Endotoxin Assay by Bioluminescence Using Mutant Firefly Luciferase

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Abstract

The Limulus reaction is an application of the defense mechanism of horseshoe crab for endotoxin detection. Endotoxin is a component of cell wall in the outer membrane of Gram-negative bacteria, and causes fever or shock when it enters the human blood stream. For endotoxin detection, gel formation or turbidity of the coagulation factor chromogen, or fluorescence modified-peptide are used. However, these conventional methods have problems with regard to their measurement time or sensitivity. We recently obtained a mutant firefly luciferase that has a luminescence intensity over 10-fold higher than that of the wild type. Therefore, we developed a new endotoxin detection method that combines the Limulus reaction and bioluminescence using mutant luciferase. The new method detects 0.0005 EU/ml of endotoxin within 15 min.

Keywords: Bioluminescence; Endotoxin; Firefly luciferase; Limulus amebocyte lysate

Endotoxin (lipopolysaccharide) is a component of cell wall in the outer membrane of Gram-negative bacteria and can be fatal to the human when it enters the bloodstream. Limulus amebocyte lysate (LAL) is a coagulation system that is induced by endotoxin [1-3]. There are several endotoxin detection methods employing the so-called Limulus reaction using LAL. Methods using turbidimetric and chromogenic end-points, as well as turbidimetric and chromogenic kinetic methods are commonly used for endotoxin detection [4-7]. However, the two end-point methods have problems with their sensitivities (detection limit is about 0.01–0.1 EU/ml) and the kinetic methods have a problem with the measurement time (over 60 min) [8].

Bioluminescence detection has a high signal-to-noise ratio compared with other optical detection methods, such as fluorescence and chromogenic detection methods. We previously constructed a genetically modified North American firefly (*Photinus pyralis*) luciferase that generates a luminescence intensity at least 10-fold greater than that the wild-type luciferase [9]. Using this mutant luciferase, it was possible to detect a single *Escherichia coli* cell [10]. In this study, we report that a bioluminescence test using mutant firefly luciferase combined with the Limulus reaction provides rapid and highly sensitive endotoxin detection (Fig. 1).

Materials and methods

Preparation of endotoxin and LAL reagent

A lipopolysaccharide prepared from *E. coli* UKT-B (a product of Wako Pure Chemical Industries, Tokyo, Japan) was used as a standard endotoxin. A standard endotoxin solution was prepared using autoclaved distilled water (121°C, 90 min) and diluted for calibration. Limulus ES Single Test Wako (Wako Pure Chemical Industries) was used as the LAL reagent. All other materials were of the highest purity commercially available. All experimental instruments and solvents were autoclaved at 121°C for 90 min.

End-point chromogenic method

Tert-butoxycarbonyl-Leu-Gly-Arg-*p*-nitroanilide (peptidyl-pNA) was used as a chromogenic substrate (Seikagaku Biobusiness, Tokyo, Japan). The endotoxin end-point chromogenic assay was performed with LAL (QCL-1000; Lonza Group Ltd., Basel, Switzerland) according to the manufacturer's instructions. The reaction mixture containing 50 µl each of endotoxin and LAL was incubated at 37°C for 10 min. Then 100 µl of peptidyl-pNA solution was added and the mixture was incubated at 37°C. After incubation for 5 min, 100 µl of 25% v/v acetic acid was added to terminate the reaction and the liberated pNA was measured at 405 nm using a DU 800 UV/visible spectrophotometer (Beckman Coulter Inc., Fullerton, CA).

Preparation of luciferase FM

We obtained a mutant North American firefly (*P. pyralis*) luciferase that generates luminescence intensity more than 10-fold higher than that of wild-type luciferase [**9**]. This mutant luciferase named luciferase FM was used for endotoxin detection in this study. The enzyme was dissolved in TMAT buffer (50 mM Tris-HCl buffer, pH 8.0, containing 1 mM magnesium acetate and 100 g/l trehalose) for the LAL assay.

Bioluminescence method

Luciferin modified peptide benzoyl-Leu-Gly-Arg-aminoluciferin (peptidyl-Luc) was designed with reference to the cleavage point in coagulogen and was synthesized at our request by ABD Bioquest Inc. (Sunnyvale, CA). It was used as the substrate for endotoxin detection by luminescence [11]. Peptidyl-Luc was dissolved in dimethylsulfoxide, diluted with TMAT buffer, and its volume adjusted to obtain a concentration of 75 µM. A reaction mixture containing 50 µl each of endotoxin and LAL was incubated at 37°C for 10 min. Then, 50 µl peptidyl-Luc was added and the mixture was incubated at 37°C. After incubation for 5 min, 50 µl each of 10 µM ATP and 250 µg/ml luciferase FM were added and mixed immediately, and luminescence intensity was measured using Lumitester C-110 (Kikkoman Corporation, Chiba, Japan).

Results

Comparison of luciferase FM with wild-type luciferase

The D-aminoluciferin detection test results are shown in Fig. 2. The symbols represent the mean of five replicates for each measurement. Wild-type luciferase was unable to detect less than 10 pM D-aminoluciferin. However, luciferase FM detected D-aminoluciferin at 1 pM, and a linear correlation between luminescence and D-aminoluciferin concentration was observed. The relative luminescence intensity of luciferase FM at 1 pM D-aminoluciferin was 41 ± 4 relative luminescence units (RLU). Luciferase FM thus showed better performance in detecting D-aminoluciferin for endotoxin detection.

Optimization of the concentration of LAL in the luciferase FM-bioluminescence assay system

When we measured the luminescence intensity of luciferase FM to D-aminoluciferin on adding the amount of LAL specified by the manufacturer, we found that the presence of LAL inhibited the bioluminescence reaction. In this condition, the luminescence intensity in the presence of LAL was decreased to 1/10 of the level measured in its absence (Figure 3). Moreover, decrease of the amount of LAL in the luciferase FM bioluminescence assay was required to achieve high sensitivity for detecting endotoxin. Therefore, we sought to optimize the amount of LAL for endotoxin detection by bioluminescence. Reaction mixtures containing 50 µl of 0.05

EU/ml endotoxin and various concentrations of LAL (0–0.48 mg/ml) were incubated at 37°C for 10 min. Then, 50 µl of peptidyl-Luc substrate was added and the mixture was incubated at 37°C. After incubation for 5 min, 50 µl each of 10 µM ATP and 250 µg/ml luciferase FM were added and mixed immediately, and luminescence intensity was measured. Figure 4 shows the relationship between luminescence intensity and the amount of LAL. Luminescence intensity at 0.2 mg/ml LAL was almost twice as high as that at 0.48 mg/ml LAL. The optimum concentration of LAL for endotoxin detection using bioluminescence was half of that specified in the manufacturer's instructions.

Comparison of the luciferase FM-bioluminescence method with the end-point chromogenic method

Endotoxin calibration using the end-point chromogenic and bioluminescence methods is shown in Fig. 5. A linear correlation between absorbance at 405 nm and endotoxin concentration was observed in the concentration range of 0.1 to 1.0 EU/ml. On the other hand, a linear correlation between luminescence intensity and endotoxin concentration was observed in the concentration range of 0.0005 to 0.1 EU/ml, when optimized for the amount of LAL. The detection limit for the end-point chromogenic method was 0.1 EU/ml for a reaction time of 15 min. On the other hand, the detection limit value of bioluminescence using luciferase FM was 0.0005 EU/ml for a reaction time of 15 min. The endotoxin detection limit for bioluminescence using wild-type

luciferase was 0.01 EU/ml (Table 1). The sensitivity of the bioluminescence method using Luciferase FM was 200-fold better than that of the end-point chromogenic method and 20-fold better than the bioluminescence method using wild type luciferase.

Discussion

Endotoxin has been considered by many to be the most important toxin involved in the pyrogenic response (increase in body temperature) and several reports showed that a low concentration of endotoxin caused fatal symptoms of shock [12]. In the pharmaceutical industry, the current requirement is for a highly sensitive measurement method that is able to detect 0.1 pg/ml (equivalent to 0.0007 EU/ml) endotoxin for human health [8].

The end-point methods involve a protease reaction between the native coagulogen or chromogenic peptide and LAL, which results in the production of increasing turbidity or color in the presence of endotoxin [4, 5]. The reaction times for these methods are 15–30 min and the detection limit for endotoxin is 0.1–0.01 EU/ml. The kinetic chromogenic method, based on the time of onset of increasing turbidity or the color reaction, depends on the amount of endotoxin and is used for the detection of small amounts of endotoxin. The endotoxin detection limit of the kinetic chromogenic method is 0.002 EU/ml in 76 min, and for the turbidimetric kinetic method, it is 0.0005 EU/ml in 138 min [6, 7, 13]. Therefore, the end-point methods are mainly used as

primary tests for endotoxin contamination in products and the kinetic methods are used for the detection of low levels of endotoxin, for example in clinical treatments [14-16]. There is a trade-off between measurement time and sensitivity to endotoxin in the chromogenic end-point method. The sensitivity and reaction time of each method are shown in Table 1. The use of a mutant luciferase elevates luminescence intensities as compared to the wild type luciferase bioluminescence method.

We found that the bioluminescence method detected 0.0005 EU/ml of endotoxin in 15 min. There is no difference in the principle of the LAL assay between either bioluminescence method and the end-point chromogenic assay. The differences between the new method and the conventional method are the use of a bioluminescence reaction that has good signal-to-noise ratio for endotoxin detection and the use of a mutant luciferase that shows high luminescence intensity. Consequently, the bioluminescence method was able to detect endotoxin at the same level or with greater sensitivity compared with the kinetic methods in the same measurement time as that in end-point methods. Moreover, the bioluminescence method had a high sensitivity for detecting endotoxin when the amount of LAL was decreased.

Recently, a laser light-scattering particle-counting method was reported as a new turbidimetric kinetic method for detecting endotoxin with high sensitivity [13, 17]. This method detects the formation of small particles of clotted enzyme related to endotoxin concentration when the reaction mixture is agitated, and can detect endotoxin at 0.00015 EU/ml in 71 min. The endotoxin detection sensitivity of this

method is about 3-fold better than that of the luciferase FM bioluminescence method. Therefore, we are attempting to improve the sensitivity of the bioluminescence method for endotoxin detection by reducing background luminescence (over 6,000 RLU) in the negative control by using a modification of peptidyl-Luc and recombinant Factor C and Factor B, as well as pro-clotting enzyme because of the nonendotoxin-specific serine protease activities exhibited by LAL. The sensitivity of the bioluminescence method should increase with the use of pure Limulus reaction component factors.

The bioluminescence method using mutant luciferase was effective for rapid and highly sensitive endotoxin detection. In addition, portable bioluminescence measurement machines such as PD-10 (Kikkoman Corporation) are widely used for checking environmental clean levels, and the principle of bioluminescence measurement used by these machines is the same as that of the endotoxin bioluminescence assay [18]. Therefore, we expect highly sensitive endotoxin detection will be readily available with the use of these portable measurement machines combined with the luciferase FM-bioluminescence method.

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References

[1] J. Levin, F. B. Bang, Clottable protein in Limulus; its localization and kinetics of its coagulation by endotoxin, Thromb. Diath. Haemorrh. 19 (1968) 186-197.

[2] N. S. Young, J. Levin, R. A. Prendergast, An invertebrate coagulation system activated by endotoxin: evidence for enzymatic mediation, J. Clin. Invest. 51 (1972) 1790-1797.

[3] N. O. Solum, The coagulogen of Limulus polyphemus hemocytes. A comparison of the clotted and non-clotted forms of the molecule, Thromb. Res. 2 (1973) 55-70.

[4] R. Nandan, D. R. Brown, An improved *in vitro* pyrogen test to detect picograms of endo-toxin contamination in intravenous fluids using *Limulus* amebocyte lysate, J. Lab. Clin. Med. 89 (1977) 910-918.

[5] K. Tsuji, P. A. Martin, D. M. Bussey, Automation of chromogenic substrate Limulus amebocyte lysate assay method for endotoxin by robotic system, Appl.

Environ. Microbiol. 48 (1984) 550-555.

[6] J. F. Remillard, M. C. Gould, P. F. Roslansky, T. J. Novitsky, Quantitation of endotoxin in products using the LAL kinetic turbidimetric assay, Prog. Clin. Biol. Res. 231 (1987) 197-210.

[7] K. Duner, A new kinetic single-stage Limulus amoebocyte lysate method for the detection of endotoxin in water and plasma, J. Biochem. Bioph. Methods 26 (1993) 131-142.

[8] M. Kojika, N. Sato, Y. Yaegashi, Y. Suzuki, K. Suzuki, H. Nakae, S. Endo, Endotoxin adsorption therapy for septic shock using polymyxin B-immobilized fibers (PMX): Evaluation by high-sensitivity endotoxin assay and measurement of the cytokine production capacity, Ther. Apher. Dial. 10 (2006) 12-18.

[9] 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.

[10] 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.

[11] S. Nakamura, T. Takagi, S. Iwanaga, M. Ntwa, K. Takahashi, A clottable protein (coagulogen) of horseshoe crab hemocytes structural change of its polypeptide chain during gel formation, J. Biochem. 80 (1976) 649-652.

[12] R. L. Danner, R. J. Elin, J. M. Hosseini, R. A. Wesley, J. M. Reilly, J. E. Parillo,

Endotoxemia in human septic shock, Chest 99 (1991)169-75.

[13] K. Mitsumoto, K. Yabusaki, K. Kobayashi, Y. Shirasawa, T. Obata, Novel endotoxin assay by laser light-scattering particle-counting method, J. Clin. Lab. Anal. 23 (2009) 117-124.

[14] K. I. Duner, A new kinetic single-stage Limulus amoebocyte lysate method for the detection of endotoxin in water and plasma, J. Biochem. Biophys. Methods 26(1993) 131-142.

[15] M. Daneshian, A. Wendel, T. Hartung, S. Aulock, High sensitivity pyrogen testing in water and dialysis solutions, J. Immunol. Methods 336 (2008) 64-70.

[16] H. Oishi, M. Fusamoto, Y. Hatayama, M. Tsuchiya, A. Takaoka, Y. Sakata, An automated analysis system of Limulus amebocyte lysate (LAL)-endotoxin reaction kinetics using turbidimetric kinetic assay, Chem. Pharm. Bull 36 (1988) 3012-3019.

[17] T. Obata, M. Nomura, Y. Kase, H. Sasaki, Y. Shirasawa, Early detection of the Limulus amebocyte lysate reaction evoked by endotoxin, Anal. Biochem. 373 (2008) 281-286.

[18] T. Lewis, C. Griffith, M. Gallo, M. Weinbren, A modified ATP benchmark for evaluating the cleaning of some hospital environmental surfaces, J. Hosp. Infect. 69 (2008) 156-163.

FIGURE LEGENDS

Fig. 1 The principle of detection of endotoxin by bioluminescence.

Fig. 2 Comparison of the luminescence intensities of luciferase FM and wild-type luciferase with D-aminoluciferin concentration. First, 50 µl of D-aminoluciferin at 0.05 pM to 50 nM are mixed with 100 µl TMAT buffer. Then, 50 µl of 10 µM ATP and 50 µl of 250 µg/ml luciferase FM or wild-type luciferase are added and mixed immediately, and luminescence intensity is measured using Lumitester C-110. Symbols: ×, Luciferase FM; $^{\Delta}$, wild-type luciferase. The data are shown as averages ± standard deviations (*n*=5).

Fig. 3 Comparison of the luminescence intensity of luciferase FM with D-aminoluciferin concentration on addition of LAL. First, 50 µl of D-aminoluciferin at 0.5 pM to 50 nM are mixed with 100 µl TMAT buffer (−LAL), or 50 µl of TMAT buffer and 50 µl of LAL (+LAL). Then, 50 µl of 10 µM ATP and 50 µl of 250 µg/ml luciferase FM or wild-type luciferase are added and mixed immediately, and luminescence intensity is measured using Lumitester C-110. □, −LAL; $^{\Delta}$, +LAL. The data are shown as averages ± standard deviations (*n*=5).

Fig. 4 Relationship between luminescence intensity and the amount of LAL in the

bioluminescence method. Luminescence intensity is measured using Lumitester C-110. The data are shown as averages ± standard deviations (*n*=5).

Fig. 5 Comparison of endotoxin detection by chromogenic and bioluminescence methods. The reaction solution contains about 0.24 mg LAL. ○, bioluminescence method; $^{\Delta}$, end-point chromogenic method. The data are shown as averages ± standard deviations (*n*=5).

Table 1 Comparison of reaction times and sensitivities of endotoxin assays

Method	Reaction time (min)	Detection limit (EU/ml)
End-point chromogenic	15	0.1
End-point chromogenic (long reaction time)	30	0.01
End-point turbidimetric	15	0.01
Kinetic chromogenic	76	0.005
Turbidimetric kinetic	138	0.0005
Laser light-scattering particle-counting	71	0.00015
Bioluminescence (wild-type luciferase)	15	0.01
Bioluminescence (mutant-type luciferase FM)	15	0.0005

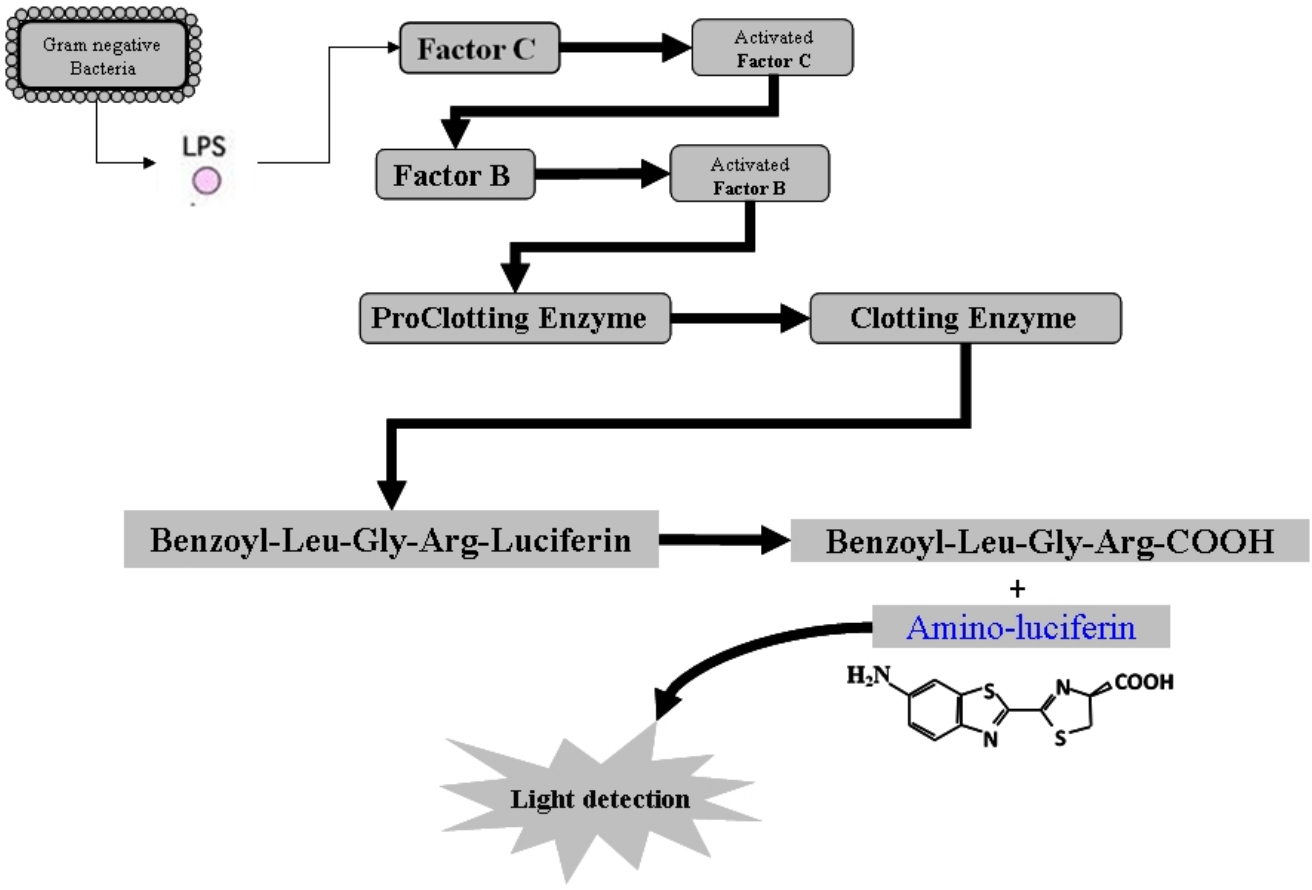
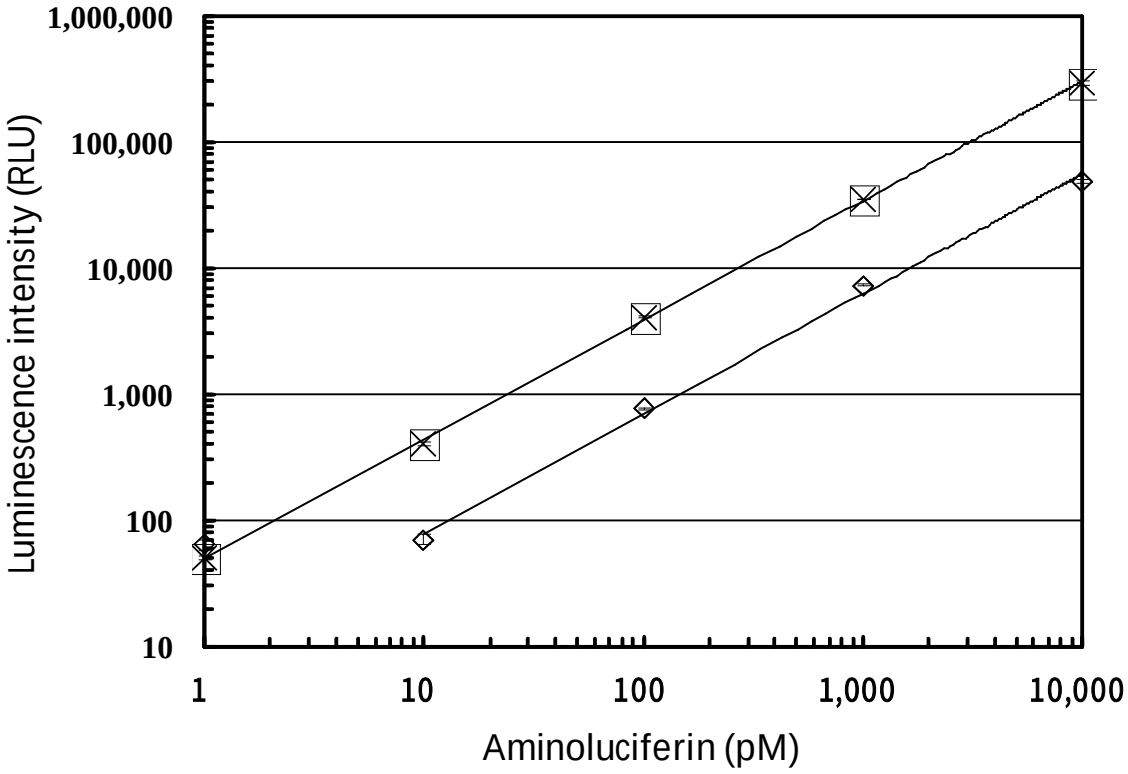

Gram negative Bacteria
LPS
Factor C
Activated Factor C
Factor B
Activated Factor B
ProClotting Enzyme
Clotting Enzyme
Benzoyl-Leu-Gly-Arg-Luciferin
Benzoyl-Leu-Gly-Arg-COOH
+
Amino-luciferin
H2N
S
N
N
S
COOH
Light detection

Fig. 1


1,000,000
100,000
10,000
1,000
100
10
Luminescence intensity (RLU)
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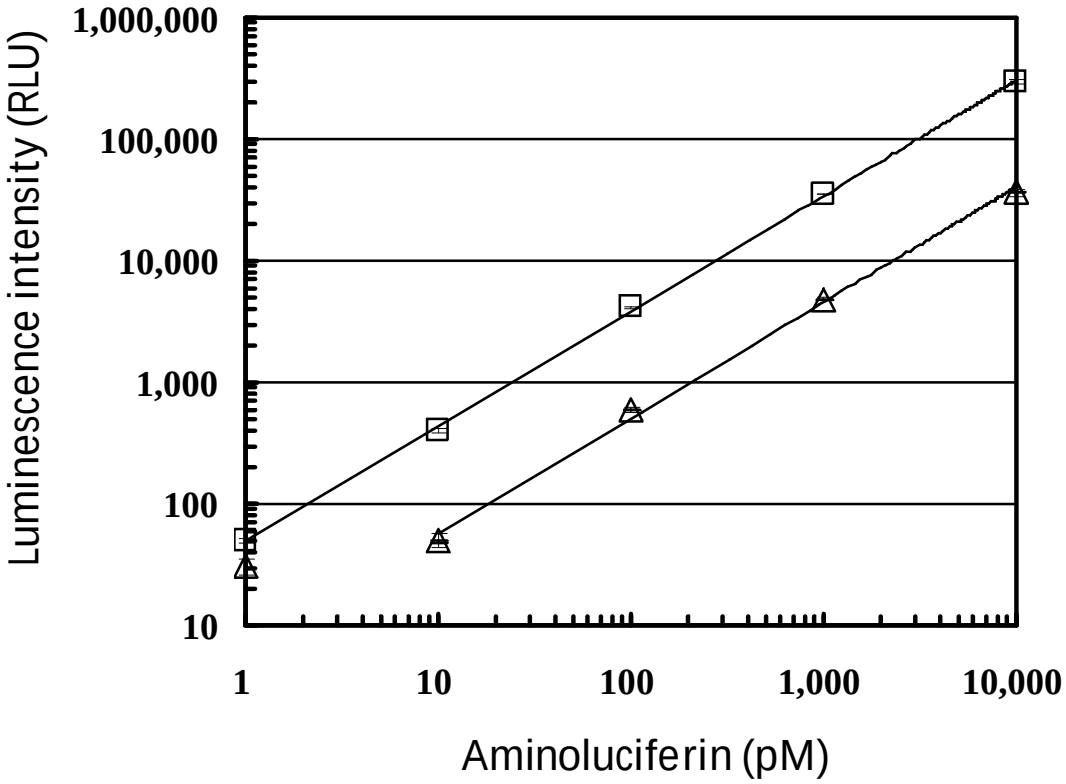
10
100
1,000
10,000
Aminoluciferin (pM)

Fig. 2


1,000,000
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Luminescence intensity (RLU)
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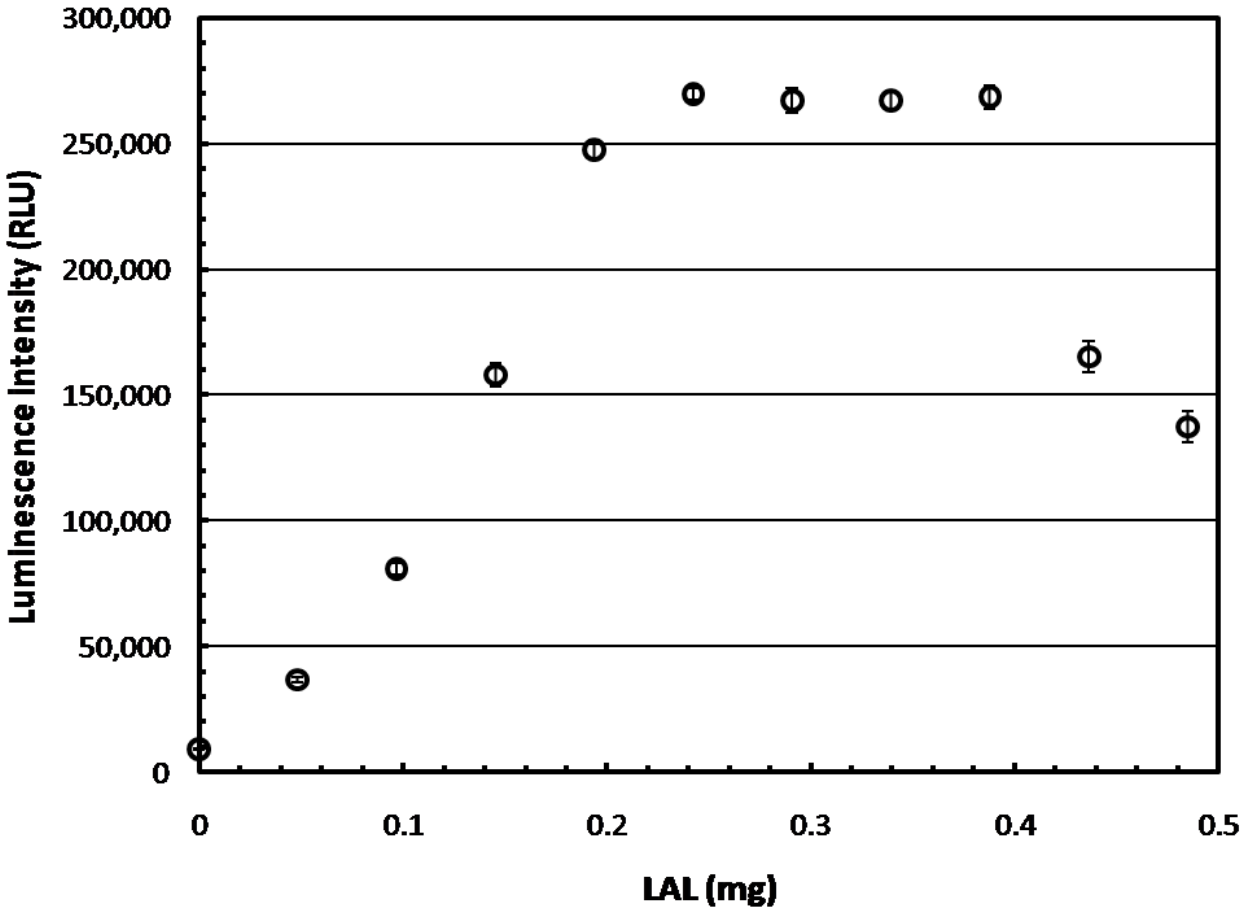
10,000
Aminoluciferin (pM)

Fig. 3


300,000
250,000
200,000
150,000
100,000
50,000
0
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0.1
0.2
0.3
0.4
0.5
LAL (mg)

Fig. 4

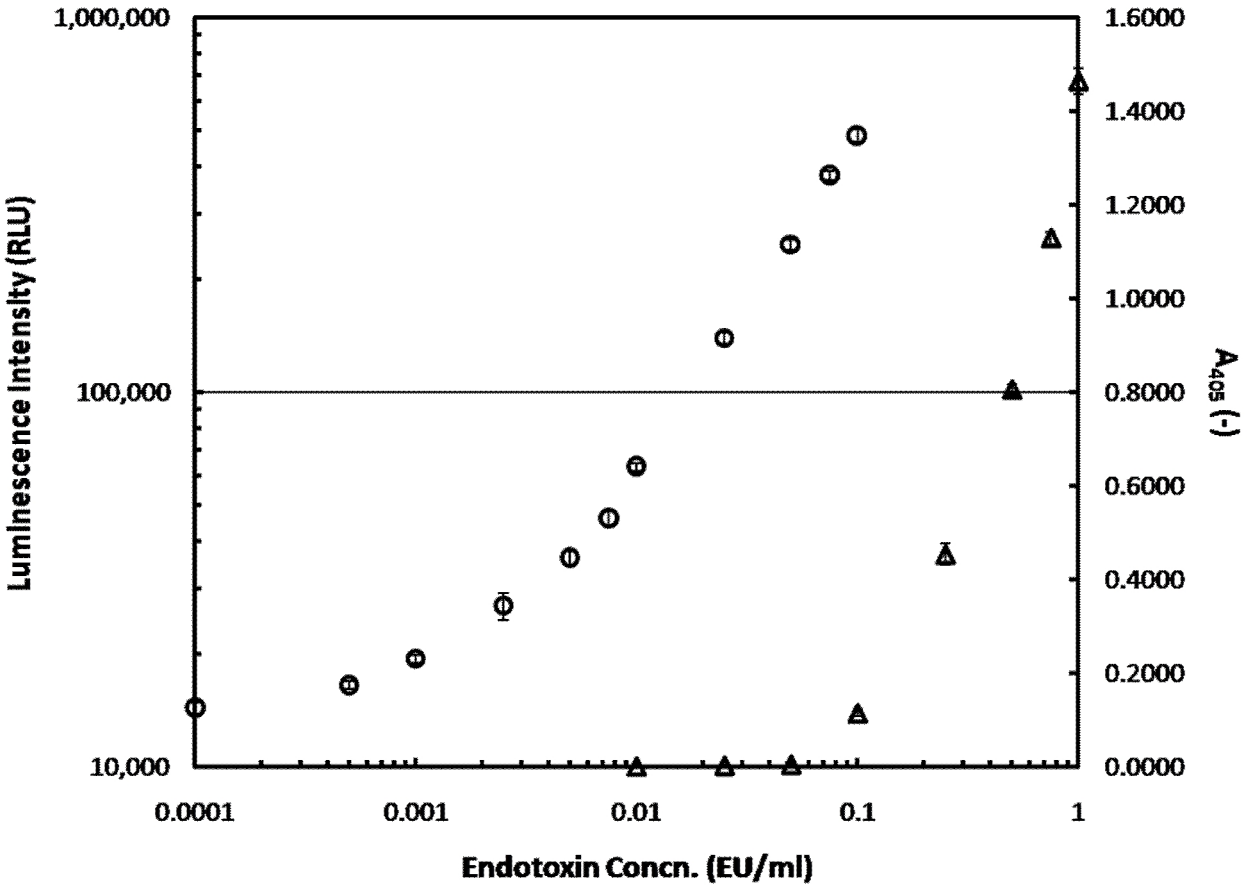
1,000,000
100,000
10,000
Lumlnescence Intenslty (RLU)
1.6000
1.4000
1.2000
1.0000
0.8000
0.6000
0.4000
0.2000
0.0000
A405 (-)
0.0001
0.001
0.01
0.1
1
Endotoxin Concn. (EU/ml)

Fig. 5