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A multienzyme bioluminescent time-resolved pyrophosphate assay

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Abstract

We have developed a high-sensitivity assay for measurement of inorganic pyrophosphate (PPi) in adenosine 5′-triphosphate (ATP)-contaminated samples. The assay is based on time-resolved measurements of the luminescence kinetics and implements multiple enzymes to convert PPi to ATP that is, in turn, utilized to produce light and to hydrolyze PPi for measurement of the steady state background luminescence. A theoretical model for describing luminescence kinetics and optimizing composition of the assay detection mixture is presented. We found that the model is in excellent agreement with the experimental results. We have developed and evaluated two algorithms for PPi measurement from luminescence kinetics acquired from ATP-contaminated samples. The first algorithm is considered to be the method of choice for analysis of long, i.e., 3–5 min, kinetics. The activity of enzymes is controlled during the experiment; the sensitivity of PPi detection is about 7 pg/ml or 15 pM of PPi in ATP-contaminated samples. The second algorithm is designed for analysis of short, i.e., less than 1-min, luminescence kinetics. It has about 20 pM PPi detection sensitivity and may be the better choice for assays in microplate format, where a short measurement time is required. The PPi assay is primarily developed for RNA expression analysis, but it also can be used in various applications that require high-sensitivity PPi detection in ATP-contaminated samples.

Keywords: Bioluminescent assay; Enzyme kinetics; Inorganic pyrophosphate; Time-resolved kinetics; Gene expression; Nucleic acid detection

Bioluminescence assays for detecting inorganic pyrophosphate (PPi)[1] at sub nanomolar concentrations can become a promising new tool for RNA expression analysis for both messenger (mRNA) and microRNA [1,2]. The bioluminescence RNA quantification method is based on detection of released PPi during RNA reverse transcription or replication [3,4]. During the polymerization reaction the PPi is released as the result of nucleotide incorporation by polymerase. The released PPi can be converted into adenosine 5′-triphosphate (ATP) by ATP–sulfurylase, and, subsequently, the ATP provides the energy for luciferase to oxidize luciferin and generate light [5]. The bioluminescence PPi assay can be extremely sensitive and potentially can detect a single target RNA molecule. This is possible because the synthesis of a single large cDNA can require incorporation of thousands of deoxynucleotides, consequently producing thousands of ATP molecules and resulting in the emission of hundreds of photons per each cDNA copy synthesized. Yet, to achieve such a level of sensitivity the development of a new generation of bioluminescence PPi assay is required. One promising approach which employs a bioluminescence regenerative cycle (BRC) system has been reported recently [1,3]. The regenerative cycle uses the ATP–sulfurylase enzyme to convert PPi to ATP. In the presence of luciferin and luciferase the consumption of the ATP molecule results in light emission and formation of a PPi molecule as by-product. This by-product PPi molecule can be reused in another cycle of ATP production and subsequent light emission. The advantage of the regenerative bioluminescence system is that each PPi molecule can initiate potentially hundreds of light emission cycles, producing steady state emission with intensity proportional to the number of PPi molecules in the sample.

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[1] *Abbreviations used:* PPi, inorganic pyrophosphate; BRC, bioluminescence regenerative cycle; Pi, inorganic phosphate; PPase, inorganic pyrophosphotase; APS, adenosine *s*-phosphosulfate sodium salt; RT, reverse transcription.

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Yet, the regenerative system also has serious drawbacks. The real biological samples unavoidably carry some residual amount of ATP molecules (ATP contamination) that along with target PPi molecules can be involved as a substrate in the regenerative cycle. In addition, the biological samples exhibit steady state luminescence (luminescence background), which is originated by non-ATP substrate(s) in samples [6]. The ATP contamination and steady state luminescence background reduce the sensitivity and distort the accuracy of PPi measurement by the regenerative cycle system.

To address the drawbacks of the regenerative cycle system we have developed an alternative high-sensitivity PPi assay, which allows, in a single experiment, measurement of the three components of the luminescence: the PPi component, the ATP component, and the steady state background luminescence. This is achieved by formulating a multienzyme assay mixture, which produces luminescence kinetics with easily distinguishable fast and slow decay components. The fast component is due to conversion of PPi to ATP, and the slow component is driven by two enzymatic reactions: one involves the consumption of ATP by luciferase and the other the hydrolysis of PPi to inorganic phosphate (Pi) by inorganic pyrophosphatase (PPase) [7]. The use of PPase allows assessment of the intensity of steady state luminescence in biological samples for increasing sensitivity and accuracy of PPi measurements.

The principle difference between our approach and the regenerative cycle system is the use of time-resolved measurement (i.e., kinetics assay) instead of the measurement of the steady state luminescence intensity by the regenerative cycle system. In this report we present the theoretical analysis of the multienzyme assay for kinetics measurements, describe the experimental procedures and algorithms for analysis of experimental data, and present results of PPi measurement at a sensitivity of about 7 pg/ml or 15 pM of PPi in ATP-contaminated samples. The assay is about three orders of magnitude more sensitive than the previously reported methods of PPi quantification (i.e., 15–20 pM vs ~20 nM sensitivity of the commercial assay) [8–12]. The kinetics assay was developed primarily for application in mRNA and microRNA expression analysis [2,4], but it also can be used for PPi measurement in other applications. It was used, for instance, to measure PPi and ATP in a patient's breath condensate, where the PPi was detected at ~0.8 ng/ml and ATP at <0.1 ng/ml in 5-μl fluid samples (unpublished results). The assay is designed for application with commercially available photon-counting luminometers and can be implemented in single-tube or microplate format to match the specific requirements of various applications.

Theoretical consideration of time-resolved PPi assay

In the simplified model of PPi kinetics assay, three linked reactions determine the kinetics of luminescence: (1) the reaction of conversion of PPi to ATP by ATP–sulfurylase, $E_{ATP\text{-}Sulf}$, (2) the reaction of consumption of PPi by inorganic pyrophosphatase, E_{PPase}, and (3) the reaction of consumption of ATP by luciferin–luciferase $E_{L\text{–}L}$ that results in photon emission [13]:

$$PPi + E_{ATP-Sulf} \overset{\gamma_{Sulf}}{\rightarrow} ATP, \quad (1)$$

$$PPi + E_{PPase} \overset{\gamma_{PPase}}{\rightarrow} 2Pi, \quad \text{and} \quad (2)$$

$$ATP + E_{L\text{–}L} \overset{\gamma_{L\text{–}L}}{\rightarrow} PPi + h\nu. \quad (3)$$

Here γ is the rate of the corresponding enzymatic reaction. In the simplified model Eqs. (1–3) only products that are essential for further discussion are shown. Under the excess of enzymes $E_{ATP\text{-}Sulf}$, E_{PPase}, and $E_{L\text{–}L}$, the concentrations of ATP and PPi in solution are given by equations for a first-order biochemical reaction,

$$d[PPi]/dt = -(\gamma_{Sulf} + \gamma_{PPase})[PPi] + \gamma_{L\text{–}L}[ATP], \quad (4)$$

$$d[ATP]/dt = \gamma_{Sulf}[PPi] - \gamma_{L\text{–}L}[ATP], \text{ and} \quad (5)$$

$$I_{h\nu} = \gamma_{L\text{–}L}[ATP], \quad (6)$$

where $I_{h\nu}$ is the intensity of luminescence measured in photons/s. Eqs. (4–6) assume that the cosubstrates, i.e., luciferin and molecular oxygen, are at constant concentration, which is valid at typical assay conditions. Fig. 1 shows examples of the luminescence vs time detected at typical experimental conditions by a mixture of ATP–sulfurylase/PPase/luciferin–luciferase in solution containing different amounts of PPi and ATP at $t = 0$. If there is a little or no ATP in solution at $t = 0$, the luminescence increases rapidly until it reaches the maximum intensity followed by a slower exponential decay to a steady state luminescence level, I_{Backgr}, as illustrated in Fig. 2. The luminescence kinetics in Fig. 2 can be considered as a sum of three components described by five parameters: the fast and slow exponential components with amplitudes A_{Fast} and B_{Slow} and rates γ_{Fast} and γ_{Slow} for the fast and slow components, respectively, and the steady state luminescence level I_{Backgr}. The background luminescence I_{Backgr} is due to slowly reacting impurities, that often are present in samples. The I_{Backgr} is not described by the simplified reaction model given by Eqs. (1)–(3) and can be introduced as an additional phenomenological parameter of the model

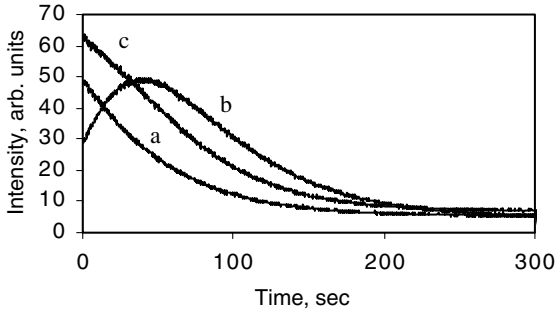

Fig. 1. Luminescence kinetics detected from three samples: (a) 1.8 fmol ATP, (b) 11.2 fmol PPi, and (c) a mixture of ATP and PPi.