Bioluminescence Regenerative Cycle (BRC) System for Nucleic Acid Quantification Assays

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ABSTRACT

A new label-free methodology for nucleic acid quantification has been developed where the number of pyrophosphate molecules (PPi) released during polymerization of the target nucleic acid is counted and correlated to DNA copy number. The technique uses the enzymatic complex of ATP-sulfurylase and firefly luciferase to generate photons from PPi. An enzymatic unity gain positive feedback is also implemented to regenerate the photon generation process and compensate any decay in light intensity by self regulation. Due to this positive feedback, the total number of photons generated by the bioluminescence regenerative cycle (BRC) can potentially be orders of magnitude higher than typical chemiluminescent processes. A system level kinetic model that incorporates the effects of contaminations and detector noise was used to show that the photon generation process is in fact steady and also proportional to the nucleic acid quantity. Here we show that BRC is capable of detecting quantities of DNA as low as 1 amol (10^{-18} mole) in 40 µlit aqueous solutions, and this enzymatic assay has a controllable dynamic range of 5 orders of magnitude. The sensitivity of this technology, due to the excess number of photons generated by the regenerative cycle, is not constrained by detector performance, but rather by possible PPi or ATP (adenosine triphosphate) contamination, or background bioluminescence of the enzymatic complex.

Keywords: Chemiluminescence, bioluminescence, gene expression, nucleic acid, assay, polymerization, enzyme kinetics, positive feedback, regeneration

1. INTRODUCTION

A significant technical challenge in the field of genomics is to enhance the performance and flexibility of methods for nucleic acid quantification. Real time quantitative polymerase chain reaction (RT-PCR)[1-4] is a common label-based assay which implements PCR thermo-cycling platforms. As an example in one method of RT-PCR during normal PCR cycle, if the target of interest is present, a designed probe which consists of an oligonucleotide with a reporter and quencher dye anneals specifically between the forward and reverse primer binding sites. The exonuclease activity of the polymerase cleaves the probe, which results in an increase in the fluorescence intensity of the reporter dye. This process occurs in every cycle and does not interfere with the accumulation of PCR product. This approach is applicable to small numbers of target DNA molecules within a sample. In contrast, nucleic acid microarrays[5-7] provide a systematic and parallel platform for multiparametric exploration of the genome. Microarray technology, in principal and practice, is the extension of hybridization-based methods which have been used to identify and quantify nucleic acids in biological samples. The basic concept comes from the specificity and affinity of complementary base-pairing for handling specific DNA or RNA target molecules, and fluorescent labeling to detect their quantity. A label-independent and sensitive method for parallel detection and quantification of nucleic acid would add to our arsenal of tools for analyzing genomes.

In this study we explore an alternative method of quantifying nucleic acid molecules, one which does not require any molecular modification or labeling and merely counts the inorganic pyrophosphate (PPi) molecules released during the polymerization of the nucleic acid by a polymerase enzyme (e.g. Klenow[8]). This technique implements a bioluminescence regenerative cycle, activated by the generated PPi molecules which, in quantity, are proportional to the

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number of target molecules. The regenerative cycle comprises ATP-sulfurylase enzyme[9-10], which converts PPi to ATP (adenosine triphosphate) by consuming APS (adenosine phosphosulfate), and firefly luciferase[11-12], which, in the presence of luciferin, consumes ATP as an energy source to generate photons as a detectable signal, and again yields PPi as a byproduct (Fig. 1). We have shown that the photon emission rate with PPi regeneration becomes steady and that it is also a monotonic function of the introduced PPi. For very low concentrations of PPi (lower than 10^{-8} M), the total number of photons generated in a fixed time interval, is proportional to the number of PPi molecules, and thus proportional to the number of nucleic acid molecules present in the solution.

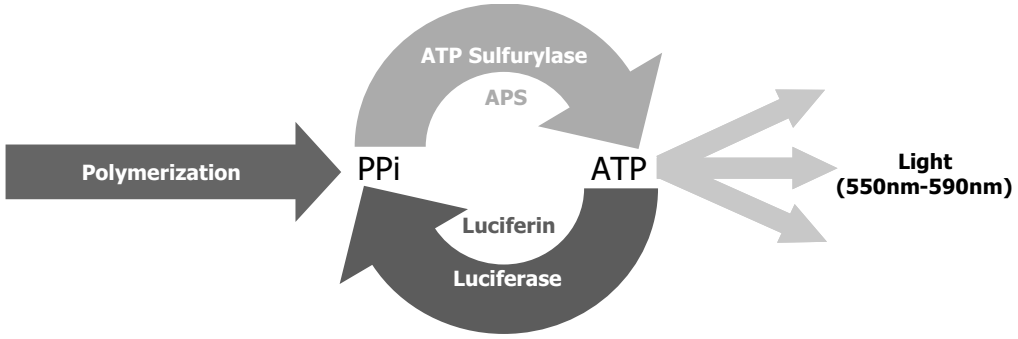

Figure 1: Bioluminescence regenerative cycle (BRC) with ATP-sulfurylase and firefly luciferase detecting the released pyrophosphate from nucleic acid polymerization.

The basic concept of enzymatic light generation from PPi molecules was initially introduced in references[13-14]. Other applications of this enzymatic detection method, including single nucleotide polymorphism (SNP) detection[15] and DNA sequencing by synthesis[16-17], have also been studied. This paper describes the additional steps that are necessary to accurately quantify extremely small numbers of nucleic acid copies in low density assays despite the presence of contaminants and other potential sources of noise. The new system has an intrinsic controllable assay dynamic range of about five orders of magnitude, and is sensitive to 10^5 copies of target nucleic acid.

2. EXPERIMENTAL PROCEDURES

2.1 Synthesis and purification of oligonucleotides

The oligonucleotides B-MBPup, (5'-Biotin CGGCGATAAAGGCTATAACGG), MBPup (CGGCGATAAAGGCTATAACGG), B-MBPR1 (5'-Biotin CTGGAACGCTTTGTCCGGGG), MBPR1 (5'- CTGGAACGCTTTGTCCGGGG) and oligo-loop, (5'- TTTTTTTTTTTTTTTTTTTGCTGGAATTCGTCAGACTGGCCGTCGTTTTACAACGGAACG TTGT AAAACGACGGC), were all synthesized and HPLC purified by MWG Biotech (High Points, NC, USA).

2.2 Template preparation

The biotinylated PCR products were immobilized onto streptavidin-coated superparamagnetic beads, Dynabeads™ M280-Streptavidin (Dynal A.S., Oslo, Norway). Single-stranded DNA (ssDNA) was obtained by removing the supernatant after incubation of the immobilized PCR product in 100 mM NaOH for 3 min. Sequencing primers MBP-up or MBP-R1 (5 pmol) were hybridized to the immobilized ssDNA strand that was obtained from an amplified product via polymerase chain reaction (PCR).

2.3 BRC assay

The immobilized single stranded PCR product was resuspended in annealing buffer (10mM Tris-acetate pH 7.75, 2mM Mg-acetate), and primers were added to the single stranded templates. Hybridization of the template and primers was performed (by incubation at 95°C for 3 min, 72°C for 5min and then cooling to room temperature) and extension conducted after the addition of exonuclease-deficient (exo-) Klenow DNA polymerase (10 U; Fermentas, NY, USA) and all 4 nucleotide triphosphates (1.4 mM nucleotides (1.4mM final concentration) to the extension mixture. After completion of the reaction, the content of each well was serially diluted for comparison of extension analysis (PPi

concentration). Extension and real-time luminometric monitoring was performed at either 37°C in an IVIS™ imaging system (Xenogen, Alameda, CA USA), or at 27°C in an Lmax™ microplate luminometer (Molecular Devices, Sunnyvale, CA, USA). A luminometric reaction mixture was added to the substrate at different concentrations (extended primed single-stranded DNA or self primed oligonucleotide). The 40µl bioluminescence assay mixture contained: 3.0 µg luciferase (Promega, USA), 50 mU recombinant ATP-sulfurylase (Sigma, USA), 0.1 M Tris-acetate (pH 7.75), 0.5 mM EDTA (Sigma, USA), 5 mM Mg-acetate (Sigma, USA), 0.1% (w/v) bovine serum albumin (Sigma, USA), 2.5 mM dithiothreitol (Sigma, USA), 10 µM adenosine 5'-phosphosulfate (APS) (Biolog, Belgium), 0.4 mg polyvinylpyrrolidone/ml (molecular weight 360000) and 100 µg D-luciferin/ml (BioThema, Uppsal, Sweden), and the emitted light was detected in real time and measured after approximately 45 sec with 1 sec and 10 sec integration times for the CCD imaging system and luminometer, respectively.

2.4 Detection Devices

To estimate the quantity of the target nucleic acid, we counted the photons generated by the BRC process. The general luciferase generation of photons has a quantum efficiency (Q.E.) of approximately 0.88 per consumed ATP molecule, and the maximum wavelength (which depends on the type of luciferase), is in the visible range of the optical spectrum (e.g., 565 nm for firefly luciferase).

A variety of photosensitive devices have been developed to detect bioluminescent signals, and these have been used to detect light from the regenerative phenomenon. These devices include photomultiplier tubes (PMTs), charge coupled devices (CCDs), and photodiodes. The photosensitive device can either be in close proximity to the BRC reaction to receive the incident photons directly, or at a distance from the reaction buffer with a light coupling device (e.g. optical fiber or mirror system) to convey photons from the sample to the detector.

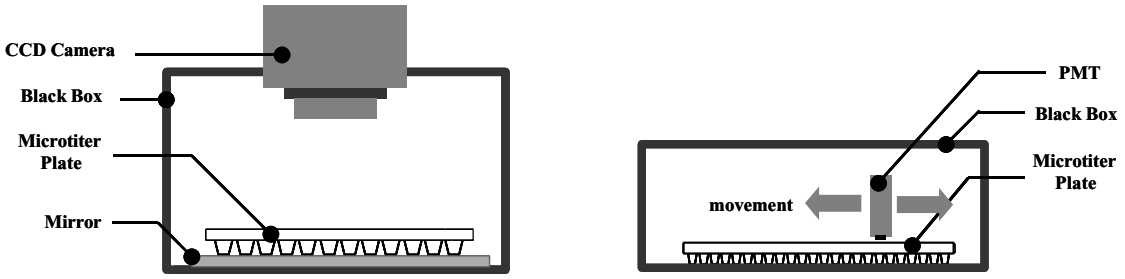

Figure 2: (a) CCD camera system which simultaneously measures the light from reference buffer and target sample, and (b) PMT imaging system in luminometer.

In our experiments we used both a cooled CCD camera imaging system (IVIS; Xenogen) and a luminometer (Lmax™; Molecular Devices) that employed a single PMT detector. The light coupling efficiencies of each system (including path loss), from the microtiter plate where the DNA samples were located, to the sensor, were approximately 0.012% and 8% for the CCD and PMT systems, respectively, as shown in Fig. 2.

In the CCD imaging system a 96-well microtiter plate with multiple DNA samples was placed 18 cm below the lens of the camera (Fig. 2a), and in the luminometer a 384-well microtiter plate was inserted in the instrument chamber, where a PMT directly moves into close proximity (1cm) of the sample for reading (Fig. 2b).

3. THEORETICAL CONCEPTS

3.1 Pyrophosphate generation

In polymerase-catalyzed reactions, PPi molecules are generated when nucleotides (dNTPs) are incorporated into the nucleic acid chain. For each addition of a nucleotide, one PPi molecule is cleaved from the dNTP by the polymerase enzyme (e.g. Klenow[8, 18]) and released into the reaction buffer (Fig. 3). For RNA and DNA molecules we have

$$(DNA)_n + dNTP \xrightarrow{Polymerization} (DNA)_{n+1} + PPi \quad (1)$$

$$(RNA)_n + dNTP \xrightarrow{Polymerization} (RNA)_{n+1} + PPi \quad (2)$$

where n is the position of the 3' end and 5' end of the primer in the DNA and RNA strand. If one assumes that the strand is completely polymerized, then the number of PPi molecules N_{PPi} which are released during this process is

$$N_{PPi} = N_{NA} \cdot (L_{NA} - L_P) . \quad (3)$$

N_{NA} is the total number of primed nucleic acid molecules present in the reaction buffer, and L_{NA} and L_P are the lengths of the nucleic acid chain and the primer, respectively.

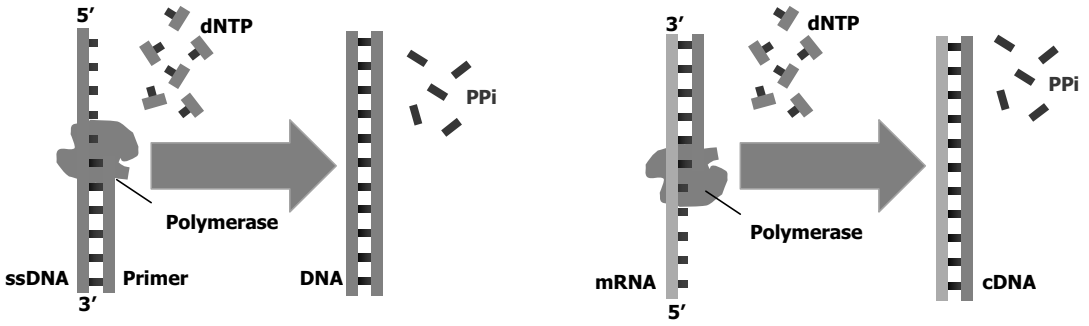

Figure 3: Pyrophosphate generation from DNA polymerization and RNA reverse transcription.

3.2 Enzymatic bioluminescence cycle

To generate photons efficiently from the pyrophosphate release process, ATP-sulfurylase enzyme[9-10] is initially introduced to the system to catalyze the transfer of the adenylyl group from ATP to inorganic sulfate. This sulfurylase enzyme is ubiquitous in nature, although its physical role depends on the metabolic lifestyle of the organism. In this case we used the enzyme to generate ATP from pyrophosphate, by consuming APS:

$$PPi + APS \xleftarrow{ATP-Sulfurylase} ATP + SO_4^{-2} \quad (4)$$

To complete the chemical process we used firefly luciferase which, with a nominal turnover rate of 0.015 [11,17], consumes the generated ATP to emit photons (λ_{max}=565 nm, Q.E. ≈ 0.88). This process uses luciferin as a substrate and generates oxyluciferin, adenosine monophosphate (AMP), CO_2 and PPi as byproducts.

$$ATP + luciferin + O_2 \xrightarrow{Luciferase} AMP + oxyluciferin + CO_2 + h\upsilon + PPi \quad (5)$$

It is apparent from (4) and (5) that the PPi molecules generated at the end of the photon emission process by luciferase can again trigger the ATP synthesis reaction by ATP-sulfurylase, which results in a substrate cycling phenomenon (enzymatic positive feedback). Because this positive feedback regulates the total amount of ATP molecules in the solution, the light emission can also be regulated without any decay. The chemical yield of PPi generation per ATP from luciferase is close to unity, therefore we were able to model this phenomenon as an ideal unity-gain positive feedback (Fig. 4), which regulates the process and inhibits any light generation decay due to substrate consumption.

It is extremely important to understand that the number of photons generated by the regenerative cycle can potentially be orders of magnitude higher than the initial number of PPi (or ATP) molecules introduced to the system. This results in increased sensitivity with longer integration times.

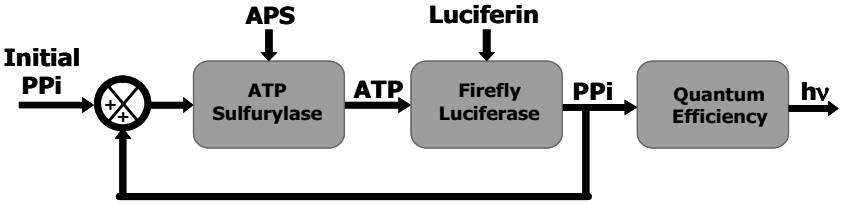

Figure 4: Bioluminescence regenerative cycle block diagram.

In Fig.5 the simulation results of a BRC system compared to an ATP assay (no enzymatic feedback) is shown. As we can see the feedback stabilizes the light intensity in contrast to the assay with no substrate cycling.

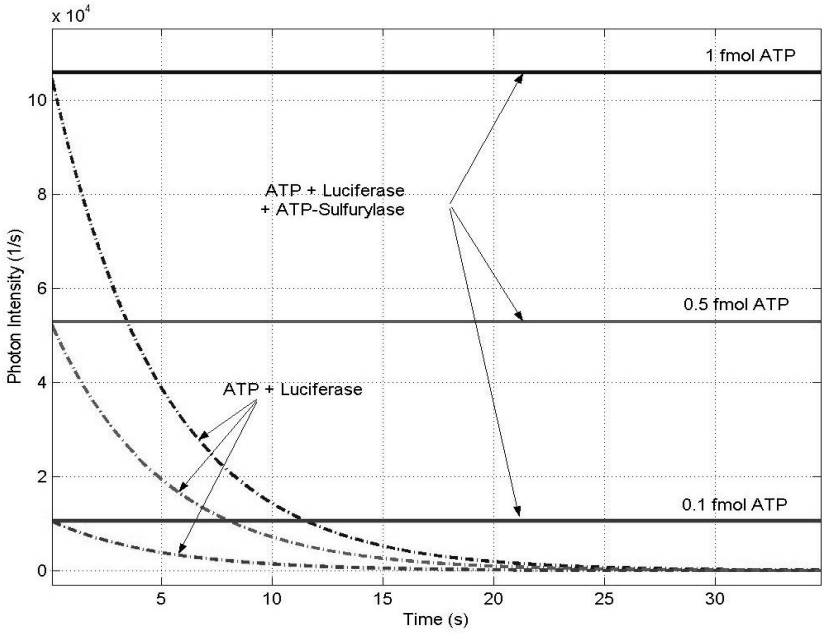

Figure 5: Simulation results based on the kinetics of the enzymes from [9] and [17] comparing light intensity of ATP assay and BRC system (Luciferin=0.1mM, APS=0.1mM).

3.3 Photon Generation Rate

The kinetics of ATP-sulfyrylase and luciferase[19] determine the steady state photon generation rate of BRC, since polymerase neither affects the luminescence process, nor the enzymatic feedback. As shown in Fig.4 the system consists of two different enzymatic processes with a unity -gain positive feedback. At saturated concentrations of APS and luciferin, we can assume that the cycle has a steady state where

$$\frac{dN_{ATP}}{dt} = \frac{dN_{PPi}}{dt} = cte\,. \tag{6}$$

Equation (6) states that the overall rates of PPi generation (luciferase) and consumption (ATP-sulfurylase) are equal, therefore if the turnover rate of luciferase and ATP-sulfurylase are k_L and k_a, respectively, then

$$k_L\left(\frac{N_{ATP}(t)}{V}\right) = k_a\left(\frac{N_{PPI}(t)}{V}\right). \tag{7}$$

The turnover rate of ATP-sulfurylase is much larger than luciferase ($k_L << k_a$), therefore

$$\frac{N_{PPI}(t)}{N_{ATP}(t)} = \frac{k_L}{k_a} \approx 0, \tag{8}$$

which means that the relative number of PPi molecules, in the reaction buffer compared to ATP is almost zero during BRC steady state. On the other hand if we assume that the initial number of PPi molecules from the polymerization of the target nucleic acid is $(N_{PPi})_0$, then the total number of ATP plus PPi at any given time in the process will be equal to $(N_{PPi})_0$. Considering (8) we can conclude that the total number of ATP molecules in the BRC steady state is then equal to the total initial number of PPi molecules, $(N_{PPi})_0$ introduced to the system:

$$N_{ATP}(t) = (N_{PPi})_0. \tag{9}$$

Equation (9) states that the photon generation process is only a function of the turnover of luciferase rather than ATP-sulfurylase. The simplified equation expressing light intensity *I*, is

$$I = \alpha \cdot \frac{d}{dt}\left(\frac{N_{ATP}}{V}\right) = \left(\frac{\alpha \cdot k_L}{V}\right) \cdot N_{ATP}(t), \tag{10}$$

or

$$I = \left(\frac{\alpha \cdot k_L}{V}\right) \cdot (N_{PPi})_0. \tag{11}$$

V is the volume of the reaction buffer, and *a* is the quantum efficiency of the bioluminescence process. Combining (11) with the number of nucleic acid molecules in the solution (3) we have:

$$I = \left(\frac{\alpha \cdot k_L}{V}\right) \cdot N_{NA} \cdot (L_{NA} - L_P). \tag{12}$$

This shows the linear proportionality of generated light intensity to the initial number of nucleic acid molecules. If we accumulate photons for a time interval *T* (integration time), then the total number of captured photons N_{ph} is

$$N_{ph} = \left(\frac{\alpha \cdot k_L}{V}\right) \cdot T \cdot N_{NA} \cdot (L_{NA} - L_P). \tag{13}$$

The total number of photons received by the detector (e.g. CCD camera) depends on the integration time and the number of molecules present in the solution. By controlling the integration time we are able to change the gain of the system and potentially increase the sensitivity. In typical chemiluminescence assays, where no enzymatic feedback is present (e.g. Pyrosequencing[16]), we introduce $N = N_{NA}$ substrate molecules which are going to be converted to αN_{NA} photons, where α is the quantum efficiency. If we introduce the same number of molecules to the BRC process and compare the total photons generated in a time interval T, then the signal amplification of the BRC system is defined by

$$A_E = \left(\frac{\alpha \cdot k_L}{V}\right) \cdot \frac{T \cdot N_{NA} \cdot (L_{NA} - L_P)}{\alpha \cdot N} = \left(\frac{k_L}{V}\right) \cdot T \cdot (L_{NA} - L_P). \quad (14)$$

3.4 Noise and background light

There are two different phenomena in BRC which ultimately limit the performance and sensitivity of the system. One is the possibility of PPi and ATP background contamination by chemicals used in the buffer solution and the other is the inherent noise of the detector (e.g. thermal and shot noise in a photodiode system). To model ATP, PPi or any other light generating chemicals in the presence of enzymatic cycle, we refer them to an equivalent number of PPi molecules, C_{PPi}. Therefore (12) can be rewritten as:

$$I = \left(\frac{\alpha \cdot k_L}{V}\right) \cdot \left[N_{NA} \cdot (L_{NA} - L_P) + C_{PPi}\right]. \quad (15)$$

Although C_{PPi} is relatively low for common bioluminescence assays (on the order of 0.1 to 1 fmoles), it can be an order of magnitude higher than the target nucleic acid concentration for gene expression applications. It is also possible for the value of C_{PPi} to vary between experiments by as much as 2.5 fold. To eliminate the measurement effects of any possible background, the light intensity of the system is also measured in the absence of any PPi generated from polymerization. This control measurement serves as a reference point for quantification of the catalytically produced PPi. If the light intensity in the reference state is I_r, by combining equations (11) and (15) the value of N_{NA} is

$$N_{NA} = \left(\frac{V}{\alpha \cdot k_L}\right) \cdot \frac{I - I_r}{L_{NA} - L_p}, \quad (16)$$

and in terms of number of photons detected

$$N_{NA} = \left(\frac{V}{\alpha \cdot k_L}\right) \cdot \frac{N_{ph} - N_{phr}}{T \cdot (L_{NA} - L_p)}. \quad (17)$$

To take into account the noise of the system, we assume that the total noise of the detector in the measurement $n(t)$ is random and has a normal distribution $N(0,s)$ (mean of zero, and standard deviation of s). Thus, the apparent light intensity in the presence of detector noise is given by

$$I(t) = \left(\frac{\alpha \cdot k_L}{V}\right) \cdot N_{NA} \cdot (L_{NA} - L_P) + n(t). \quad (18)$$

Integrating (18) over a time interval T,

$$N_{ph} = \int_T I(\tau) d\tau = \left(\frac{\alpha \cdot k_L}{V}\right) \cdot N_{NA} \cdot (L_{NA} - L_P) T + \int_T n(\tau) d\tau. \quad (19)$$

As we can see in (19) the number of photons generated in a time interval T consists of a deterministic value plus the measurement noise integrated over a time interval T. The new noise term has a normal distribution with mean and standard deviation of zero and $\sigma/\sqrt{T}$ respectively, so the signal to noise power becomes:

$$\left(\frac{S}{N}\right)=\left(\frac{\alpha\cdot k_L N_{NA}\cdot(L_{NA}-L_P)}{V\sigma^2}\right)\cdot T \tag{20}$$

Now in order to calculate the error distribution of the measurement, initially we express the calculated number of nucleic acids, N'_{NA}, as

$$N'_{NA}=\left(\frac{V}{\alpha\cdot k_L}\right)\cdot\frac{\int_T I(\tau)d\tau}{(L_{NA}-L_p)\cdot T}=\left(\frac{V}{\alpha\cdot k_L}\right)\cdot\frac{N_{ph}-N_{phr}+\int_T(n_1(\tau)-n_2(\tau))d\tau}{(L_{NA}-L_p)\cdot T}, \tag{21}$$

where $n_1(t)$ and $n_2(t)$ are the noise introduced by the detector in the actual and reference experiment. We assume that $n_1(t)$ and $n_2(t)$ are uncorrelated but both have the same normal distribution of *N(0,s)*. Now (17) can be rewritten as

$$N'_{NA}=N_{NA}+n'(t), \tag{22}$$

and $n'(t)$, the error distribution, is ultimately defined as

$$N'_{NA}-N_{NA}=n'(t)\rightarrow N\left(0,\sqrt{\frac{2}{T}}\cdot\frac{V\sigma}{\alpha\cdot k_L(L_{NA}-L_p)}\right). \tag{23}$$

4. RESULTS

This study was first designed to demonstrate that an enzymatic regenerative cycle for the luciferase reaction was possible and then to assess the performance characteristics of an assay based on this reaction. In order to observe the kinetics of the BRC and compare to other ATP- based assays, we introduced different ATP concentrations into the enzymatic complex and the light intensity for multiple experiments was measured. Our first observation was that the total photon generation was orders of magnitude higher than ATP molecules present in the solution (Fig 6a, 7a). If luciferase were the only enzyme controlling the photon generating kinetics, then we would expect, in the best case, a single photon per ATP molecule. This proves that a regenerative process is in fact present in our enzymatic system. By measuring the light intensity over a long interval we observed that, although the light intensity was constant, a very slow exponential decay with a time constant of approximately 7-8 min did exist. This observation also fits well with our hypothesis, that the energy source for photon emission comes from APS and luciferin, which eventually would be consumed and ultimately become the limiting factors for the luciferase and ATP-sulfurylase kinetics.

By measuring the light intensity from different samples , we observed a backgound signal, even without the addition of excess PPi from DNA polymerization. The level of this background, which we used as a reference for all our measurements, was comparable to up to 0.1 fmol of ATP in 40 µl. This background varied almost up to 2.5 fold between independent reaction mixes, but remained constant within a given experiment. There are two possible explanations for the background signal. First, there may be PPi or ATP residue from either luciferse or ATP-sulfurylase mixes, or there may be intrinsic chemiluminescence of luciferase at high concentrations. The background light in our experiments was always subtracted from the sample signal, and the total value correlated to the sample was derived. By using this method we were able to successfully differentiate between nucleic acid concentrations ranging over six orders of magnitude, from 1amol (10^{-18} mol) to 0.1 pmol (10^{-12} mol) (PCR product and self-primed oligo; Fig. 6 and 7).

By using the CCD camera for measurements we were able to detect a signal form 10 amole to 100 fmol of the target molecule using both the self-primed oligonucleotide and a 230bp PCR product (Fig.6). The background luminescent light, in the presence of the BRC assay, was approximated to be 80 amoles of equivalent ATP molecules in 40 µl

reaction buffer, which corresponds to a concentration of 2.5 pM of substrate for luciferase. The integration time was 1sec.

The luminometer had worse noise performance, which was compensated by increasing the integration time from 1 sec to 10 sec. The performance of the two detectors with the modified integration time was then comparable (Fig 7) and we were able to detect 1amol to 100 amol of target in 20 µl for both the self-primed oligonucleotide (oligo-loop) and the 230bp PCR product (MBP) with luminometer.

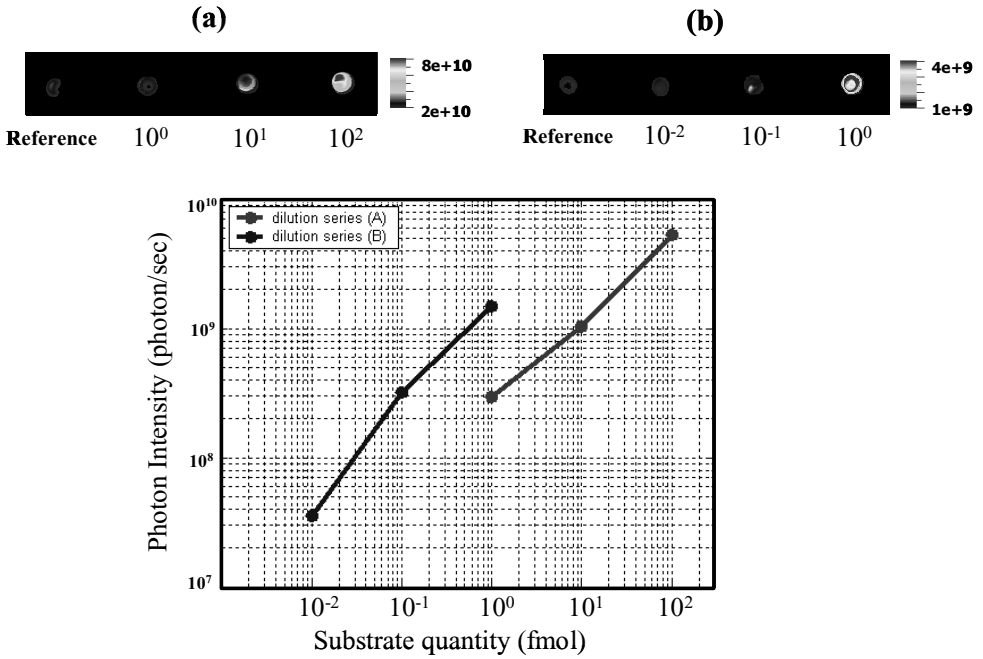

Figure 6: Photon intensity (photon/sec) measured by CCD imaging systems in a 96-well microtiter plate format from (a) 1 to 100 fmol of 40bp oligo-loop and (b) 10amol to 1fmol of 230bp PCR product (Maltose binding protein – MBP).

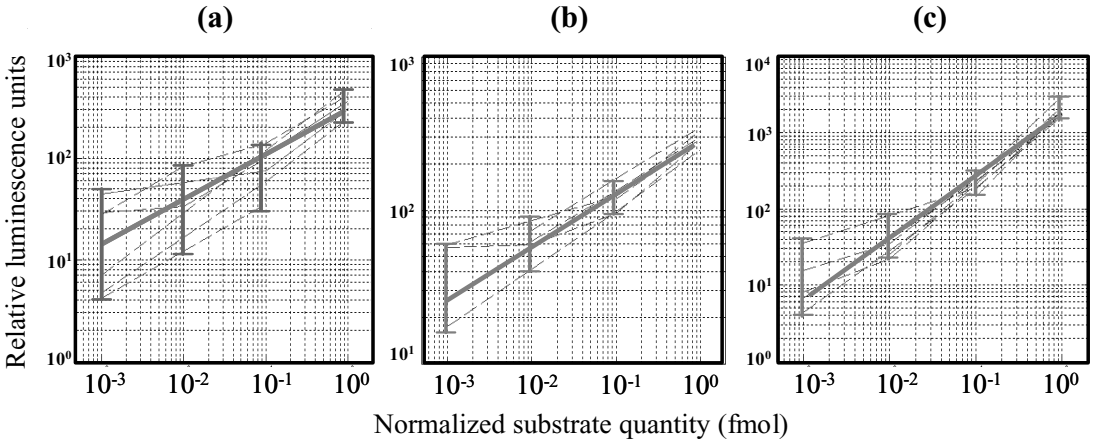

Figure 7: Relative luminescence units measured by luminometer. Results are independent but identical normalized 1fmol to 1amol dilution series (incorporated dNTPs) for (a) ATP (b) 40bp oligo-loop (c) 230bp PCR product (Maltose binding protein – MBP)

5. CONCLUSION

In BRC the bioluminescence photon emission rate was steady and a monolithic function of the introduced PPi. The regeneration characteristic of the BRC system stabilized the light intensity and made the photon emission rate relatively steady for long periods of time. This system could potentially generate orders of magnitude more photons than most luciferase assays. The steady photon emission in this system is also a linear function of the nucleic acid concentration for less than 10^{-8} M, and by adjusting the integration time we are able to achieve a dynamic range of 5 orders of magnitude for the nucleic acid quantity using commercially available CCD imaging systems and luminometers. This methodology can be used as an alternative label-free optical detection methodology for parallel in vitro nucleic acid quantification for detecting DNA at concentrations of 1 amol. Direct applications of BRC are gene expression, DNA or RNA quantification and single nucleotide polymorphism (SNP) detection. The practical challenges of this approach are reducing background light, pre-experiment enzyme and substrate purification, and biochemical optimization for the above applications.

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