Chapter 2
Bioluminescent Analysis

Bioluminescent analysis is part of the kinetic type of analysis, where proportionality of the analyte concentration to the reaction rate is used as a basis.

2.1 Basics of Kinetic Analysis

According to the law of mass action, for any given reaction the rate (v) of reaction is proportional to the concentration of all reactants:

$$A + B \rightarrow P$$
$$v = k[A][B], \tag{2.1}$$

where A and B are reactants, $[A]$ and $[B]$ are their respective molar concentrations, P is a product, and k is the reaction-rate constant. This equation is the basis for kinetic analysis.

The progressive curve of the reaction is obtained to determine the reaction rate (i.e., the time it takes for products to accumulate, $[P]$ versus time of the disappearance of reactants, or $[A]$ or $[B]$ versus time). The instantaneous slope of this curve represents the reaction rate for this point in time (Fig. 2.1). Mathematical equation for instantaneous reaction rate definition is

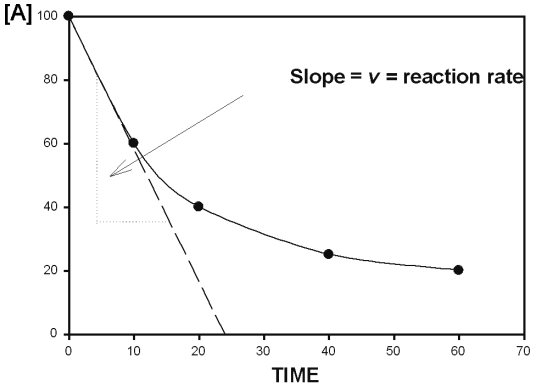

Figure 2.1 Representation of a progressive curve for a reaction that is dependent on substrate concentration (A) versus time, and a determination of an initial reaction rate (v) as a slope of a tangent-to-progressive curve at time zero.

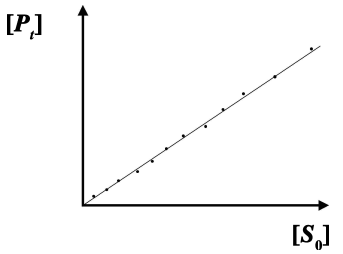

Figure 3.4 Example of a standard curve for "fixed-time" format of kinetic analysis: concentration of the formed product at fixed-time $(P)_t$ versus initial concentration of the substrate $(S)_0$.

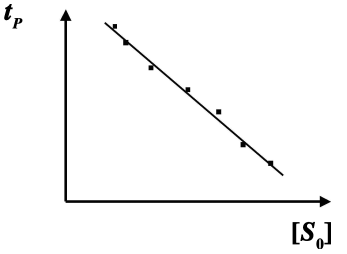

Figure 3.5 Example of a standard curve for "fixed-concentration" format of kinetic analysis: time for the forming product reached fixed-concentration t_P versus initial concentration of the substrate $(S)_0$.

For bioluminescent analysis, the fixed-time format is used most often when a bioluminescent intensity signal is integrated for a predetermined period of time. An integrated signal is in turn used as a measure of amount of product formed during this time and subsequently as a measure of initial substrate concentration.

3.2 Analytical Applications of the Bioluminescent Reaction Catalyzed by Firefly Luciferase

Firefly luciferase catalyzes luciferin oxidation by oxygen in the presence of ATP-Mg. Though it is a complex enzymatic reaction that involves three different substrates, in the case of fixed-concentrations of two substrates, e.g., luciferin and oxygen, it can be treated as a simple one-substrate enzyme reaction presented in Eq. 3.1. For the majority of other enzymes the reaction follows the Michaelis-Menten mechanism, thus at the conditions of $[E] << [S]$ there is a linear dependence of reaction rate (v) on the enzyme concentration ([luciferase]) and a hyperbolic depen-dence on the concentration of substrates ([ATP]) (Fig. 3.6, Eq. 3.2):

$$v = k'_{\text{cat}}\,[\text{luciferase}][\text{ATP}]/(K'_m + [\text{ATP}]), \qquad (3.3)$$

where k'_{cat} is the effective catalytic reaction constant which is a function of both luciferin and oxygen concentrations, and K'_m is the effective Michaelis constant, which also depends on luciferin and oxygen concentrations.

At the substrate levels below the Michaelis constant (for ATP K_m is in the range 0.01– 0.1 mM, depending on conditions) the reaction rate is proportional to its concentration:

$$v = (k'_{cat} / K'_m)[\text{luciferase}][\text{ATP}], \tag{3.4}$$

Taking into consideration that one of the products of firefly bioluminescence is a photon of light, the rate of product accumulation (reaction rate) can be monitored by measuring the rate of photon emission, which is the bioluminescent intensity. Thus, the bioluminescent intensity is equal to the reaction rate and is proportional to the enzyme and substrate concentrations, according to Eq. 3.4.

At ATP concentrations below 0.01 mM the intensity of bioluminescence (I) is proportional to the concentration of the luciferase itself and the substrate ATP:

$$I \sim [\text{luciferase}][\text{ATP}]. \tag{3.5}$$

This is the basis of bioluminescent ATP-assay using the reaction catalyzed by firefly luciferase. An example of the graph bioluminescence intensity versus ATP is presented in Fig. 3.6.

Firefly luciferase is absolutely specific regarding its substrates. Even minor changes in the structure of ATP and/or luciferin result in an almost 100% decrease of reaction rate and, subsequently, bioluminescence intensity. Because the quantum yield of this bioluminescent reaction is 0.88, on average almost 1 photon is emitted per reaction cycle. Instruments are available that can detect even single photons, theoretically making the sensitivity of the bioluminescent ATP assay extremely high (1 amol = 10^{-18} mole). In practice, the sensitivity is lower, due to ATP instability and the background bioluminescent signal of the reagent.

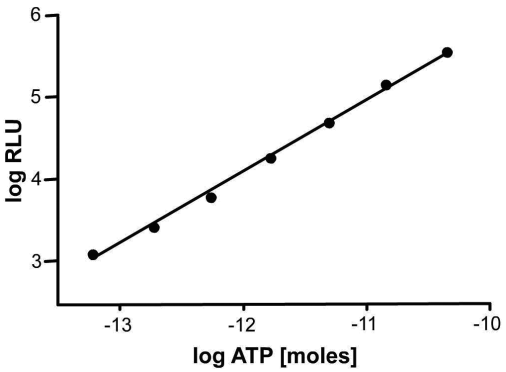

Figure 3.6 Dependence of bioluminescence intensity in relative light units (RLU) on ATP in moles.

Chapter 6
Hygiene Monitoring

ATP bioluminescent techniques are increasingly used for measuring the efficiency with which surfaces and utensils are cleaned. In late 1990s, a survey of 500 food manufacturing businesses in the UK revealed that 48% of respondents used swabbing followed by bacterial culture, while 27% used ATP bioluminescence. To adequately sanitize a food contact surface, it must first be washed to remove any food residues that could act as a nutrient source for the subsequent growth of microorganisms. After washing, the surface is sanitized to kill the residual microflora. If either process is not done properly, food particles and/or microorganisms can remain and may constitute a risk to the quality and safety of foods to be processed afterward. Normal swabbing and plate-counting procedures only detect microbial contamination of the surface and may not indicate whether the surface has been properly cleaned. ATP bioluminescence detects contamination from both sources within 2 min and is considered a more reliable indicator of the overall hygienic condition of the area tested.

Several studies have compared the results obtained by standard microbiological techniques and ATP bioluminescence for assessing surface cleanliness. Some reported a good correlation between these methods,[10,29,30] while others have obtained a poor correlation.[31–33] Such discrepancies in findings could be explained either by the different nature of the surface and surface contamination (presence of spores for example) or the inability/inconsistency of swabs to pick up microorganisms effectively. Loss in bacterial viability during drying could also have an impact on both ATP bioluminescence and plate-count results. In addition, the presence of detergents, sanitizers, or other chemicals may interfere with bioluminescent reactions,[34] leading to false-positive or false-negative results. Despite these difficulties ATP bioluminescence has been used successfully as an initial step in hygiene monitoring, especially within HACCP (hazard analysis critical control point) plans. Most of the ATP-based reagent kits produced for rapid surface cleanliness tests (Enliten Total ATP Rapid Biocontamination Detection kit;* Clean-Trace Rapid Cleanliness kit;** PocketSwab, Charm Sciences, Inc.+) rely on the use of a baseline cut-off value that should be determined for every tested environment. The procedure involves the following steps.

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* Promega, WI.
** Biotrace, UK.
\+ Lawrence, MA.