

# Reciprocating flow ATP amplification system for increasing the number of amplification cycles

Tetsuya Satoh <sup>a,b</sup>, Kosuke Tsuruta <sup>b</sup>, Yasuharu Shinoda <sup>b</sup>, Ryuichi Hirota <sup>b</sup>,

Kenichi Noda <sup>a,b</sup>, Akio Kuroda <sup>a,b</sup>, Yuji Murakami <sup>a,b\*</sup>

<sup>a</sup>*Research Institute for Nanodevice and Bio Systems, Hiroshima University,*

*Higashi-Hiroshima, Hiroshima 739-8530, Japan*

<sup>b</sup>*Department of Molecular Biotechnology, Graduate School of Advanced Sciences of Matter,*

*Hiroshima University, Higashi-Hiroshima, Hiroshima 739-8530, Japan*

\*To whom correspondence should be addressed.

Department of Molecular Biotechnology, Graduate School of Advanced Sciences of Matter,

Hiroshima University, Higashi-Hiroshima, Hiroshima 739-8530, Japan

Tel: +81 82 424 4517

Fax: +81 82 424 7047

E-mail: [yuji@hiroshima-u.ac.jp](mailto:yuji@hiroshima-u.ac.jp)

## **Abstract**

We constructed a novel ATP amplification reactor using a reciprocating flow system to increase the number of ATP amplification cycles without an increase in back pressure. We previously reported a continuous-flow ATP amplification system that effectively and quantitatively amplified ATP and increased the sensitivity of a quantitative bioluminescence assay. However, it was difficult to increase the number of amplification cycles due to back pressure in the system. Because addition of immobilised adenylate kinase (ADK) and pyruvate kinase (PK) columns increased back pressure, the maximum number of ATP amplification cycles within column durability was only four. In this study, ATP amplification was performed using a reciprocating flow system, and 10 cycles of ATP amplification could be achieved without an increase in back pressure. As a result, ATP was amplified more than 100-fold after 10 cycles of reciprocating flow. The gradient of ATP amplification was approximately  $1.76^N$ . The back pressure on the columns was 0.03 MPa in 1-10 ATP amplification cycles, and no increases in back pressure were observed.

*Keywords:* ATP amplification; reciprocating flow system; bioluminescence assay

## Introduction

The firefly luciferase bioluminescence assay for detection of ATP is a well-established technique [1]. Because it is useful for rapidly detecting and quantifying a broad range of ATP concentrations, the assay has been employed in many areas of industry [2-4]. Thus, improvements in technology are important, particularly for increasing the intensity of bioluminescence. At low concentrations of ATP, relatively low bioluminescence is too weak to be detected by a luminometer and hinders development of technologies employing the bioluminescence assay.

Several new methods to increase sensitivity have recently been reported [5-8]. For example, Fujii *et al.* developed a modified luciferase that generates more than 10-fold greater luminescence intensity than the wild-type and could be used to detect amol ( $10^{-18}$  mol) levels of ATP [8].

We have also enhanced the sensitivity of ATP detection by developing an ATP amplification reaction employing (i) adenylate kinase (ADK) to convert AMP + ATP into two molecules of ADP, (ii) pyruvate kinase (PK) or polyphosphate kinase (PPK) to convert ADP back to ATP (ATP amplification) and (iii) a commercially available firefly luciferase (Fig. 1). This technique was effective for increasing the intensity of bioluminescence [9]. ATP was amplified depending on initial ATP concentrations, and the intensity of bioluminescence increased 100-fold. The bioluminescence assay coupled with ATP amplification was able to detect amol

levels of ATP and 1 colony forming unit (CFU) of bacteria, providing sufficient sensitivity for some industrial applications [9].

We have also recently reported on a continuous-flow ATP amplification system that allows us to control the number of ATP amplification cycles. That system is an effective way of performing reproducible ATP amplification and increasing the sensitivity of ATP quantification by the bioluminescence assay [10]. However, the overall assay time and complication are simply added to the standard luciferase assay, because ATP amplification is an additional pretreatment step. In the continuous flow system, it was difficult to increase the number of amplification cycles due to back pressure in the system. Because addition of immobilised ADK and PK columns increased the back pressure, the maximum number of ATP amplification cycles within column durability was only four.

The fundamental pressure loss of a tandem repeat method in continuous flow was also the main problem in the optimization of column length with the microchip format, and the maximum number was six [12]. These continuous amplifications were able to amplify ATP, and contributed to understanding for the optimization of the homogeneous ATP amplification reaction. The amplified ATP, however, contained contaminants that inhibited luciferase, which resulted in a slight overall improvement of lower detection limit. It is necessary to develop a new method to avoid the fundamental pressure drop increase for the future development of a portable hygiene inspection apparatus with simple photosensor and an analytical instrument for

biochemistry research. Even though highly purified reagents were used, the amplification reagents in previous research still contained a certain level ADP especially in reagent AMP.

A repeated injection method was examined to avoid the back pressure increase. In this method, the product from a system with a single ADK-PK column was repeatedly injected back into the system. A sample of ATP was exponentially amplified as a continuous-flow ATP amplification system. However, the repeated injection of sample carries the risk of ATP contamination, and this method is also impractical due to a complicated flow system. In this study, we constructed a novel ATP amplification reactor using a reciprocating flow system. Modified purification method for reagent was also described.

### **Principle**

Two immobilised PK areas and one ADK area were prepared in the order of the reaction steps (PK-ADK-PK) (Fig. 2). Separately immobilised enzymes catalyse their reactions independently, and the reaction is controlled by flow rate to reach equilibrium. The first area of immobilised PK is not involved in the amplification reaction when the reaction mixture flows in the regular way, but is in the reverse way (Fig. 2). ATP is amplified 2-fold by passing over the immobilised ADK area and the second PK area that drives the equilibrium of the reactions catalysed by ADK and PK toward ADP and ATP, respectively. Next, the amplified ATP is again amplified 2-fold with reverse flow. The number of ATP amplification cycles can be further increased in a reciprocating flow system by repeating this cycle. In the previous

continuous ATP amplification system, ADK- and PK-immobilised columns were added to increase the number of ATP amplification, resulting in the increase of the back pressure. While the back pressure remains constant in a reciprocating flow system, because the additional area is not required to increase the number of the cycles. Therefore, the reciprocating flow ATP amplification system would successfully increase the number of ATP amplification cycles without limitation and thus the sensitivity of bioluminescence assay.

## **Materials and methods**

### *Chemicals*

PK, ADK, ATP and phosphoenolpyruvate (PEP) were obtained from Sigma-Aldrich (Missouri, USA). AMP was obtained from Tokyo Chemical Industry (Tokyo, Japan). The bioluminescence assay kit (CLS II) containing luciferin and luciferase was obtained from Roche (Basel, Switzerland). ATP degradation enzyme was obtained from Kikkoman (Noda, Japan).

### *Removal of ATP and ADP in substrates*

AMP was purified by a method combining recrystallization and reverse phase chromatography. First, 1 g of commercially available AMP was added to 20 ml pure water and suspended. The precipitate was obtained by centrifugation and added to 30 ml pure water. The suspension was dissolved in pure water completely by adding pure water and heating at 98°C. AMP crystals were obtained by slow cooling and centrifugation. The crystals were washed

with methanol and evaporated. Second, 5 mM of recrystallized AMP aqueous solution was purified using a ODS column (TSKgel ODS-120T, Tosoh, Tokyo, Japan) with buffer A: 100 mM phosphate buffer (pH 6.0), and buffer B: 100 mM phosphate buffer (pH 6.0) containing 25% (v/v) methanol. AMP was eluted after 30 min with a linear gradient of 0–100% buffer B. The eluate was diluted 50% (v/v) with pure water and repurified by the above method.

PEP was manually injected into the column immobilised with ATP degradation enzyme. A bottle of ATP degradation enzyme was suspended in 1 ml of a buffer containing 0.5 M NaCl and 0.2 M NaHCO<sub>3</sub> (pH 8.3) and immobilised on 1-ml HiTrap NHS-activated HP columns (GE Healthcare Ltd., Amersham, UK). Immobilisation of ATP degradation enzyme was performed as follows. The column was washed with 6 ml of ice-cold 1 mM HCl. Immediately, 1 ml of ATP degradation enzyme solution was injected into the columns. After 30 min of incubation at 25°C, 6 ml of a blocking buffer containing 0.5 M ethanolamine and 0.5 M NaCl (pH 8.3) was injected into each column in order to inactivate the excess NHS-active groups. Following additional incubation at 25°C for 30 min, the columns were washed with 6 ml of washing buffer containing 30 mM KCl, 5 mM (CH<sub>3</sub>COO)<sub>2</sub>Mg and 50 mM HEPES-NaOH (pH 7.4). Next, 1 ml of 50 nM PEP was injected into the column. After 3 h of incubation of the column at room temperature, injection of 1 ml of buffer containing 30 mM KCl, 50 mM (CH<sub>3</sub>COO)<sub>2</sub>Mg and 50 mM HEPES-NaOH (pH 7.4) extruded PEP from the column.

Purified AMP and PEP were employed in subsequent experiments.

### *Enzyme immobilisation and column preparation*

PK and ADK were each immobilised on 1-ml HiTrap NHS-activated HP columns according to the above method. Buffer C was prepared with 30 mM KCl, 50 mM  $(\text{CH}_3\text{COO})_2\text{Mg}$  and 50 mM HEPES-NaOH (pH 7.4). PK column was washed with 20 ml pre-washing buffer containing 0.5 mM PEP in buffer C and 30 ml buffer C at a flow rate of 1.0 ml/min. The ADK columns were washed with 50 ml buffer C at a flow rate of 1.0 ml/min. The ADK and PK columns were then washed with 40 ml reaction buffer containing 0.1 mM AMP and 0.5 mM PEP in buffer C at a flow rate of 0.5 ml/min. The flow rates in all washing procedures and subsequent experiments were controlled by a syringe pump (IC3200, KD Scientific Inc., MA, USA).

### *Enzymatic reaction and bioluminescence assay*

The PK and ADK columns were prepared by the method described above. Ten ml of reaction mixtures containing 100 nM ADP, 0.1 mM AMP and 0.5 mM PEP in buffer C for PK, and 50 nM ATP, 80  $\mu\text{M}$  AMP and 0.5 mM PEP in buffer C for ADK, were injected into columns with flow rates ranging from 0.1 to 4.0 ml/min at room temperature. For each reaction, 100  $\mu\text{l}$  of reaction mixture was collected from the outlet.

The amount of ATP and ADP in each fraction was measured as follows. For ATP, 5  $\mu\text{l}$  of each fraction was mixed with 45  $\mu\text{l}$  of bioluminescence assay mixture, and bioluminescence was measured using a luminometer (C-100, Kikkoman). For ADP + ATP, 40  $\mu\text{l}$  of each fraction



was mixed with 20  $\mu$ l of a solution containing 10 U/ml PK, 0.5 mM PEP in buffer C, followed by incubation at 37°C for 1 h. When the PK reaction reached equilibrium, 45  $\mu$ l of reaction mixture was added to 5  $\mu$ l of the bioluminescence assay mixture, and bioluminescence was measured using the luminometer. The amount of ADP in each fraction was calculated by subtracting the amount of ATP from that of ATP + ADP.

#### *Reciprocating flow ATP amplification reaction and bioluminescence assay*

PK and ADK columns prepared by the above method were connected in the order of the reaction steps (PK-ADK-PK). Ten ml of reaction mixtures containing various amounts of ATP, 0.1 mM AMP and 0.5 mM PEP in buffer C were injected into the PK-ADK-PK column in regular flow at 2.0 ml/min at room temperature. The reaction mixture was then collected in a syringe from the outlet and injected again from the outlet as reverse flow. The number of amplification cycles was increased by repeating regular and reverse flow. For each reaction, 10  $\mu$ l of reaction mixture was collected in the syringe. From each reaction, 5  $\mu$ l was mixed with 45  $\mu$ l of bioluminescence assay mixture, and bioluminescence was measured using the luminometer. The back pressures on the PK-ADK-PK columns for all cycles were measured by a digital pressure gauge (GC67, Nagano Keiki Co., Ltd., Tokyo, Japan).

## **Results and discussion**

### *Removal of ATP and ADP*

Commercially available AMP and PEP are often contaminated with ATP and ADP. A 1 mM solution of commercial AMP was found to contain 7.6 nM ATP and 200 nM ADP, and a 1 mM solution of commercial PEP was found to contain 12 pM ATP and 25 pM ADP. Purification of AMP by anion exchange chromatography has been reported [10]. Although this method effectively removed ATP and ADP, the AMP was diluted during this purification. No dilution of AMP was observed in the purification method using reverse phase chromatography and the purity was at the same level as anion exchange chromatography. Thus, this method was effective for production of high concentrations of purified AMP. Following purification and treatment, ATP and ADP contamination in the 1 mM solutions of AMP and PEP was reduced to less than 1 pM.

#### *Optimization of flow rate*

To achieve reproducible ATP amplification, ADK and PK reactions in each column must reach equilibrium. Therefore, the reaction time of immobilised ADK and PK must be sufficient to reach equilibrium in each enzymatic reaction. Manipulating the flow rate allowed us to control the reaction time, as flow rate is inversely proportion to residential substrate time. On each immobilised column, reactants reached equilibrium with a flow rate of 2.0 ml/min. Thus, a flow rate of 2.0 ml/min was employed in subsequent experiments.

#### *Back pressure on a reciprocating flow system*

A continuous-flow system had the limitation of the number of ATP amplification cycles due to back pressure. Because the pressure drop increased by adding immobilised ADK and PK columns, the back pressure of five sets of columns exceeded the column durability. Therefore, the number of amplification cycles was limited by column durability. The back pressure was a critical factor in increasing the number of ATP amplification cycles in a continuous-flow system.

In this study, the flow system was improved by a reciprocating flow system, and no increase in the back pressure was observed (Fig. 3). The back pressure on the PK-ADK-PK column was 0.12 MPa in the amplification reaction. A reciprocating flow system thus avoids the limitation of the number of amplification cycles, and it should theoretically be able to perform a quantitative ATP amplification reaction without the fundamental pressure limits.

#### *Magnesium concentration in the PK reaction*

Preliminary reciprocating flow ATP amplification was performed with a low magnesium concentration reaction mixture containing various amounts of ATP, 0.1 mM AMP and 0.5 mM PEP, 30 mM KCl, 5 mM  $(\text{CH}_3\text{COO})_2\text{Mg}$  and 50 mM HEPES-NaOH (pH 7.4). The amplification gradient is theoretically  $2^N$  ( $N$  = number of ATP amplification cycle), and experimentally  $\mathbf{a}^N$ . The base part of the amplification gradient,  $\mathbf{a}$ , is the gradients of logarithm of the bioluminescence intensity with respect to the number of amplification cycles. It is better that the value of  $\mathbf{a}$  is independent to initial ATP concentration, but the preliminary results show

that higher initial ATP gave lower value of **a**. The PK reaction is inhibited by high concentration of ATP, and the inhibition of PK varies depending on ATP concentration at low magnesium concentrations [11]. The activities of PK in the presence of 5 mM ATP and the absence of ATP were measured with various magnesium concentrations. At low magnesium concentrations, the activity of PK was lower in the presence of 5 mM ATP than it was in the absence of ATP (Fig. 4). However, no differences in activities were observed with 50 mM magnesium. These data suggest that an increase in magnesium concentration should reduce the variation in PK activity and the amplification gradient.

#### *Reciprocating flow ATP amplification reaction*

ATP was amplified in the reciprocating flow system (Fig. 5). Although only 4 cycles of ATP amplification were performed in a continuous-flow system, 10 cycles of the ATP amplification reaction were accomplished in a reciprocating flow system. The bioluminescence was amplified quantitatively by repeating regular and reverse flow. The amplification gradients of the bioluminescence assay were quantified as  $1.74^N$  ( $R^2 = 0.9952$ ) when 5 nM ATP was added into the reaction initially,  $1.77^N$  ( $R^2 = 0.988$ ) for 500 pM ATP and  $1.76^N$  ( $R^2 = 0.9757$  among  $N = 5 - 10$ ) for 50 pM ATP, and no significant differences were observed among these gradients. Although 50 pM of ATP was not detectable by the bioluminescence assay without amplification, it could be detected by increasing the amplification cycles. These data suggest that a reciprocating flow ATP amplification system was effective in increasing the number of

ATP amplification cycles and performance of quantitative ATP amplification. The data also suggest that the reagents for amplification still contains ATP or ADP that is not detectable by the standard luciferase assay but detectable with the reciprocating amplification pretreatment. Further purification of the reagents may improve the lower detection limit of the ATP assay.

#### *Reciprocating flow ATP amplification system for bioluminescence assay*

Various amounts of ATP were amplified by 10 cycles of the reciprocating flow ATP amplification system (Fig. 6). The bioluminescence was amplified more than 100-fold after 10 cycles of amplification, and there was a significant linear correlation between initial ATP and bioluminescence.

The lower ATP quantification limit of the bioluminescence assay without amplification was 0.5 nM (sample volume: 5  $\mu$ l) due to the detection limit of the luminometer, and the limit with continuous flow pretreatment was 50 pM due to the pressure drop limit. However, the amplified 5 pM sample could be quantified after 10 cycles of ATP amplification. The sensitivity of the bioluminescence assay was increased approximately 100-fold after 10 cycles of ATP amplification. Thus, a reciprocating flow system can increase the sensitivity of the bioluminescence assay better than a continuous-flow system.

#### *AMP concentration in the reaction mixture*

Commercially available AMP was purified and employed in this study. The purification effectively removed ATP and ADP from AMP. However, it was difficult to remove ultra low

concentration of ATP and ADP (less than 1 pM) in AMP solution even after this purification, which gave background ATP and resulted in a poorer ATP quantification limit. On the other hand, the amplification reaction requires an excess of AMP. Thus, the AMP concentration in a reaction mixture for ATP amplification reaction determined the allowance range of the amount of ATP for amplification. Therefore, AMP concentration is a key parameter in this ATP amplification system.

Decreasing AMP concentration allowed us to improve the lower detection limit of the bioluminescence assay with ATP amplification. We observed a significant difference in bioluminescence between background ATP and the amplified 0.5 pM ATP (initial) after 10 cycles of reciprocating flow ATP amplification with a reaction mixture containing 0.01 mM AMP. The value of bioluminescence was  $1.13 \times 10^3$  relative luminescence unit (rlu) for background ATP,  $1.78 \times 10^3$  rlu for 0.5 pM ATP and  $1.91 \times 10^3$  rlu for 5 pM ATP. These data suggest that decreasing AMP concentration could increase the sensitivity of the bioluminescence assay with ATP amplification.

## **Conclusion**

We devised and tested a novel ATP amplification reactor using a reciprocating flow system, and this allowed us to increase the number of ATP amplification cycles without increases in back pressure. Although only 4 cycles of ATP amplification could be performed in a continuous-flow system, 10 cycles of ATP amplification reaction were accomplished in a

reciprocating flow system. Furthermore, this system is an effective means of increasing the sensitivity of ATP quantification by the bioluminescence assay. Our method is able to amplify ATP without the fundamental pressure limitation and may allow us to achieve a highly sensitive bioluminescence assay.

### **Acknowledgements**

This work was supported in part by the Interdisciplinary Research on Integration of Semiconductor and Biotechnology Program, Hiroshima University, of the Ministry of Education, Culture, Sports, Science and Technology of Japan.

## References

- [1] M. DeLuca, W.D. McElroy, Kinetics of the firefly luciferase catalyzed reactions, *Biochemistry* 26 (1974) 921–925.
- [2] D. Champiat, N. Matas, B. Monfort, H. Fraass, Applications of biochemiluminescence to HACCP, *Luminescence* 16 (2001) 193–198.
- [3] P. Nyren, Enzymatic method for continuous monitoring of DNA polymerase activity, *Anal. Biochem.* 167 (1987) 235–238.
- [4] M. Ronaghi, Pyrosequencing sheds light on DNA sequencing, *Genome Res.* 11 (2001) 3–11.
- [5] J.A. Cruz-Aguado, Y. Chen, Z. Zhang, N.H. Elowe, M.A. Brook, J.D. Brennan, Ultrasensitive ATP detection using firefly luciferase entrapped in sugar-modified sol–gel-derived silica, *J. Am. Chem. Soc.* 126 (2004) 6878–6879.
- [6] T. Sakakibara, S. Murakami, K. Imai, Enumeration of bacterial cell numbers by amplified firefly bioluminescence without cultivation, *Anal. Biochem.* 312 (2003) 48–56.
- [7] K. Noda, T. Matsuno, H. Fujii, T. Kogure, M. Urata, Y. Asami, A. Kuroda, Single bacterial cell detection using a mutant luciferase, *Biotechnol. Lett.* 30 (2008) 1051–1054.
- [8] H. Fujii, K. Noda, Y. Asami, A. Kuroda, M. Sakata, A. Tokida, Increase in bioluminescence intensity of firefly luciferase using genetic modification, *Anal. Biochem.* 366 (2007) 131–136.



- [9] T. Satoh, J. Kato, N. Takiguchi, H. Ohtake, A. Kuroda, ATP amplification for ultrasensitive bioluminescence assay: detection of a single bacterial cell, *Biosci. Biotechnol. Biochem.* 68 (2004) 1216–1220.
- [10] T. Satoh, Y. Shinoda, M. Alexandrov, A. Kuroda, Y. Murakami, Continuous-flow ATP amplification system for increasing the sensitivity of quantitative bioluminescence assay, *Anal. Biochem.* 379 (2008) 116–120.
- [11] H. Holmsen, E. Storm, The adenosine triphosphate inhibition of the pyruvate kinase reaction and its dependence on the total magnesium ion concentration, *Biochem. J.* 112 (1969) 303–316.
- [12] T. Satoh, Y. Shinoda, S. Tokonami, R. Hirota, K. Noda, A. Kuroda, Y. Murakami, Continuous-flow ATP amplification system on a chip, *Sens. Actuators*, accepted.

## Figure legends

Fig. 1. ATP amplification reaction. ADK, PK and excess AMP and PEP were prepared in the reaction mixture. The ATP amplification reaction started when ATP was added to the reaction mixture and ended when endogenous AMP was converted to ATP.

Fig. 2. Principle of a reciprocating flow ATP amplification reaction. ATP is amplified 2-fold by being passed over the immobilised ADK area and the second PK area under conditions driving the equilibrium of the reactions catalysed by ADK and PK toward ADP and ATP formation with regular flow. Next, amplified ATP is again amplified 2-fold with reverse flow. The number of ATP amplification cycles is increased by repeating regular and reverse flow, and the number of cycles is controlled by the number of flow cycles.

Fig. 3. Comparison between a reciprocating flow system (●) and a continuous-flow system (□). The back pressure increased by adding immobilised ADK and PK columns in a continuous-flow system. However, no increase in the back pressure was observed on the PK-ADK-PK column in a reciprocating flow system.

Fig. 4. Effect of variation in the magnesium concentration on the activity of PK in the absence (●) and presence (□) of 5 mM ATP.

Fig. 5. Correlation between the number of reciprocating flow ATP amplification cycles and bioluminescence. Initial concentration of samples of (●) 5 nM, (■) 500 pM, (▲) 50 pM, and (○) without ATP was applied to the reciprocating flow amplification.

Fig. 6. Comparison of a bioluminescence assay (■) with and (×) without reciprocating flow ATP amplification. The previous data of continuous flow ATP amplification(◆) using different luciferase and less purified AMP [10] were plotted for comparison. The gradient of fitting lines is set to 1 to show proportional relation.

Figure 1

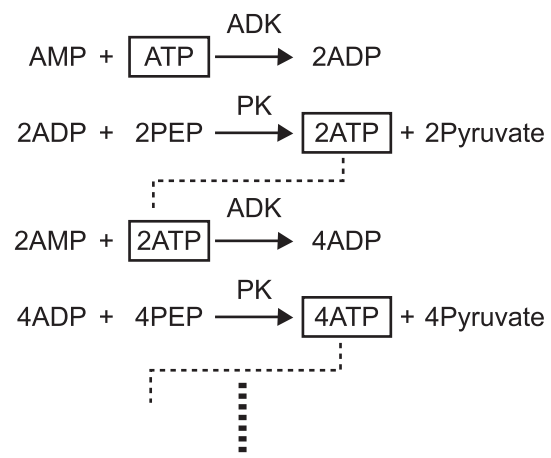


Figure 2

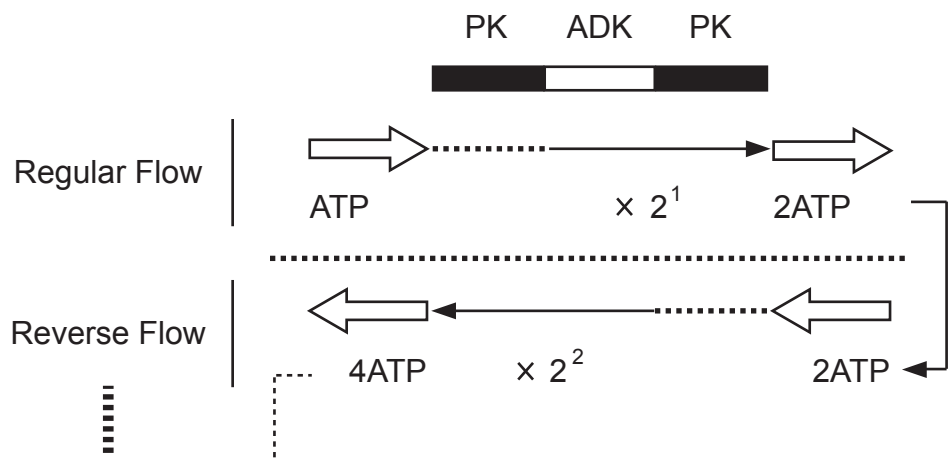


Figure 3

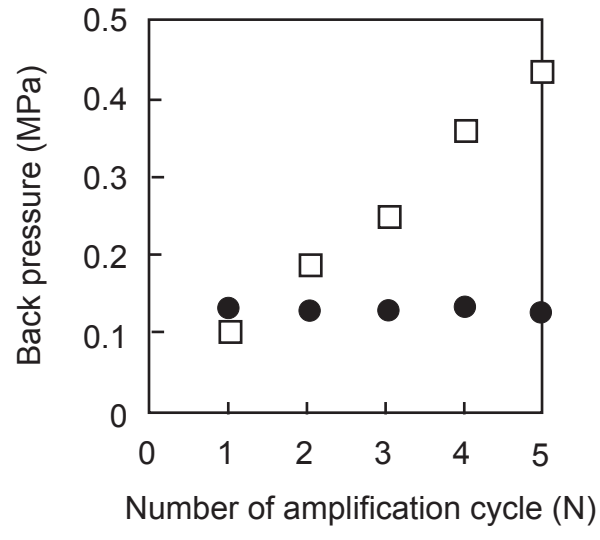


Figure 4

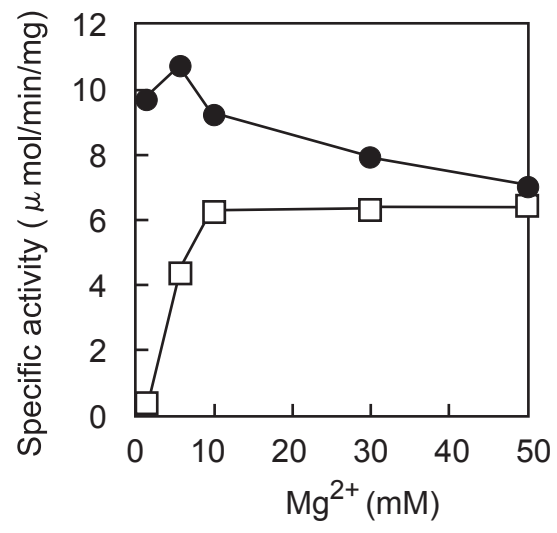


Figure 5

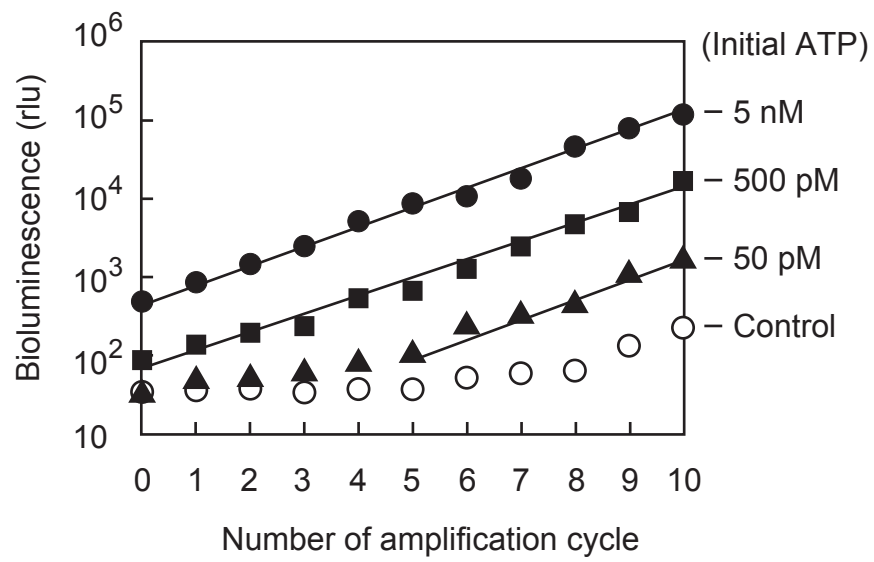




Figure 6

