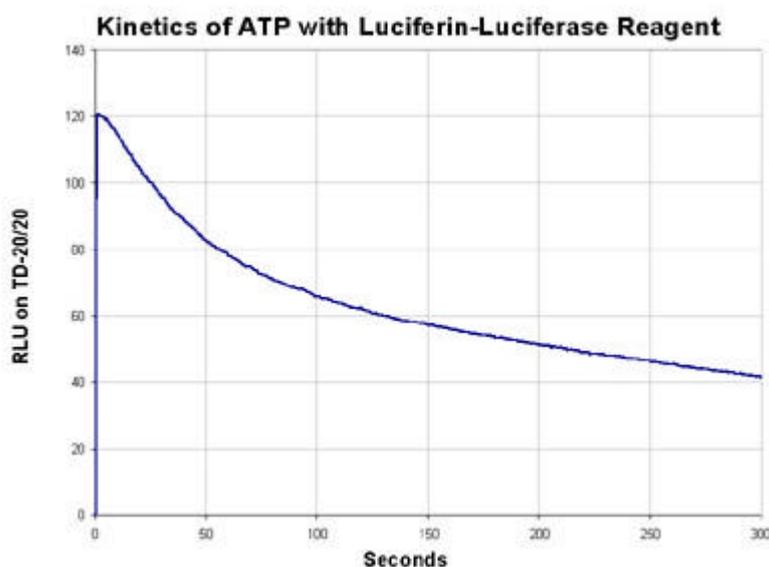


Figure 1a.

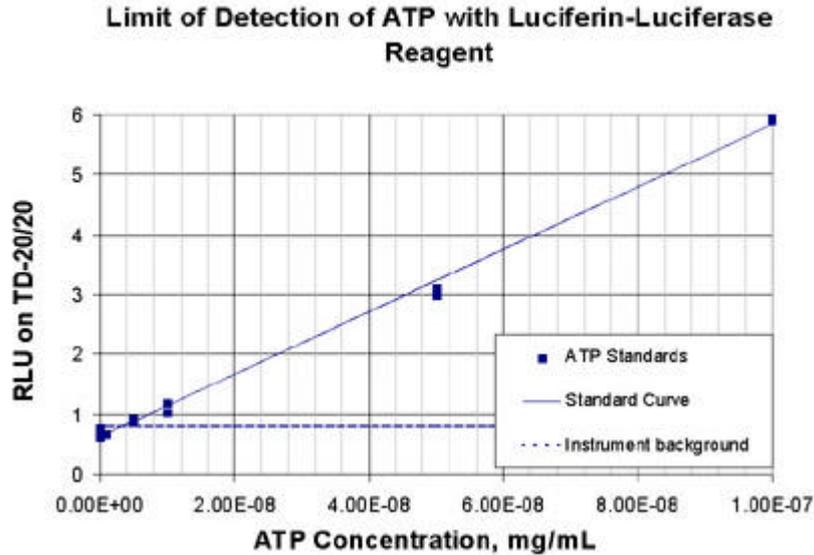


Figure 1b.

In luminescence, there are two components of light that reach the detector. The first component is proportional to the concentration of the limiting reactant in the chemiluminescent reaction. The second component, known as background, is an approximately constant light level due to various factors such as the phosphorescence of plastics, impurities in the reagents, etc.. This background light component is much lower in luminometry than in other analytical techniques such as spectrophotometry and fluorometry.

Wide dynamic range and low instrument cost are also distinct advantages of luminometry. Samples can be measured across decades of concentration without dilution or modification of the sample cell. Figure 2 covers over 5 decades of luciferase measurement using Turner Designs' TD-20/20 Luminometer.

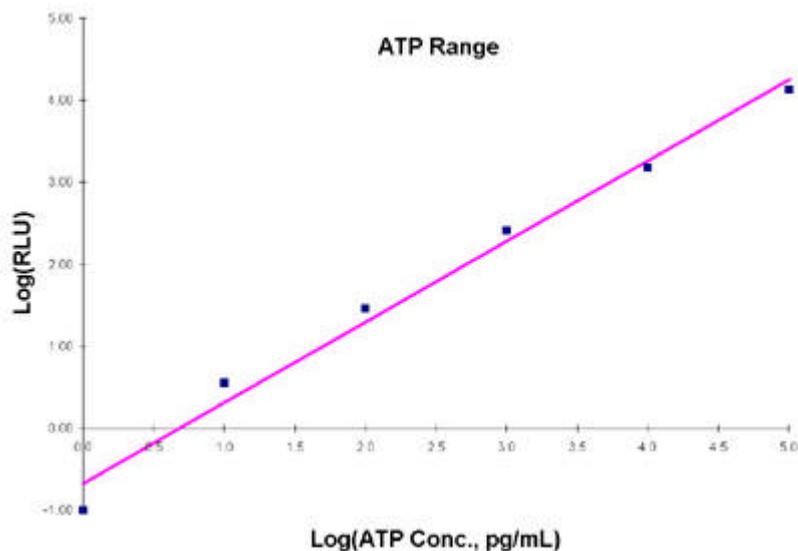


Figure 2.

On a final note, the use of luminometry in research and quality control has increased dramatically in recent years. Advances in molecular biology and an increased interest in ever-more-sensitive assays have led to the development of novel luminescence systems for a wide variety of applications in genetic research, food technology, and environmental monitoring.

3. Instrumentation

Light, as we see it, consists of billions of tiny packets of energy called photons. Photons, packets of light, emitted from bioluminescent and chemiluminescent reactions are typically measured using a luminometer. Luminometers are simple, relatively inexpensive instruments designed to measure sample light output. Light output is measured by integrating, or measuring the area under the chemical reaction's light emission curve for a set period of time. All luminometers consist of a sample chamber, detector, signal processing method, and signal output display. Figure 3 shows a typical luminometer.

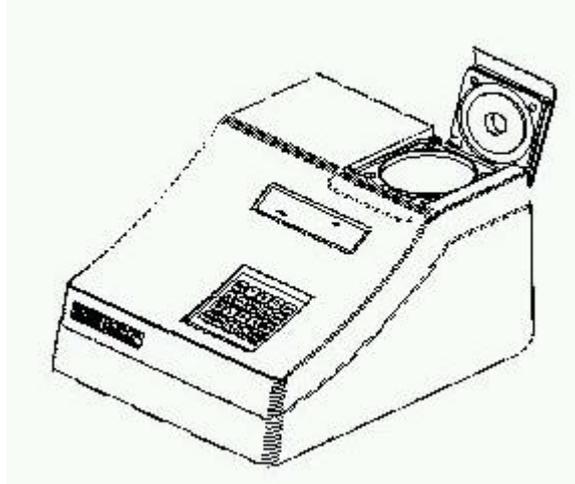


Figure 3.

3.1 Sample Chamber

The luminometer sample chamber, which holds a test tube, microplate, or other type of sample container, presents the luminescent sample to the detector. The chamber must be sealed from ambient light in order to minimize potential interferences. The sample chamber should be positioned as close to the detector as possible to maximize optical efficiency. High optical efficiency is desirable for an optimum signal-to-noise ratio, which allows rapid and precise measurements.

3.2 Detector

Photodiodes and photomultiplier tubes (PMTs) are the detection devices commonly found in inexpensive commercial luminometers. Improvements in photodiodes have made them effective for some applications, however PMTs continue to be the detector of choice for measuring extremely low levels of light.

PMTs function such that an incident photon triggers an amplified cascade of electrons. This amplification is very fast and essentially free of externally generated electrical noise. It is the PMT's quick response and amplification that make it extremely useful for luminescence measurements.

PMTs are positioned either to the side ("side-on" configuration) or underneath ("end-on" configuration) the sample cell. The bottom-viewing or end-on configuration of the photomultiplier tube ensures uniformity of light collection from even the smallest sample. Figure 4 shows both side-on and end-on PMT configurations.



Figure 4. Side-on PMT and End-on PMT

3.3 Photon Counting vs. Current Measuring

Most of today's commercial luminometers are either photon-counting or current-measuring in their signal processing and readout design. A photon-counting luminometer counts individual photons with a PMT and a current-measuring luminometer measures the electrical current that results when photons strike the PMT. Because not all of the light emitted from the sample is directed toward the PMT, neither a photon-counting or current-measuring luminometer detects all of the photons from the sample. Both photon-counting and current-measuring instruments have a response that is proportional to the sample light output. A photon counter will read in "photons per second", while a current-measuring luminometer will read in arbitrary light units, usually referred to as "relative light units" or RLUs. When comparing readings between these two types of instruments, reading ratios and hence, concentrations will be identical; however, due to different methods of measurement, the actual numeric output will be different.

A major disadvantage of photon-counting instruments is their limited dynamic range. While very low light levels can be measured, the maximum measurable light level in photon counting is relatively low. Another disadvantage of photon counters is that they are not able to count fast enough to respond to the high light levels and short durations of fast reactions. However, photon counters are less sensitive to quenching effects (quenching results in decreased measurements even though analyte concentrations are increasing). This is due to the fact that photon counters measure the number of pulses whereas direct current systems measure both the number and magnitude of pulses. Finally, the ability to distinguish noise in photon counting is a definite advantage, but properly designed current-measuring instruments can be just as efficient in this regard.

3.4 Instrument Comparison

There are several subjective comparisons that can be made between two instruments. However, when evaluating performance, there are two key criteria that can be used for comparison. These are the coefficient of variation and the lower limit of detection. The coefficient of variation is the percent of change over a set of readings -- this implies the consistency of the instrument performance. The lower limit of detection is the minimum sample quantity that generates an instrument response above the blank noise. This is also referred to as sensitivity. The coefficient of variation and the lower limit of detection should be obtained using known standards on a specific instrument, with the reagent system, injector system, and cuvette holder of interest. The best way to compare instruments is side-by-side using the same standards and reagents. If instruments in different laboratories are to be compared, be sure to use equivalent reagents and standards. The lower limit of detection is becoming increasingly important in luminescence analyses. Detecting lower light levels means using less reagents, potentially yielding significant cost savings. A sound way of evaluating the lower limit of detection is to run replicate analytical blanks and obtain a standard deviation on the blank. Assuming a normal distribution, the lower limit of detection can be taken as a differential exceeding the mean blank by two standard deviations (95% confidence level), or more conservatively, by three standard deviations (99% confidence level).

4. Variables

In order to effectively use chemiluminescent reactions for quantitative chemical analysis, the analyst should have an understanding of the key experimental variables affecting luminescence measurements. Variables worth discussing include sample volume, rates of reaction, concentration of reactants, reagent injection and mixing, cuvette material and size, and temperature control.

4.1 Sample Volume

Light output is directly proportional to the sample volume when the concentrations and ratio of the reactants are held constant. In the case of a luciferase assay, if the ATP/Luciferin-Luciferase ratio is held constant and the sample volume is doubled, the light output will double.

The optimal and maximum allowable sample volume will differ depending on the physical constraints of the luminometer used. For the Turner Designs TD-20/20 Luminometer, sample size including reagent volume can range from 1 ul to 800 ul. The most important parameter is the reproducibility of sample volume. Fifty ul to 400 ul will generally give the best results. The sample plus reagent volume should not exceed 800 ul. Beyond 800 ul, the volume exceeds the instrument's physical constraints and sensitivity (in proportion to volume) diminishes. Also, it should be noted that a 5:1 reagent to sample volume ratio is recommended to ensure proper mixing and yield results. For assays that involve more than one reagent addition, follow the instructions from the reagent manufacturer.

4.2 Rates of Reaction

In some reactions, light output varies slowly with time. These reactions are commonly referred to as "glow" reactions. An example of a glow reaction is the 1, 2-dioxetane substrate for alkaline phosphatase, CSPD®, in which light output increases slowly at 0.5% per minute. Testing of at least one sample is needed to find the optimal incubation time before measurement should be taken. Figure 5 shows the light emission curve for this reaction. In the case of glow reactions, procedure development is relatively simple and the timing of reagent addition and reagent/sample mixing are not as critical as in faster reactions.

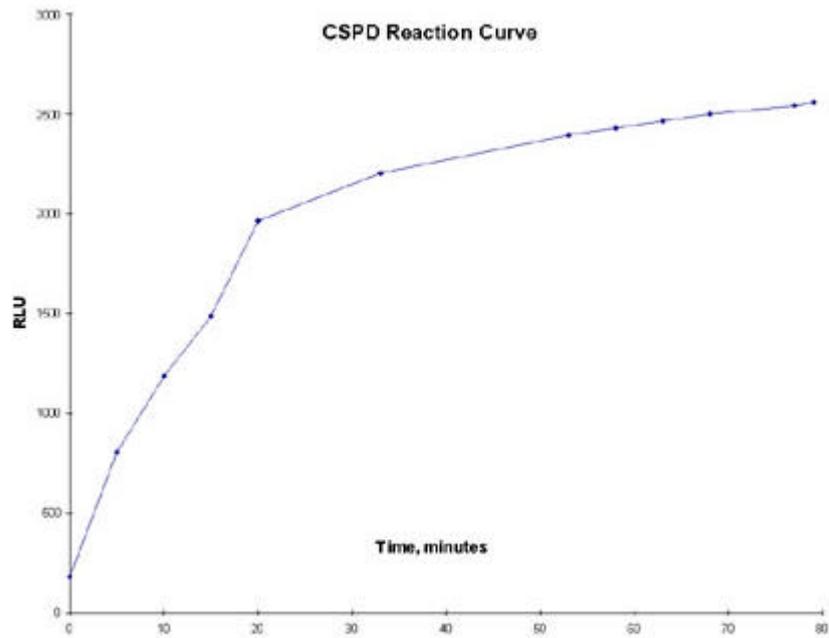


Figure 5.

An example of a faster reaction is the luminol reaction. Under proper conditions, it causes a flash of light so intense that it can briefly be seen in a partially-darkened lecture hall. Some reactions, such as the aequorin reaction, are extremely rapid. Aequorin reaches its peak light output in about 1.6 seconds and is reduced by one half in another 1.2 seconds. In fast or "flash" reactions, mixing must be complete in a period which is relatively short compared to the reaction time or, alternatively, the degree of mixing must be repeatable from sample to sample. Figures 6 and 7 illustrate the light emission curves for the luminol and aequorin reactions.

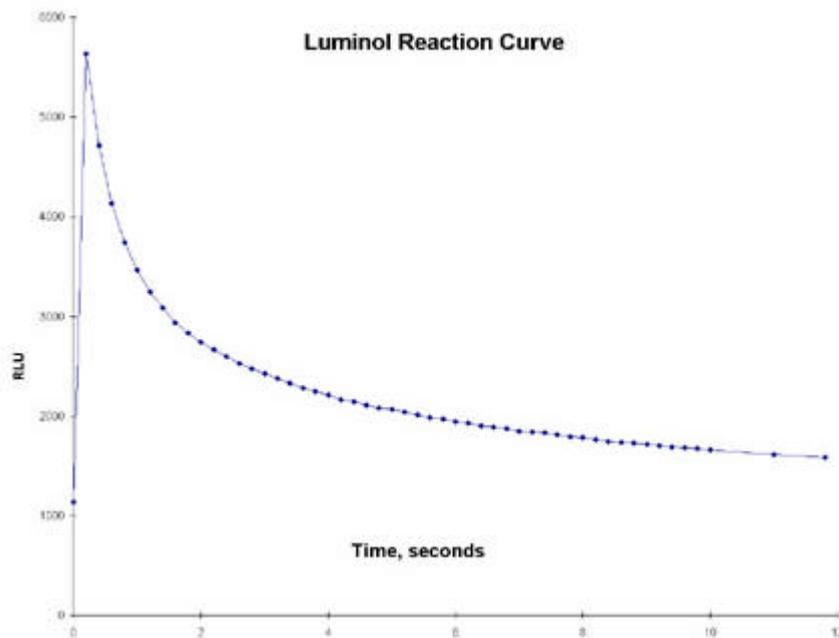


Figure 6

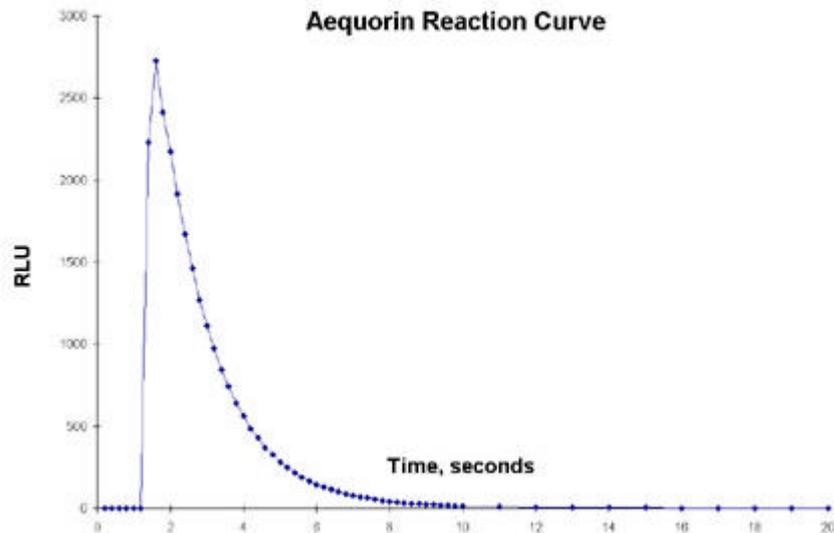


Figure 7

4.3 Concentration of Reactants

The concentration of the luminescent material directly affects the amount of light produced in a luminescent reaction. For example, 100 ul of an ATP/luciferin-luciferase solution will have greater light output than 200 ul of an ATP/luciferin-luciferase solution of one half the concentration even though the same quantity of reactants is measured in each case.

The light output is directly proportional to the concentration-limiting reactant in the system. In the ATP/luciferin-luciferase system, when the total sample volume is held constant and ATP is the limiting reactant, the light output is proportional to the ATP concentration. When luciferase is the limiting reactant, the light output is proportional to the luciferase concentration.

When the reagent is not the limiting reactant and the reaction kinetics are other than first-order, the light emitted from the reaction is proportional to a power of the concentration of the reagent. The value of the exponent is a constant for the system and is the effective kinetic order of the reaction.

The light output of the reaction is also proportional to the ratio of reagent volume to total volume in the same way.

$$L \propto \left[\frac{C_r V_r}{V_t} \right]^N$$

L = the total light reaching the detector

C_r = the concentration of the reagent, in excess

V_r = the volume of the reagent

V_t = the volume of the reagent plus the volume of the unknown (total volume)

N = the effective order of the reaction

It is possible to maximize the absolute sensitivity of the assay by using a highly concentrated reagent and by using a large volume of reagent compared to the volume of the unknown. A reagent volume to total volume ratio of 5:1 is quite effective. Little improvement is gained beyond a 7:1 ratio.

4.4 Reagent Injection and Mixing

In order to obtain precise chemiluminescence measurements, it is necessary to inject and mix reagent and sample in a repeatable way. One must choose either a laboratory pipette for manual injection and mixing or an auto-injection system for automatic injection and mixing. Automatic reagent injection systems are currently available as a standard or optional feature with most commercial luminometers.

Automatic injection systems are generally used with rapid, "flash" reactions so that the timing of reagent injection and light measurement can be carefully synchronized. With the emergence of several "glow" assays in recent years, many analysts have chosen manual injection for reasons of simplicity and cost. However, manual reagent addition and mixing are more prone to error than automatic reagent addition and mixing. Automatic reagent injection is independent of operator technique and therefore yields highly repeatable injection volumes, injection rates, and mixing. Also, samples can be analyzed more quickly when using an automatic injection system. Finally, mixing can be critical to the performance of some assay systems such as the Dual-Luciferase® Reporter Assay System (DLR) from Promega Corporation. Even auto-injectors need to be set up properly for optimization of the DLR reaction.

4.5 Cuvette Material and Size

Any material that transmits light and is compatible with the sample is suitable for luminescent measurement. Glass and polypropylene are all commonly used in luminescent analyses. Polystyrene is not recommended because it can build up static electricity which may contribute to elevated background levels. More transparent cuvette materials will allow more light to reach the detector, resulting in better detection limits.

Commercial luminometers use various test tube sizes: 8 x 50 mm, 12 x 50 mm, 12 x 75 mm, or 12 x 100 mm; as well as microfuge tubes, 28 mm scintillation vials, and 35 mm culture dishes. A greater variety of sample containers can be used with luminometers with end-on detectors, which have no minimum sample height requirement, than with luminometers with side-on detectors.

4.6 Temperature Control

Since temperature affects reaction rates and therefore, affects the rate of light emission from chemiluminescent reactions, it can be a critical variable in luminescent measurements. Some chemiluminescent reactions have a very high temperature coefficient (rate of change of emitted light as a function of temperature) and are therefore extremely sensitive to temperature fluctuations. In the case of the ATP/firefly luciferin-luciferase reaction, the temperature coefficient appears to be very low near room temperature. The reagents do, however, deteriorate rapidly at temperatures just above 30°C. It should also be noted that sample and reagents must reach final equilibrium temperature before they are mixed. Temperature effects vary from system to system, so the analyst should know the temperature coefficient of the reaction under study. In most cases, if control of the laboratory temperature is good and the instrument temperature rise is low, (1°C above ambient temperature and stable to a fraction of a degree), no other temperature control is needed.

5. Extraneous Light

It is important to be aware of the sources of extraneous light potentially interfering with luminescent measurements. Clear tubing such as that used to carry reagents or samples into the sample chamber will "pipe" light in from the outside. Also, spilled reagents in the sample chamber can be a common source of extraneous light. Phosphorescence, although short-lived in glass and polypropylene sample tubes, may also interfere with measurement since it can be induced in almost any material by exposing it to room light. In order to achieve accurate results, it is important for the analyst to minimize extraneous light in luminescent measurements.

6. Standards & Instrument Calibration

Analytical standards for luminescent analyses are solutions containing known concentrations of the analyte under study. They are used for final calibration of an analytical luminescence procedure.

The reagent systems for bio- and chemiluminescent reactions tend to be far more complex than those used for spectrophotometric and fluorometric analyses and, therefore, require more careful control. This problem is a primary concern of reagent manufacturers. They must produce a uniform product. Manufacturers have found that the best comparison standard is a stable light source with a light output comparable to that of the bio- or chemiluminescent reaction which can be installed in place of the cuvette in the sample chamber. There are three basic approaches to this problem: use radioactive material in a scintillation cocktail, use a simple and stable chemiluminescent reaction as a standard, or use a secondary light standard. Storage, disposal, handling problems, limited lifetime, and the fact that no two standards have the same output are well known problems with radioactive materials. If a simple chemiluminescent reaction is used as a standard, it must be well characterized and be of consistent quality and purity. Also, it should be insensitive to temperature, quenching, and solvent viscosity.

7. Testing Instrument Sensitivity or Comparing Instruments to Determine Relative Sensitivity

The limit of detection of an instrument is usually identified as the smallest measurable signal over the noise (instrument noise and reagent blank noise) of the system. A sensitive instrument will have a clear distinction between the blank noise and the sample signal. Typically, the system noise is determined by taking a statistically significant number of readings (10 is low, but acceptable) of the blank and calculating the average plus three standard deviations of the background signal. A sample that measures consistently above the noise level is 'detectable'.

8. Applications Include...

Numerous bioluminescent and chemiluminescent reactions are studied using luminometry. Luminometry is commonly used in biotechnology research, environmental testing, industrial applications, and clinical research. The most widely used applications are the measurement of gene expression using reporter gene assays and the determination of intracellular ATP.

Reporter Gene Assays: Molecular and cellular biologists use reporter genes to study gene expression and regulation. Reporter genes are introduced into cellular DNA and used to associate a specific molecular event with a measurable property. In this case, the molecular events involve gene function and the measurable property is luminescence. There are several luminescent reporter genes currently on the market. These include firefly luciferase, *B*-galactosidase, *B*-glucuronidase (GUS), alkaline phosphatase, and human growth hormone (hGH). Luminometry is ideal for gene expression studies because of its extraordinary sensitivity, low cost, speed, and ease of use.

ATP Determinations: All living cells contain ATP. ATP can be extracted from cells and assayed with firefly luciferase. In this luminescent reaction, ATP is the limiting reagent. Therefore, the light reaching the luminometer's photomultiplier tube is proportional to the amount of ATP in the sample and, correspondingly, to the number of cells from which it was extracted. ATP luminometric assays have been used for decades. The speed and sensitivity of luminometry make it an ideal technique for ATP determinations. ATP measurements are used in:

- ? *Microbial Contamination of Water, Foods & Pharmaceuticals:* The luminometric assay for ATP measures all the micro-organisms present in a sample. This principle has been used to measure bacterial contamination in both drinking water and industrial water used for oil field injection, cooling systems, and paper processing. Luminometric ATP determinations have also been used for the microbiological control of pharmaceuticals, cosmetics, milk, and various other foods.
- ? *Biomass Estimations:* Microbiologists use ATP determinations to measure total live biomass in soil and sediments. Related studies include determining the effect of pollutants on fresh water and marine micro-organisms.
- ? *Clinical Research:* Clinical research applications include: assessing the effect of antibiotics on microbial growth and understanding the effect of various drugs on mammalian cells.

9. References

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10. Notes

Reagents were from the following sources:

- ? CSPD® - Clontech Laboratories, Inc.
- ? Aequorin (AquaLite®) - Sealite Sciences, Inc.
- ? ATP - Turner Designs
- ? Luciferin-Luciferase - Turner Designs
- ? Luminol - SIGMA Chemical Company

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- ? CSPD® is a registered trademark of Tropix Inc.
 - ? AquaLite® is a registered trademark of Sealite Sciences, Inc.
 - ? Dual-Luciferase® is a registered trademark of Promega Corporation