

# Luminescent Determination of ATP Concentrations using the Clarity<sup>™</sup> Luminescence Microplate Reader

Detection down to the low attomole range.

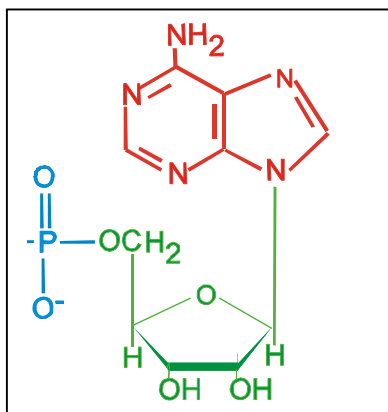
## Abstract

All living things utilize ATP as a means for storing metabolic energy. Because of this, the detection and quantitation of ATP can be used as a means to detect and/or quantitate microorganisms such as bacteria and somatic cells. The assay used in these studies relies on the ATP-dependence of the firefly luciferase reaction to detect live organisms. Here we describe the use of the Clarity<sup>™</sup> Luminescence Microplate Reader (BioTek Instruments) to measure ATP levels in solution using an ENLITEN<sup>®</sup> kit from Promega.

## Introduction

Adenosine triphosphate (ATP) plays a fundamental role in cellular energetics, metabolic regulation and cellular signaling, and therefore many different means to measure this important compound have been developed. Early methods focused on hydrolyzable phosphorus [1]. These methods were improved by Parnas *et al.* who used a phosphatase to liberate adenylic acid from ATP, with the subsequent amount of ammonia release being proportional to the ATP present [2]. Bucher described the reaction between 3-phosphoglycerate and ATP catalyzed by phosphoglycerate kinase [3]. This reaction was coupled with a dephosphoylation reaction that involved the oxidation of NADH to NAD<sup>+</sup>, which is then quantitated by measuring the change in absorbance at 340 nm [3]. The use of firefly bioluminescence to measure ATP was first proposed by McElroy when he discovered that ATP was essential for light production [4]. The use of firefly luciferase as a means to measure ATP levels has been further advanced through the use of recombinant proteins [5].

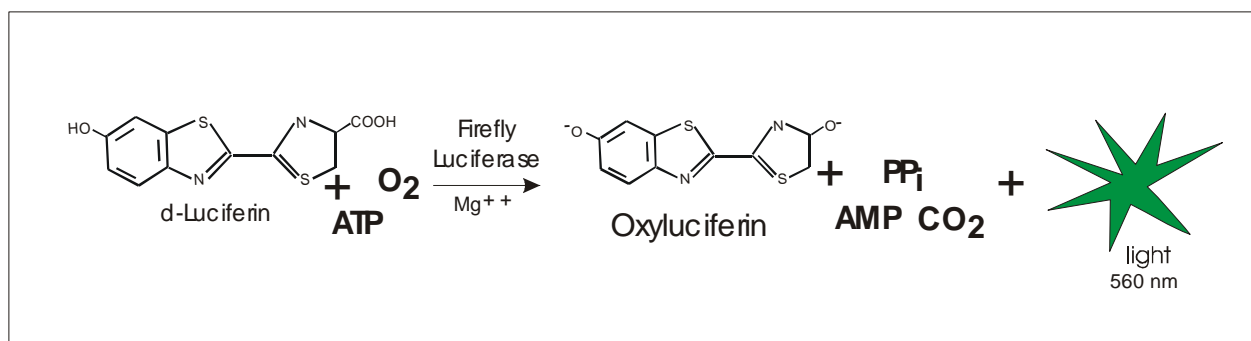
The ATP molecule is composed of three components. At the center of the molecule is a ribose sugar moiety. Attached to one side is an adenine-base, while on the other side is a string of phosphate groups which are the key to the activity of ATP. The energy stored within the molecule is contained in the phosphate bonds. Energetically unfavorable reactions can proceed if they are linked to the hydrolysis of ATP to ADP and/or AMP.



**Figure 1. Structure of ATP Molecule.** ATP consists of an adenine base (red), a ribose sugar (green) and a phosphate chain (blue).

The quantitation of ATP can be used for a variety of different purposes. Because ATP is the “coin” for energy transfer for almost all living organisms yet rapidly degrades in the absence of viable organisms, its existence can be used to identify the presence of viable organisms. Measurement of ATP has been used for the detection of bacteria on surfaces [6], quantification of bacteria in water [7], or somatic cells in culture. In addition, ATP levels in brain tissue have been measured [8,9].

Firefly luciferase is a monomeric 61 kD enzyme that catalyses a two-step oxidation of luciferin, which yields light at 560 nm (Figure 2). The first step involves the activation of the protein by ATP to produce a reactive mixed anhydride intermediate. In the second step, the active intermediate reacts with oxygen to create a transient dioxetane, which quickly breaks down to the oxidized product oxyluciferin and carbon dioxide along with a burst of light [5]. When ATP is the limiting component, the intensity of light is proportional to the concentration of ATP. Thus the measurement of the light intensity using a luminometer such as the Clarity™ Luminescence Microplate Reader permits the quantitation of ATP.



**Figure 2. Bioluminescent Reactions Catalyzed by Firefly Luciferase.** Firefly luciferase, using ATP, catalyses the two-step oxidation of luciferin to oxyluciferin, which yields light at 560 nm. In this reaction, ATP is hydrolyzed to AMP and pyrophosphate.

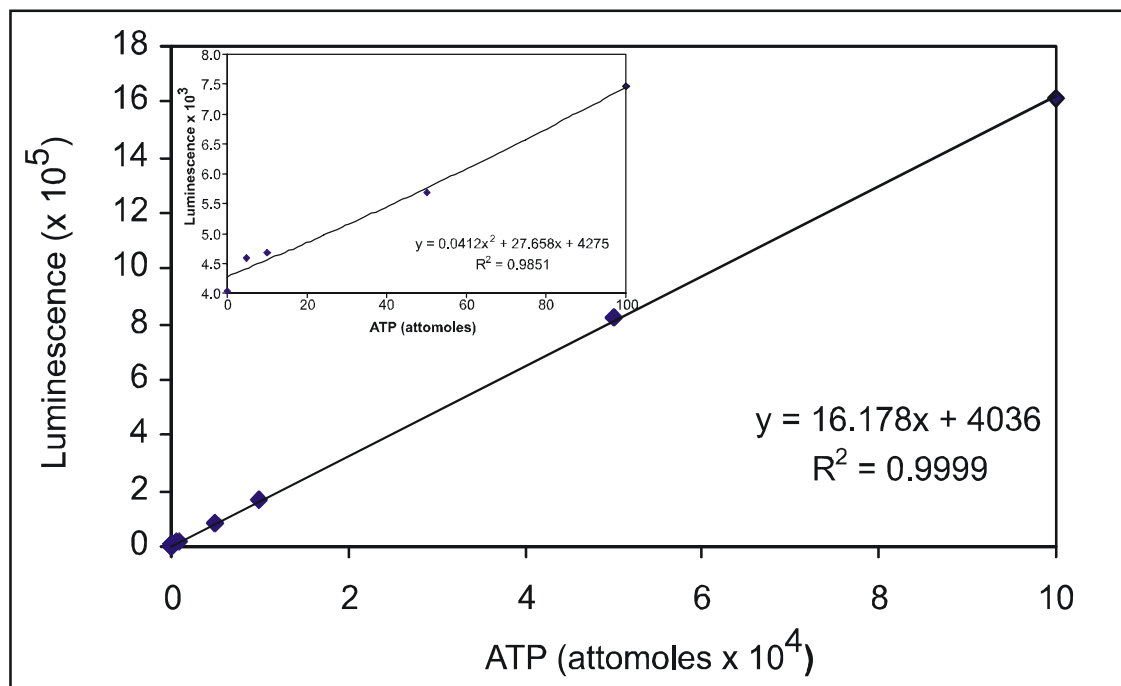
## Materials and Methods

ENLITEN ATP assay kits were obtained from Promega (Madison WI). These kits include all the reagents necessary to carry out the tests: ATP stock solution, ATP free water for dilutions of standards, as well as lyophilized rLuciferase/Luciferin reagent. Using the kit-supplied reaction buffer, reagents were rehydrated according to the kit instructions. All experiments used Corning Costar 3600 white opaque microplates.

A series of dilutions ranging from 0 to  $1 \times 10^{-13}$  M of the ATP stock standard were made using ATP free water supplied in the kit. Using sterile disposable tips, 10  $\mu$ l Aliquots of concentration were pipetted into a clean Costar 3600 opaque white 96-well microplate. Once all of the samples were pipetted, a Clarity™ Luminescence Microplate Reader was programmed to dispense 100  $\mu$ l of rLuciferase/Luciferin reagent. The reader was programmed to dispense and read in “well mode” such that the reagent would be dispensed to a well, delay for 2 seconds, then the luminescence measured for 10 seconds. The subsequent data was then exported to Microsoft® Excel™ for data analysis.

## Results

When ATP levels were measured using a luminescent reaction a linear response from 0 to 100,000 attomoles per well was observed (Figure 3). Unknown samples can be interpolated with a high degree of confidence, as the correlation coefficient ( $r^2$ ) of a linear regression is 0.9999. When the lower ATP concentrations are plotted (0 to 100 attomoles/well) a slight tailing of signal is observed, with samples containing ATP consistently having higher signal than the 0 control. A polynomial curve fit was used to describe the data and provide good predictive capability with these very low concentrations. Using a Student T-Test, samples as low as 5 attomoles per well can be statistically distinguished from the 0 standard (Figure 3).



**Figure 3. Linearity of Luminescent Response to ATP.** Serial dilutions of ATP were made using ATP-free water as the diluent. The dilutions were then assayed using an ENLITEN kit (Promega, Madison, WI) and the subsequent luminescence plotted against ATP concentration. The inserted graph depicts the signal generated at low concentrations of ATP.

## Discussion

These data demonstrate that the Clarity™ Luminescence Microplate Reader is an ideal platform to measure ATP levels using luminescence. The reader is linear over several decades of ATP concentration. With a detection limit in the low attomole range, the reader is capable of detecting even the smallest amount of bacterial contamination.

As a result of the extreme sensitivity of both the assay and the reader, there are several difficulties associated with the measurement of ATP using firefly luminescence. The measurement of ATP in solution is also particularly prone to problems associated with contamination. The presence of ATP is often used as a means to detect bacterial contamination. In order to prevent exogenous ATP it is important that aseptic technique be employed as much as possible. The use of gloves, sterile pipette tips and a HEPA filtered biosafety cabinet when preparing the samples and performing the ATP assay is highly recommended. Plates that are used should be kept covered and free of contamination as much as possible. Buffers or solutions should be sterile and free of ATP. It is also recommended that the reader fluid path be chemically sterilized with 70% ethanol, followed by rinsing with sterile deionized water immediately prior to running the assay. Another problem that can be experienced is plate autofluorescence. The white plates commonly used for luminescence experiments will often fluoresce. These plates absorb energy from the ambient light and then emit the energy as light during the luminescence measurement. This phenomenon can be a problem if the plates have been exposed to bright light for a particularly long period of time. Store unused plates in a dark environment and when pipetting samples into the plate do so in a reduced light if possible, in order to minimize plate autofluorescence. One can also “dark adapt” the plate by incubating it in the dark for approximately 10 minutes.

The Clarity™ Luminescence Microplate Reader has been specifically designed for the detection of chemi- and bioluminescence. It can be employed for all measurements of glow and flash luminescence in 96- or 384-well microplates. Clarity™ utilizes high precision reagent injectors in combination with an ultra sensitive photon counting photomultiplier tube (PMT) detector, which are controlled using external PC software. Clarity™ is available with up to four reagent injectors, two of which are intimately associated with the detector. Each injector uses microprocessor-controlled syringes to deliver exact amounts from 10 to 150 µl of reagent through chemically inert tubing to a disposable injector tip adjacent to the detector. Three different modes (e.g. linear, orbital, and cross) are available for shaking of microplates.

Clarity™ comes with Clarity PC software package which runs on the Microsoft® Windows® operating system. The software is structured in the form of protocols, allowing automation of all steps from defining measurement parameters to reporting final results. Users can create custom protocols for immediate use or store them for later availability. The Clarity protocol interface allows users to modify parameters such as injection volume, delay time and measurement duration. The software easily formats to interchange 96- and 384-well microplates. Any combination of wells can be read.

Clarity™ is ideal for the bench top, with a footprint of 15.4” (W) x 16.4” (D) and a height of 10.2” (38.5 x 41.0 x 25.5 cm respectively). Clarity™ has a robotic friendly plate carrier and can be integrated into robotic systems using technical documentation provided. Both RS-232 and USB serial ports are available for PC communication.

## References

1. Kerr, SE, and L. Daoud, (1935) A Study of the Organic Acid-soluble Phosphorus of the Erythrocytes of Various Vertebrates, *J. Biol. Chem.* **109**: 301.
2. Parnas, J.K., P. Ostern, and T. Mann (1934)  $\gamma$ ber die Verkettung der chemischen Vorgange im Muskel, *Biochim Z*, **272**:64.
3. Bucher, T. (1947)  $\gamma$ ber ein phosphat $\eta$ bertragendes Garungsferment. *Biochim. Biophys. Acta* **1**:292
4. McElroy, W.D. (1947) The Energy Source for Bioluminescence in an isolated System. *Proc. Natl. Acad. Sci. USA* **33**:342.
5. de Wet JR, Wood KV, Helinski DR, DeLuca M, (1985) Cloning of firefly luciferase cDNA and the expression of active luciferase in Escherichia coli, *Proc. Natl. Acad. Sci USA* 82:7870-7873.
6. ENLITEN® Total ATP Rapid Biocontamination Detection Kit Manual, Part Number TB265, Promega Corporation, 2800 Woods Hollow Rd. Madison, WI 53711-5399
7. Lee, J.L. and R.A. Deininger (2001) Rapid Quantification of Viable Bacteria in Water Using an ATP Assay, *Amer. Labor. News* October, 2001 pp24-26.
8. Khan, H.A. (2003) Bioluminometric assay of ATP in mouse brain: Determinant factors for enhanced test sensitivity, *J. Bioscience* **28(4)**:379-382.
9. Drew, B and C. Leeuwenburgh (2003) Method for measuring ATP production in isolated mitochondria: ATP production in brain and liver mitochondria fo Fischer-344 rats with age and caloric restriction, *Am J. Physiol. Regul. Integr. Comp. Physiol.*, 285:R1260-R1268.

**Paul Held PhD**  
**Senior Scientist & Applications Lab Manager**

Rev. 06/22/04