Construction of a sensitive and specific lead biosensor using a genetically engineered bacterial system with a luciferase gene reporter

Esmail Nourmohammadi
Mashhad University of Medical Sciences
Saman Hosseinkhani
Tarbiat Modares University Faculty of Biological Sciences
Reza Nedaeinia (molecular_biology@mail.mui.ac.ir)
Isfahan University of Medical Sciences https://orcid.org/0000-0001-9922-7181
Hoda Khoshdel-Sarkarizi
Mashhad University of Medical Sciences
Mozhdeh Nedaeinia
Islamic Azad University Marvdasht
Maryam Ranjbar
Islamic Azad University Najafabad Branch
Neshat Ebrahimi
Cedars-Sinai Medical Center
Zahra Farjami
Mashhad University of Medical Sciences
Mohammad Nourmohammadi
Mashhad University of Medical Sciences
Ali Mahmoudi
Mashhad University of Medical Sciences
Mohammad Goli
Islamic Azad University Khorasgan Branch
Gordon A.Ferns
Brighton and Sussex Medical School
Majid Sadeghizadeh
Tarbiat Modares University Faculty of Biological Sciences

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Abstract

Background: A bacterial biosensor refers to genetically engineered bacteria that produce an assessable signal in the presence of a physical or chemical agent in the environment.

Methods: We have designed and evaluated a bacterial biosensor expressing a luciferase-reporter gene controlled by *pbr* and *cadA* promoters in *Cupriavidus metallidurans* (previously termed *Ralstonia metallidurans*) containing *CH34* and *pI258* plasmids of *Staphylococcus aureus*, respectively, and that can be used for the detection of heavy metals. In the present study, we produced biosensor plasmids designated *pGL3-luc/pbr*-biosensor and *pGL3-luc/cad*-biosensor, that were based on the expression of luc+ under the control of the *cad* promoter and the *cadC* gene of *S. aureus* plasmid *pI258* and *pbr* promoter and *pbrR* gene from plasmid *pMOL30* of *Cupriavidus metallidurans*.

Results: We found that the biodegradable *pGL3-luc/pbr*-biosensor could be used to measure lead concentrations between 1-100 μM in the presence of other metals, including: zinc, cadmium, tin and nickel. The latter metals did not result in gene expression of the reporter. The *pGL3-luc/cad*-biosensor was able to detect lead concentrations between 10 nM to 10 μM.

Conclusions: This biosensor was found to be a specific for measuring lead ions in both environmental and biological samples.

Background

Ecological heavy metal pollution is a common problem that can exert damage to human health as well as the environment [1]. Because heavy metal pollutants may lead to harmful ecological outcomes [2], developing sensitive, efficient, rapid and cost-effective methods are necessary to efficiently screen for the presence of harmful metals in the environment. Lead (Pb) is a toxic heavy metal that is extensively utilized around the world [3, 4]. It has been estimated that the world production of lead is more than 3 million tons per year. It causes widespread environmental contamination in the air, water, soil, and food [5]. This element can find its way through human bodies as well as animals, entering the food chain; in fish and shrimp it can accumulate in the bone, liver, gills, kidney, ovary, and muscle [6].

Environmental lead may result in an increase in vascular endothelial growth factor (VEGF) and blood concentrations [7, 8], and can lead to neurological, cardiovascular complications [9]. The reproductive system may also be affected by developmental disorders that are highly likely to occur in children [10-13]. Lead can cross the placenta and cause damage to the developing fetal nervous system[14].

The assessment and monitoring for environment heavy metal contamination are very important to prevent harm to human health. Currently, classical analytical methods, such as spectrometry, FIAAS (Flow injection atomic absorption spectrometry), ion chromatography, and electrochemical techniques, are the main methods used for measuring environmental heavy metals pollution. The main disadvantage of these methods is the necessity for sample digestion under high temperature and pressure, or acidic

conditions in which metal ions in solution are released [15]. Therefore, simpler methods for evaluating heavy metals are required. More importantly, heavy metals are found to be present in biological systems either in bioavailable/toxic or non-available/nontoxic forms, and current measuring methods are unable to distinguish between toxic and nontoxic fractions of these elements [16]. Furthermore, these methods are both time-consuming and costly [17]. Biosensors have been developed that are an effective alternative to conventional detecting systems. These may be highly sensitivity and simple to use [18]. Cell-based biosensors are a type of biologic sensors as they contain a reporter gene under the control of a promoter that is sensitive to the presence of an agent, such as environmental contaminants that include heavy metals. Biosensors are used in various designs with different reporters and promoters. At low concentration of heavy bioavailable metals, bioluminescence signals are likely to be suitable [19, 20]. They can also be applied to monitoring bioavailable concentrations of heavy metal [21-27] and piezoelectric biosensors [28-30] as enzyme-based electrochemical biosensors. One of the most obvious advantages of this method is the ability to measure the bioavailable heavy metal at very low concentrations. It is also a cost-effective and timesaving method [18]. In these biosensors, the expression of a reporter gene is controlled by a promoter , such as *pbrR* promotor in the pMOL30 plasmid of *Cupriavidus metallidurans* CH34 and *cadC* promotor in *pI258* plasmid of *Staphylococcus aureus* that is sensitive to heavy metals. Most of these promoters originate from bacteria that have resistance systems against heavy metals [31, 32]. In this study, we have designed and evaluated the luciferase reporter gene expression of bacterial biosensor under the control of *pbr* and *cadC* promoters in Cupriavidus metallidurans *CH34* and *pI258* plasmids of Staphylococcus aureus, respectively, for the measurement of lead.

Results

Sequencing

In order to ensure the integrity of the sequencing by Miligen Company, the promoter region was sequenced in the received plasmid (Fig. 1C and 1D).

Colony confirmation with PCR reaction

PCR was performed using primers designed for the *pbr* and *cadA* promoters, and the promoter sequence and regulatory gene were amplified with 634 bp for *pbr* and 601 bp for *cadA* (Fig. 2).

Biosensor activity of *pGL3-luc/pbr*

The expression of the luciferase gene, in the presence of different concentrations of lead, showed that 1 µM of lead was the lowest concentration that could stimulate the promoter and could be distinguished from the basal expression of luciferase, and the highest expression was seen at 100 µmol/L. A good biosensor should have two characteristics: specificity and sensitivity. According to the data obtained from our experiments, this biosensor had a high specificity, and luciferase gene was only expressed in the presence of lead. -

Biosensor specificity for lead in the presence of different concentrations of zinc ($ZnCl_2$), tin ($SnCl_2$) and cadmium ($CdCl_2$)

The biosensor was cultured in the presence of different concentrations of zinc, tin and cadmium, and did not stimulate the *pbr* promoter and expression of the reporter gene (Fig.3). Data obtained from the expression of the luciferase gene in the presence of various concentrations of tin, zinc and cadmium, indicated that these heavy metals did not stimulate the *pbr* promoter.

Biosensor activity in the presence of different concentrations of Lead ($PbCl_3$)

The results revealed that lead was the only metal that stimulated the *pbr* promoter. In the absence of lead, the regulator gene prevents the promoter from activation. Lead ions bind to the regulator gene and inhibits its binding to the operator. As a result, the promoter is activated and the luciferase is expressed. The minimum detectable concentration of this biological sensor was approximately 1 μM and a maximum is 100 μmol/L. The expression of luciferase was no longer linear for value of lead from 100 to 200 μmol/L (Fig. 4A).

The expression of *pGL3-luc/ pbr*-biosensor reporter gene at different times

In order to identify the appropriate time for biosensor growth, a biosensor was cultured at different concentrations of lead for different durations (Fig. 4B). The maximum expression of the luciferase gene was 12 h (Fig. 5A).

The difference in the growth rate of *pGL3-luc/ pbr*-biosensor compared to *E. coli* strain DH5α

The sensor bacteria had a recombinant plasmid containing the *pbr* promoter region and the *pbrR* regulatory gene. These bacteria have a greater resistance to lead than *E. coli* DH5α without plasmid. This resistance may be related to the *pbrR* regulatory gene (Fig. 5B). The resistance genes of metals have heavy metal binding motifs, they can result in the non-toxicity of these metals inside the cell, because of these proteins, the relative resistance of the cell to heavy metals.

The activity of *pGL3-luc/cad*-biosensor at the different concentrations of lead

The lowest and highest concentrations of lead that could stimulate expression of the reporter gene were 10 nmol/L and 10 μmol/L respectively (Fig. 6 and 7A).

Expression of the Luciferase gene in the presence of 1 micro Molar concentration of Lead at different times

The sensor bacteria were incubated at 0.2 OD (1 μmol/L concentration) at different times in the incubator. The expression of luciferase was measured at different times (Fig. 7B). As shown in Fig. 7B, the concentration of 1 υM lead can cause luciferase expression. The amount of expression raised by progressing time, just due to during 2 h the amount of expression is high enough to measure the change

in Luciferase, and in biological sensors the pollution is measured at low rates, we chose 2 h for culture *the pGL3-luc/cad*-biosensor.

Discussion

There are several advantages to the use of bacterial biosensors, including speed, simplicity and cost. Biological sensors containing *cadA* and *pbr* promoter regions have been designed by other researchers, the optimization of this cell biological sensor with ability to measure lead comparing *cadA* and *pbr* promoters in a bioassay system was considered in this study. The use of biosensors or biological cell sensors containing a reporter gene controlled by promoters susceptible to the heavy metal ions can provide an efficient method to trace particular pollutants in the environment and in a biological solution [33]. The present study assessed a biosensor system for detecting lead ions through construction of a luminescent bacterial sensor containing the *luc*$^{+}$ regulated by the cad promoter and *ca*dC gene in plasmid *pI258* of S. aureus and the *pbr* promoter and *pbrR* gene in *pMOL30* plasmid of *Cupriavidus metallidurans*. Pb specific bacterial biosensors were formerly defined using reporter genes including *lacZ*, *lux,* and *luc* in the transcription fusion constructs [34-36]. In our study, the luciferase reporter gene was used. Luciferases, as a set of heterogeneous enzymes, are able to produce light as a byproduct of catalyzing reactions. They are reporter genes extensively used by prokaryotic and eukaryotic organisms due to their high sensitivity and ease of detection. The quantification of the emitted light, i.e. bioluminescence, is of great importance; it can also be measured uing a liquid scintillation counter, a luminometer, or even an X-ray film [35]. It was concluded that *pGL3-luc/ pbr*-biosensor can detect Pb^{+2} in the range of 1–100 µM through expression of firefly luciferase and is highly specific, with no expression of reporter in the presence of other metals such as Sn^{+2}, Ni^{+2}, Cd^{+2} are present. Moreover, 50 times higher sensitivity was observed in this biosensor when compared with the previous biosensors reported by Chakraborty et al [32]. The *R. mettalidurans* CH34 strain has several resistance systems that can reduce the concentration of toxic substances to their non-toxic levels. A highly specific system for resistance to lead is known in plasmid *pMOL30* [37]. It effectively reduced the concentration of lead ions and is equipped with specific mechanisms for the transfer and separation of lead. The *pbr* operon includes *pbrA*, *pbrB*, *pbrC* and *pbrD* genes in which *pbrD* has a role as a chaperone to accumulate lead in the cell and pbrA eliminates Lead ions [37]. Our results show that the *pGL3-luc/ pbr*-biosensor is not expressed in the presence of cadmium, zinc, or tin, indicating high sensitivity and specificity of the designed system for lead detection. One of the most important heavy metal transfer systems in *Staphylococcus aureus* is located in the plasmid *pI258*. The plasmid has an operon *cadA* that encodes an ATPas of type P, which causes resistance to metals such as cadmium, lead, zinc, copper, and tin. The expression of the *cadA* operon is controlled by the cadC homodimeric protein. This protein is able, in a binary manner, to bind to the promoter and metal ions, such as cadmium, lead, zinc, and tin. The *cadC* belongs to *ArsR / SmtB*, a regulating protein family [38]. In our research, The luciferase gene was used as a reporter and *E. coli* strain of DH5α as a host. Our results showed that the *pGL3-luc/ cad*-biosensor can detect at least 10 nM of lead and the lead toxicity was not observed until a concentration of 300 µM. However, the maximal expression of the reporter gene was performed at 10 µM. Our results are supported by the report of Liao et al that showed the regulating role

of cad promoter and the cadC gene in plasmid *pI258* of *S. aureus*, the fluorescence emission was intensified with increasing Cd(II), Pb(II), and Sb(III) ions concentrations[39]. For Pb(II), just like our result in *pGL3-luc/cad*-Biosensor, to induce GFP expression significantly, 10 nM was the low, and 10 µM was the maximum concentration of lead that induced significantly GFP expression [39]. The metalloregulatory α_3N thiolate-rich site in cadC displays a practical selectivity for larger, softer heavy metal like Pb(II), Cd(II), although smaller boundary metal ions such as Zn(II) accommodated [40].

Conclusion

Our results show that the maximum expression of reporter gene was found in the presence of 100 µM of Lead in *pGL3-luc/ pbr*-biosensor and 1 µM of lead in pGL3-*luc/cad*-biosensor. In this study, the specificity and sensitivity of the two heavy metal susceptive probes, *pbr* and *cadA,* was investigated. Sensors composed of these two promoter regions were able to detect the concentration of lead between 1-100 µM and 10 nM to 10 µM of, respectively. For other heavy metals such as Mercury, Copper, Nickel, Manganese, Zinc and Cadmium, different biological sensors can be made and their presence in the environment can be measured with very high accuracy. By developing these sensors, the time identified to environmental pollution can be minimized.

Methods

Chemicals

Analytical reagents, media and buffer solutions like TBE-EDTA buffer (Tris-borate-Ethylenediaminetetraacetic acid), NaOH (Sodium hydroxide), $CaCl_2$ (calcium chloride), boric acid, Tris base, and agarose were all purchased from Merck (Germany). Fermentas (Lithuania) supplied the restriction endonucleases *Nco1* and *Hind3*, *T4* DNA ligase, and molecular ladder 10000-300bp. We also supplied the DNA polymerase (TaKaRa LA Taq®. DNA Polymerase), dNTP and $MgCl_2$ from Takara (Beijing, China). In addition, the plasmid extraction kit and primers were brought from Bioneer (Seoul, South Korea).

Construction of biosensor plasmid

pMOL30 (X71400 AJ278984) and *PI258* (GQ900378.1) containing the *pbrR* gene (634 bp) and *CadC* gene (601bp) (Accession number: *pbrR*: WP_003103716.1 and *CadC*: WP_000726009, respectively, were synthesized and supplied by Millegen company. To ensure the accuracy of synthesized plasmid, the promoter region was sequenced. *PGl3*-control as a vector containing the Luciferase gene and *E.coli* strain *DH5α* as the host were used in our study. To obtain a large amount of pMA-T plasmid (a synthetic plasmid) which contains p-promoter sequences and the regulatory gene was sent to MilliGen, after evaluation at the NCBI site, for the synthesis of sequences. Synthesized sequences consisted of both *pbr_pMA-T* plasmids containing the promoter sequence of the pRR operon and the *pbrR* regulator gene including; *cadA_pMS-RQ-Bs* plasmid containing the promoter region of the *cadAp* and the *cadA* gene

regulating gene), it was cloned to *E. coli* host. Afterwards, *pMA-T* was extracted using plasmid extraction kit, and its quantity and quality were both examined by spectrophotometry and agarose gel, respectively, before they got digested by *HindIII* and *NcoI*. The promoter regions with the regulator genes were also purified from the gel electrophoresis. The received sequence and *pGL3*-control vector were cut using the same restriction enzyme (Nco1 and Hind3) and ligation reaction at 37°C for 3–4 h with ligase enzyme. The firefly luciferase gene was placed under the control of the received promoter sequences and recombinant plasmids of *cad* and *pbr* promoters were named *pGL3-luc/ pbr*-biosensor and *pGL3-luc/Cad*-biosensor, respectively. Recombinant plasmids *pGL3-luc/ pbr*-biosensor (Fig. 1A) and *pGL3-luc/Cad*-biosensor (Fig. 1B) were transferred to the *DH5α* bacteria using the chemical method of CaCl2 and then were screened using selective plates containing antibiotic Ampicillin. After plasmid extraction, PCR was performed to detect colonies containing the promoter region of *pbr* and *cadA* using primers designed for the cloned fragments. After these processes, recombinant plasmids were used to evaluating and measuring different concentrations of heavy metals.

Culture of bacteria and measuring biosensor activity of Luciferase enzyme

To study the efficiency of promoters the detection of heavy metals, a luciferase enzyme measurement performed in the presence of lead and other heavy metals such as tin, zinc and cadmium. In this process, *E.coli* stains carrying *pGL3-luc/Cad*-Biosensor and *pGL3-luc/ pbr*-Biosensor were cultured in Luriae Bertani (LB) broth that contained 100 µg/mL ampicillin at 37˚C, overnight. Then 50 µl from overnight grown culture of *pGL3-luc/ pbr*-Biosensor for 12 h and *pGL3-luc/Cad*-Biosensor with optical density (OD_{600}) 0.8 for 2h were cultured in the presence of heavy metals at different concentrations [41]. Next, the culture was centrifuged at 5000 rpm for 10 min at 4°C metals for bacterial sedimentation. Then the medium was removed, and lysis buffer was added to the plate and sonicated at low temperature. Then, the amount of luciferase expression was measured by a luminometer (Berthold Company).

Statistical analysis

All the experiments were repeated at triplicate to minimize error. The Student t- test was used to compare the results with the level of significance ($P \leq 0.05$). Linear regression was used to model the standard curve.

Declarations

Compliance with ethical standards

Ethical approval

This article does not contain any studies with human participants or animals performed by any of the authors.

Informed consent

For this type of study, formal consent is not required.

Competing interests

The authors have declared no conflict of interest.

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Consent for publication

All authors have given consent for publication

Availability of data and materials

All data generated or analyzed during this study are included in this published article

Author Contributions

MS and SH designed research; EN and RN performed research; HKS and NE analyzed data; EN and MNo wrote the manuscript; AM , MN and ZF performed statistical analysis; MG and MR contributed new reagents or analytical tools. GAF revising the manuscript critically for important intellectual content. All authors read and approved the manuscript.

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None

Abbreviations

Pb: Lead; VEGF: Vascular endothelial growth factor; Flow injection atomic absorption spectrometry: FIAAS; LB: Luriae Bertani; RLU: Relative luminescence units.

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Figures

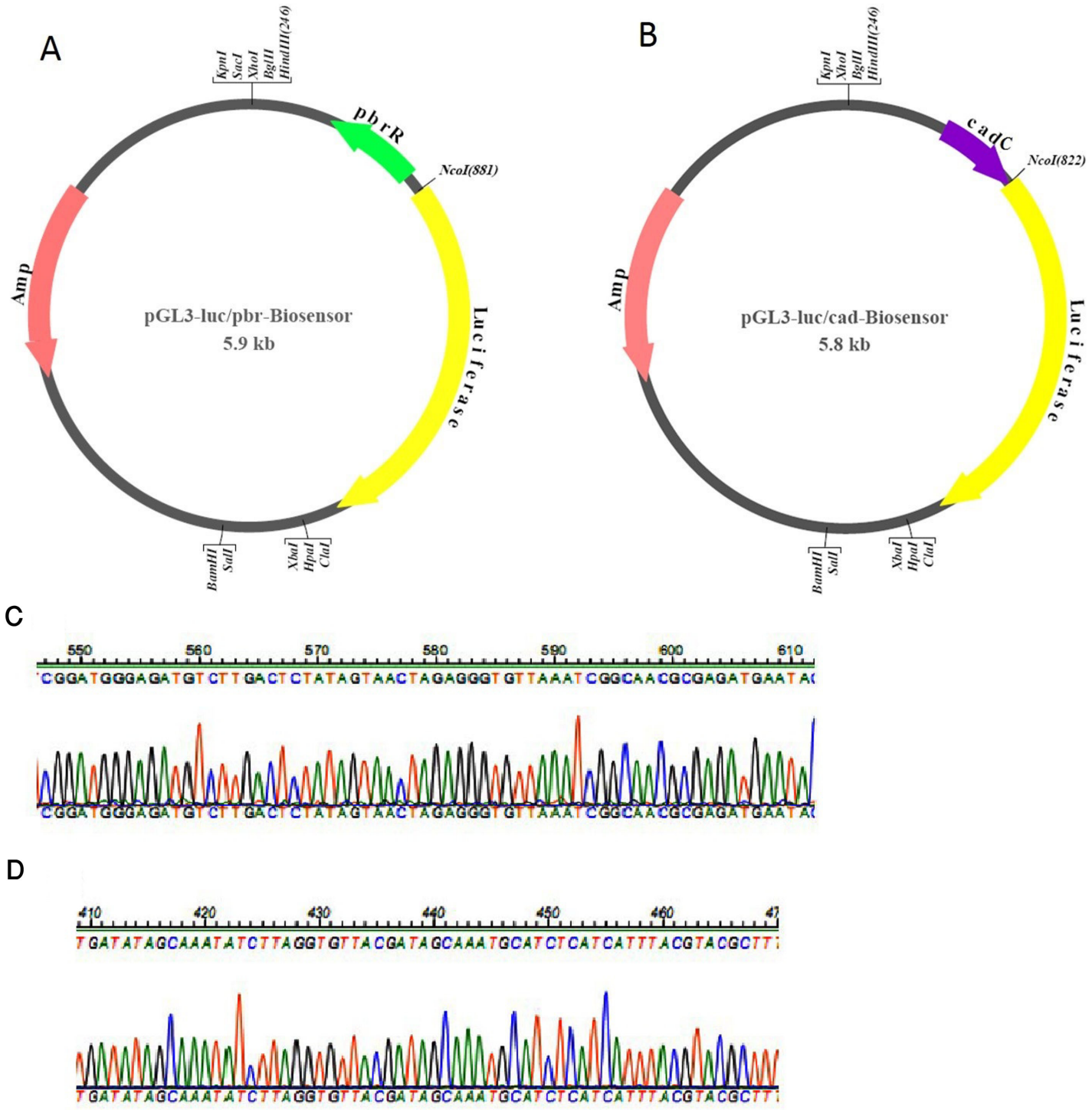

Figure 1

A Recombinant plasmid (pGL3-luc/pbr-Biosensor). B Recombinant plasmid (pGL3-luc/cad-Biosensor). pGL3-luc/pbr-biosensor and pGL3-luc/Cad- biosensor were transferred to the E.coli strain DH5α using the chemical method of Cacl2 and then were screened using selective plates containing antibiotic ampicillin. C Sequencing and integrity of synthesis sequence. D PGL3-luc/pbr-Biosensor pGL3-luc/cad-Biosensor. The promoter region was sequenced in the received plasmid.

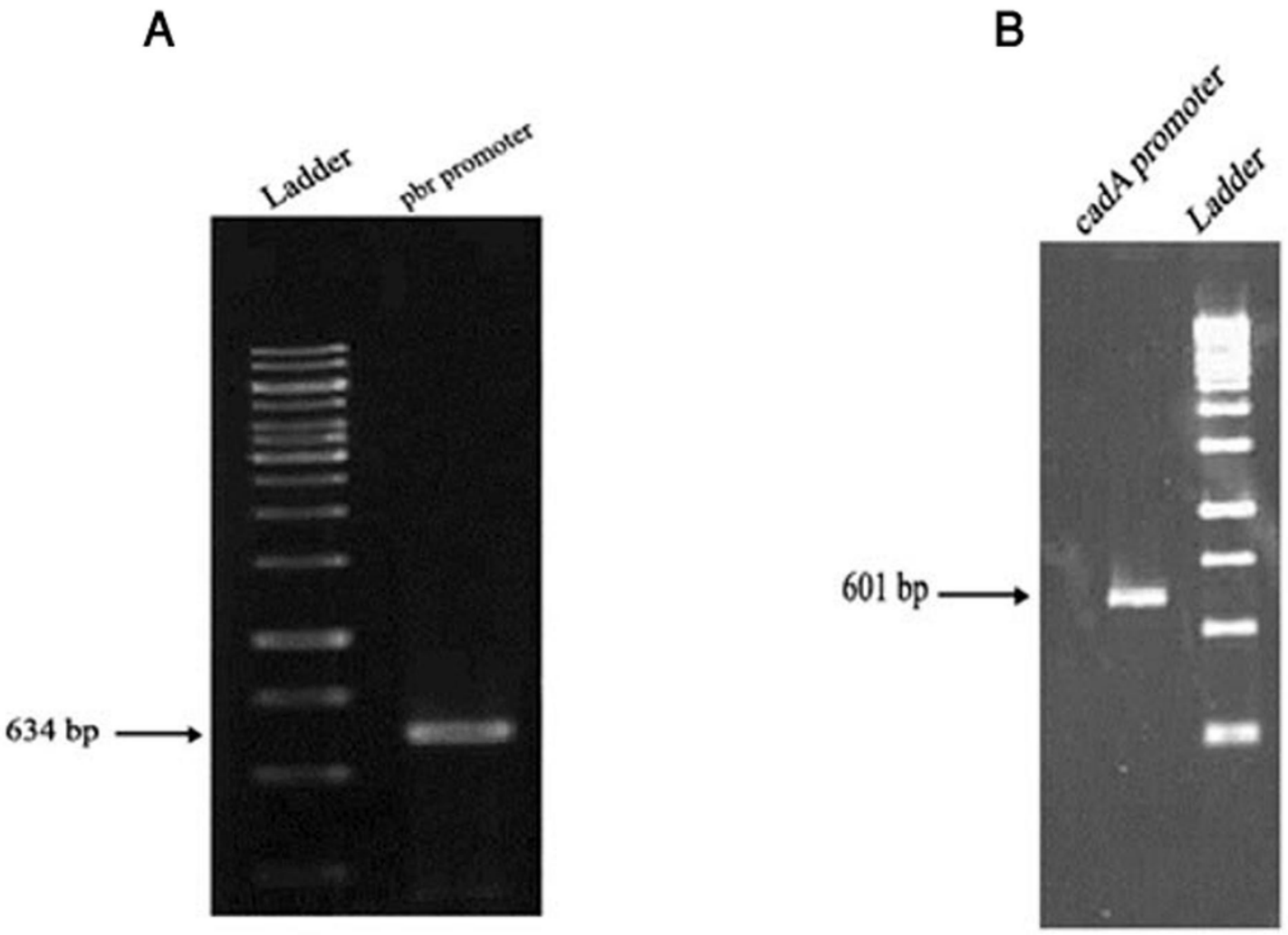

Figure 2

A The proliferation region of the pbr promoter with 634 bp. B cadA promoter with 601 bp. The promoter sequence and regulatory gene were amplified with 634 bp for pbr and 601 bp for cadA. 1 kb DNA Ladder (containing 14 linear double-stranded DNA fragments).

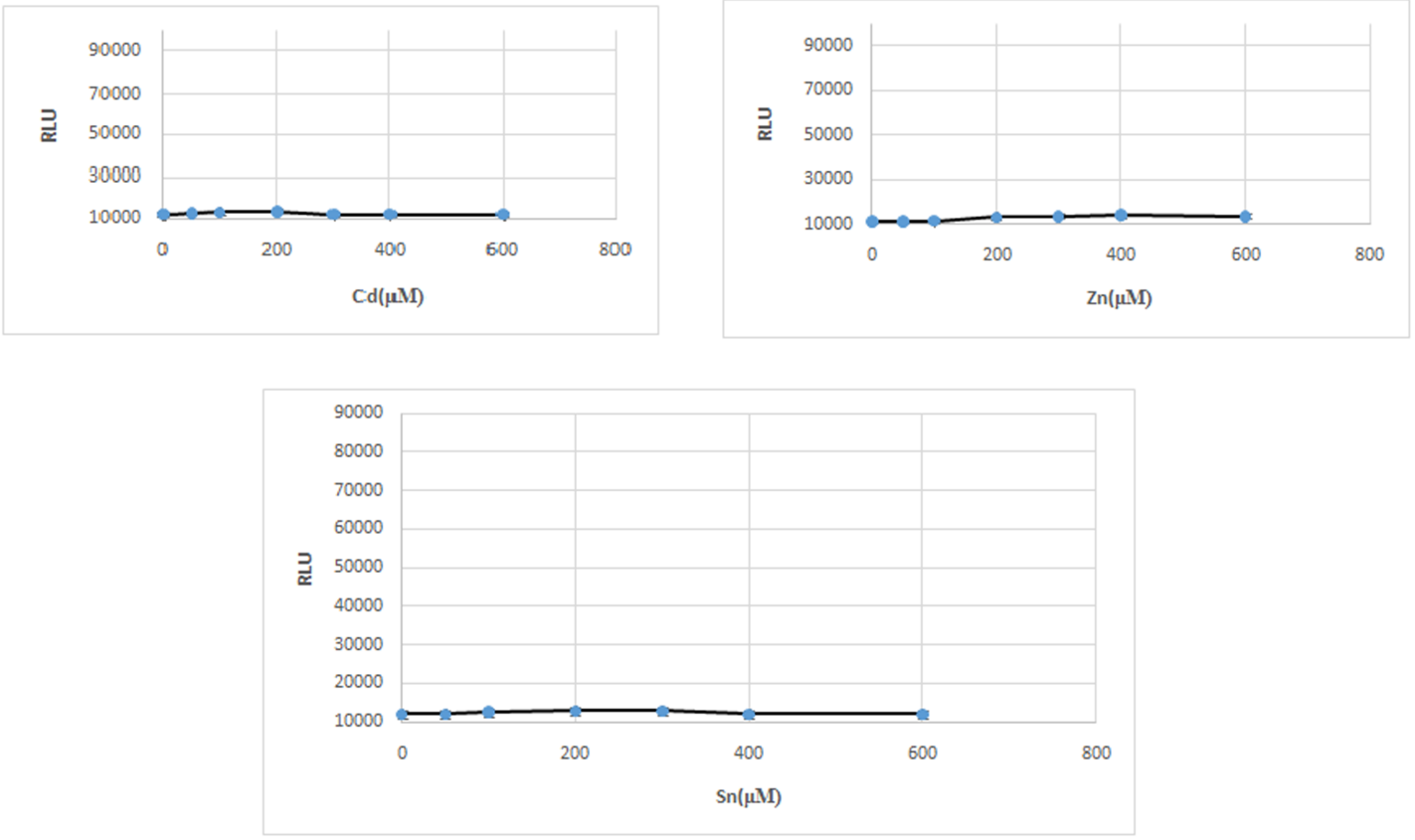

Figure 3

Expression of luciferase gene in different concentrations of zinc, Tin and Cadmium. Heavy metal had no effect on the stimulation of the pbr promoter.

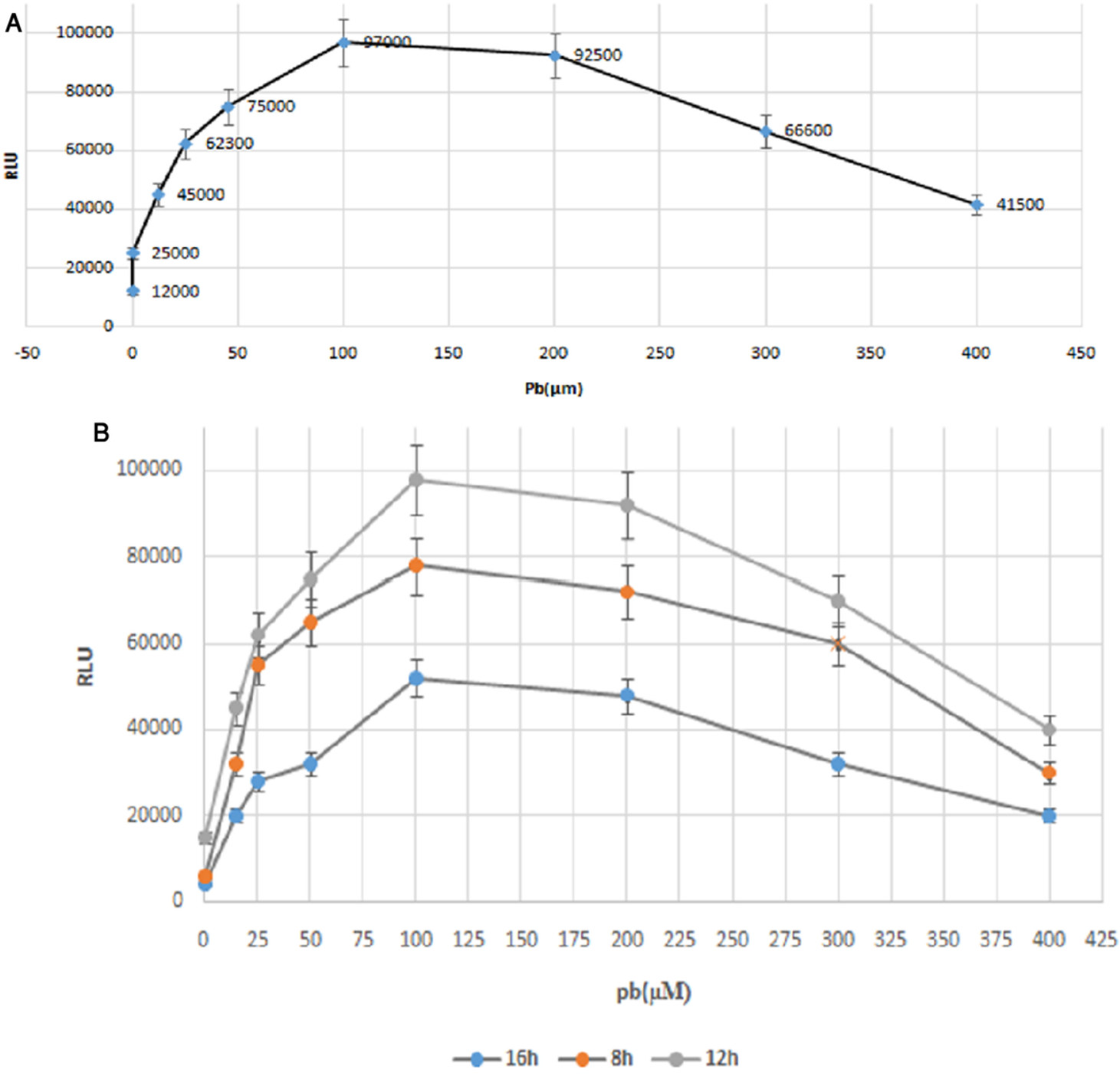

Figure 4

A Luciferase expression in different concentrations of lead. The expression of luciferase was decreased with a slight gradient from 100 to 200 micro molar. Relative luminescence units (RLU). B The expression of pGL3-luc/pbr-biosensor reporter gene at different times.

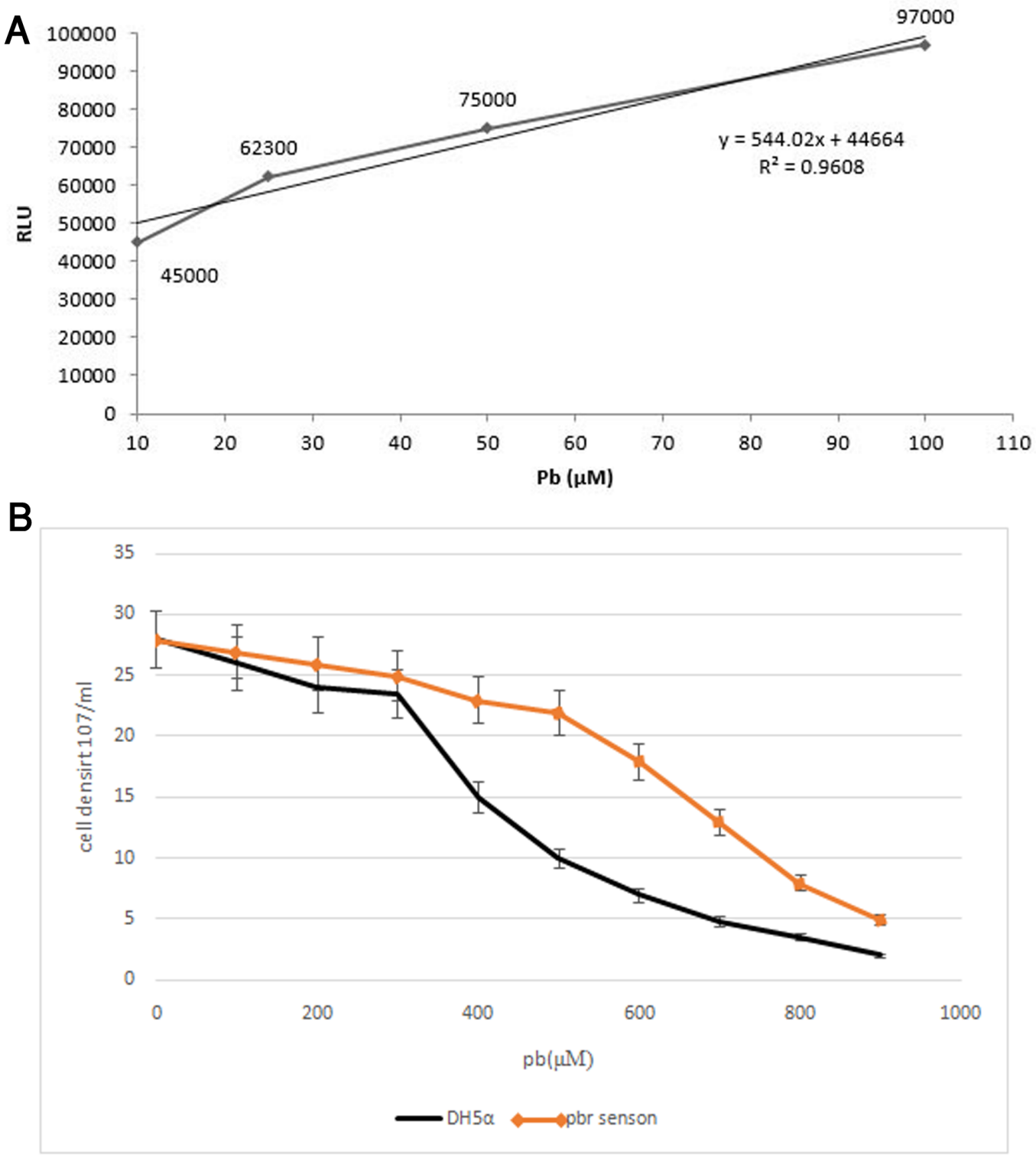

Figure 5

A Linear expression ranges of Luciferase in the presence of lead with regression coefficient R2 = 0.960. The maximum expression of the luciferase gene was 12h. B Difference in the growth rate of pGL3-luc/pbr-biosensor compared to E. coli strain DH5α. Resistance may be related to the pbrR regulatory gene.

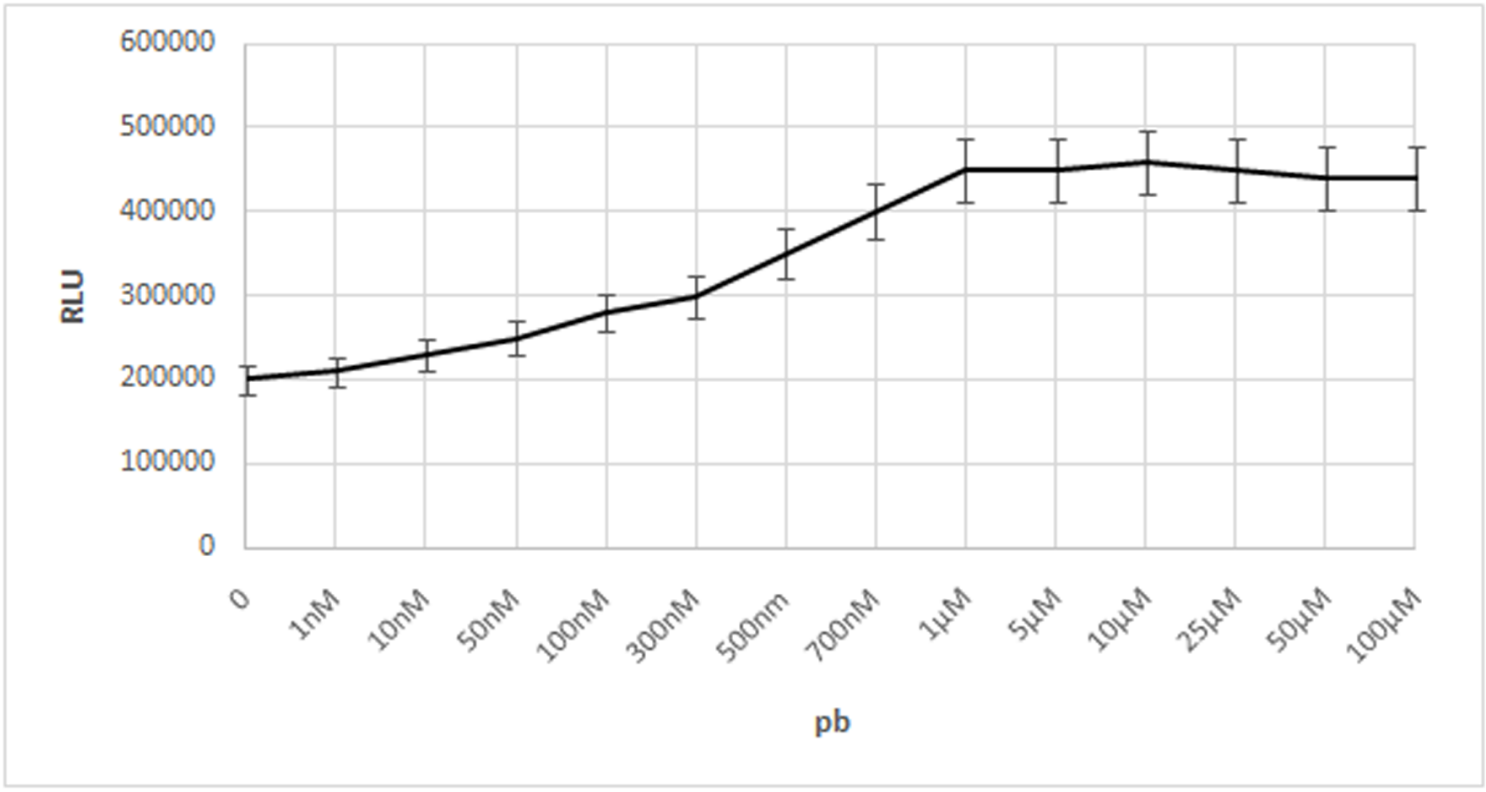

Figure 6

Expression of luciferase gene in different concentration of lead.

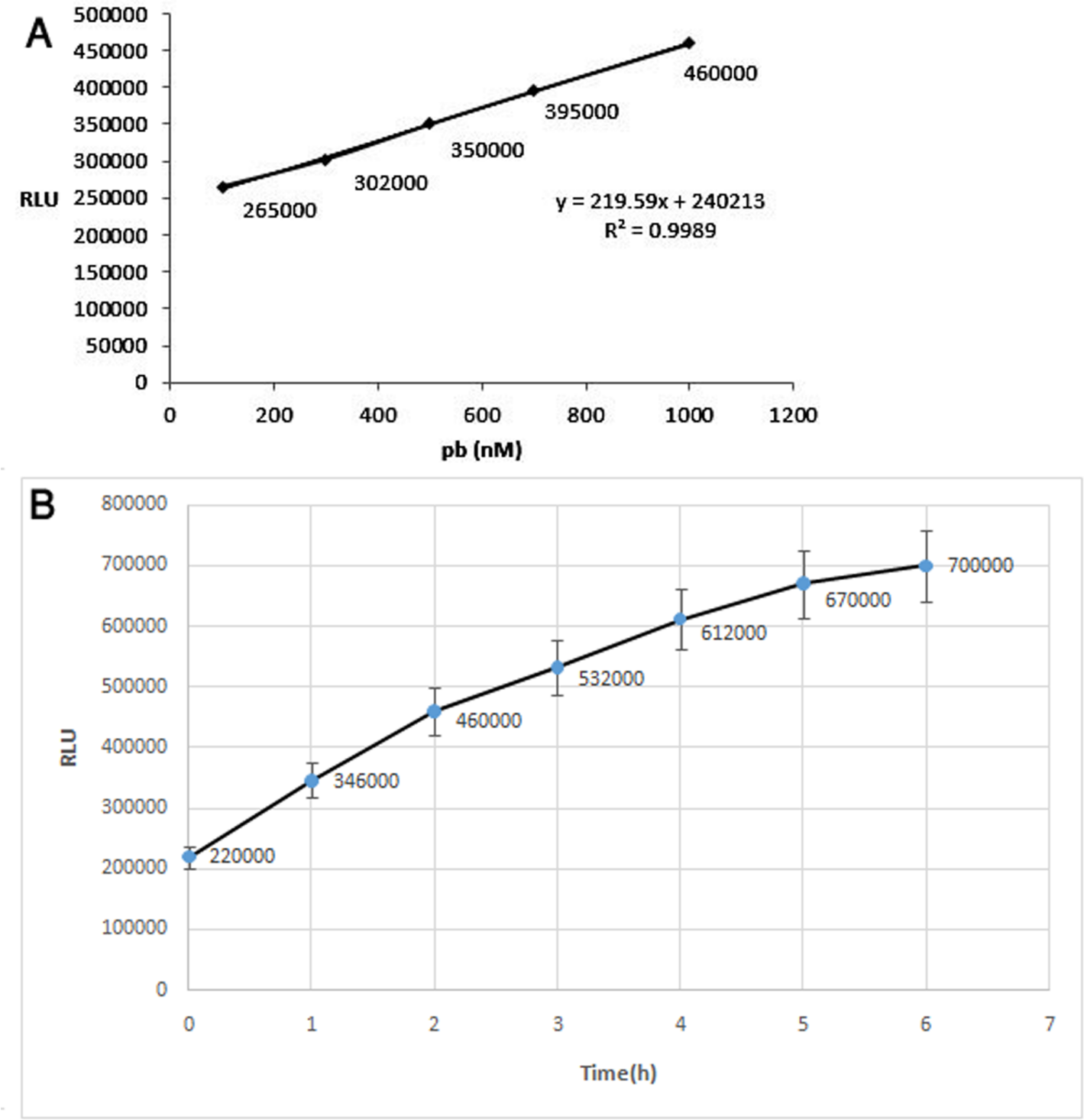

Figure 7

A linear expression ranges of Luciferase expression between 100-1000 nM concentrations of lead. B The expression of luciferase at different times at 1 μM Pb concentration. During 2h, the amount of expression is high enough to measure Luciferase, in biological sensors; the pollution is measured at low rates.

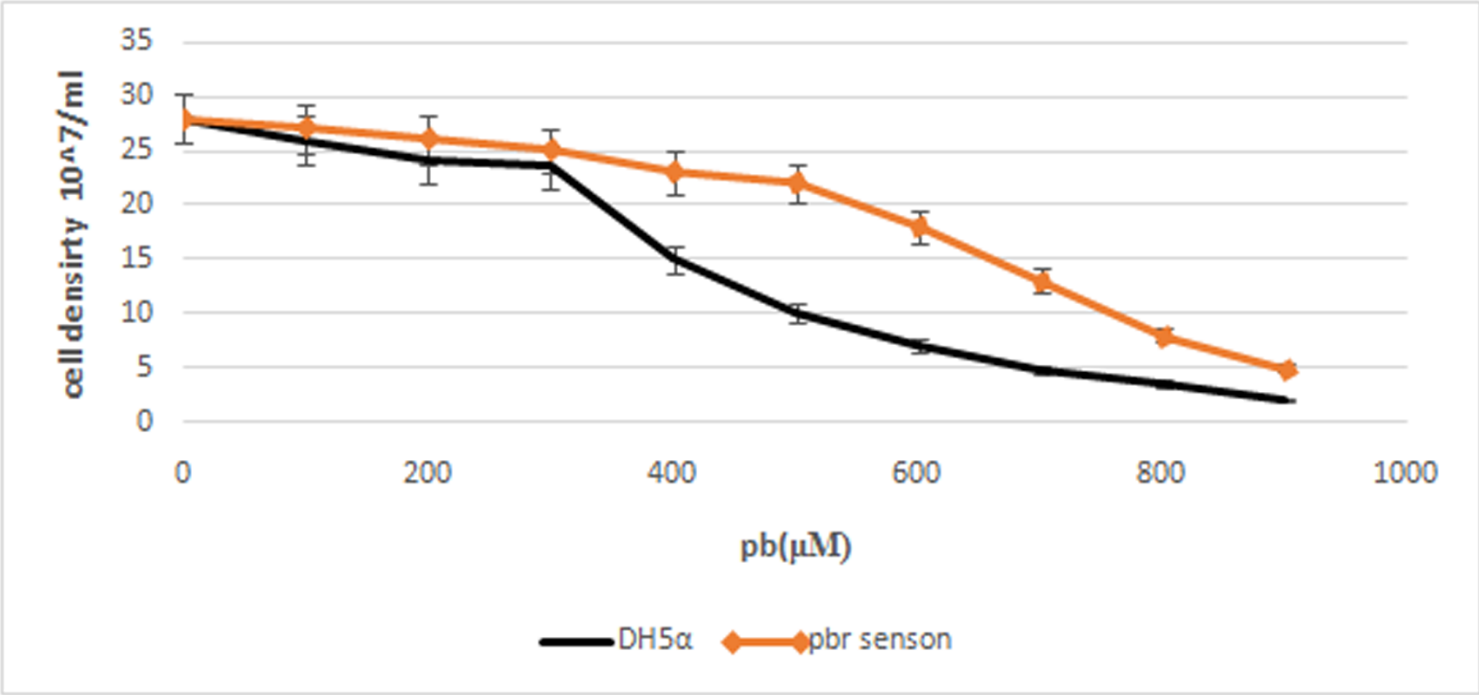

Figure 8

Difference in the growth rate of pGL3-luc/pbr-biosensor compared to E. coli strain DH5α. Resistance may be related to the pbrR regulatory gene.

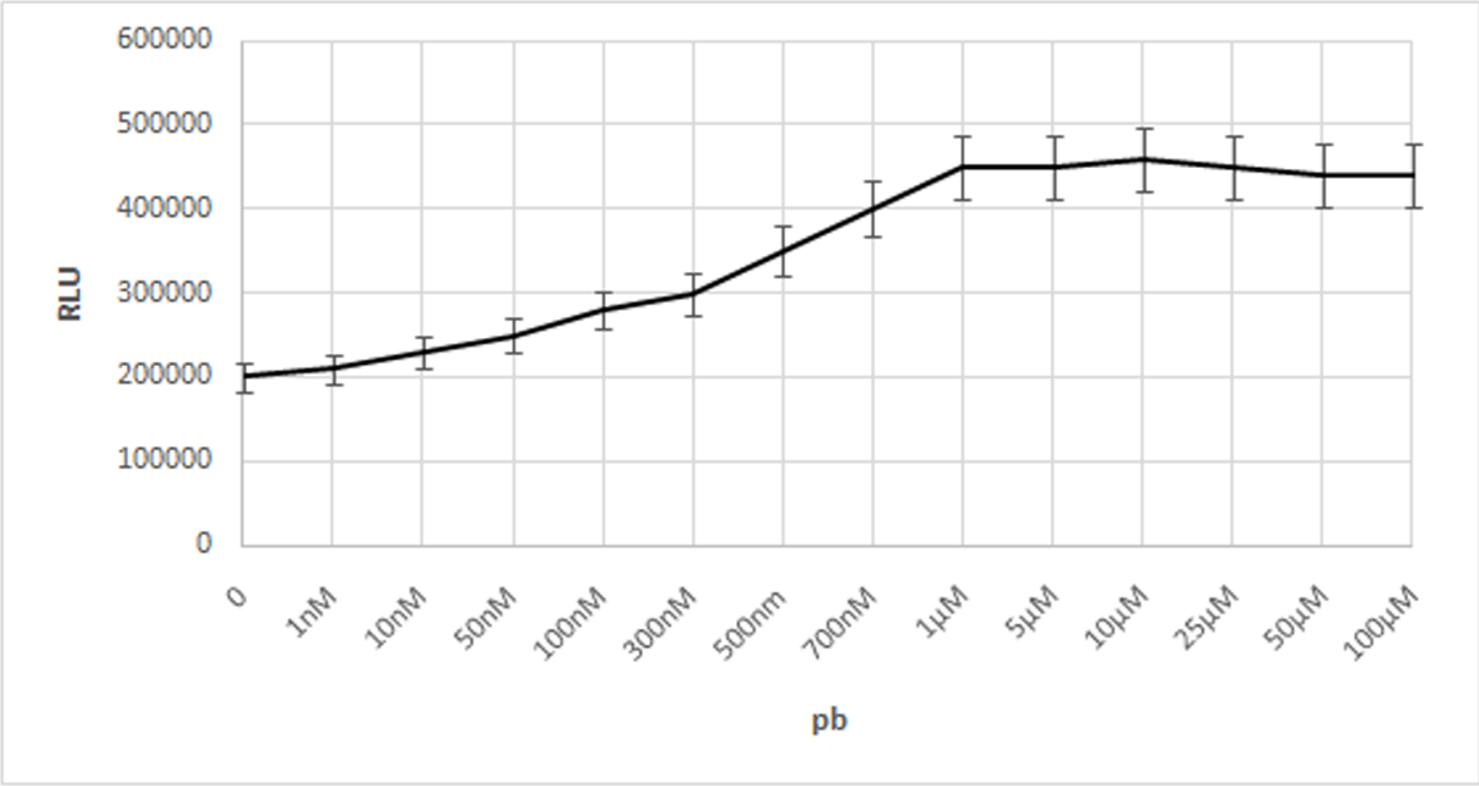

Figure 9

Expression of luciferase gene in different concentration of lead. Relative luminescence units (RLU).

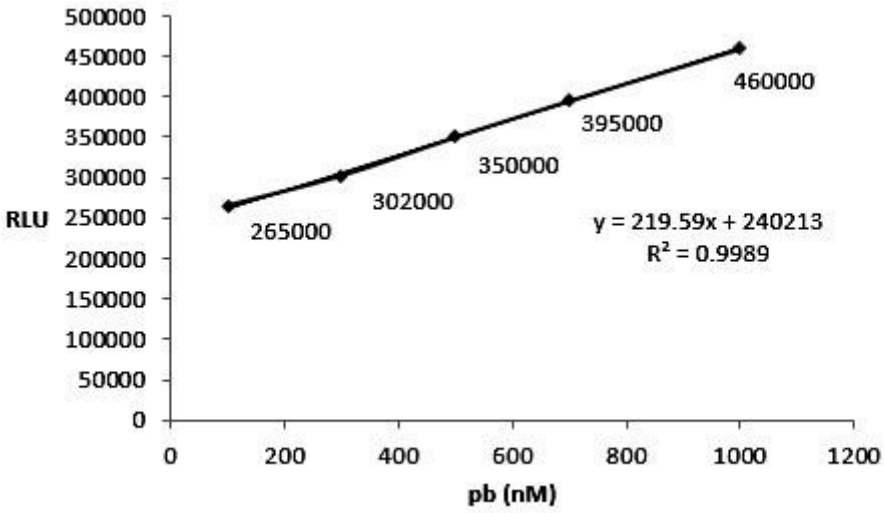

Figure 10

linear expression ranges of Luciferase expression between 100-1000 nM concentrations of lead.

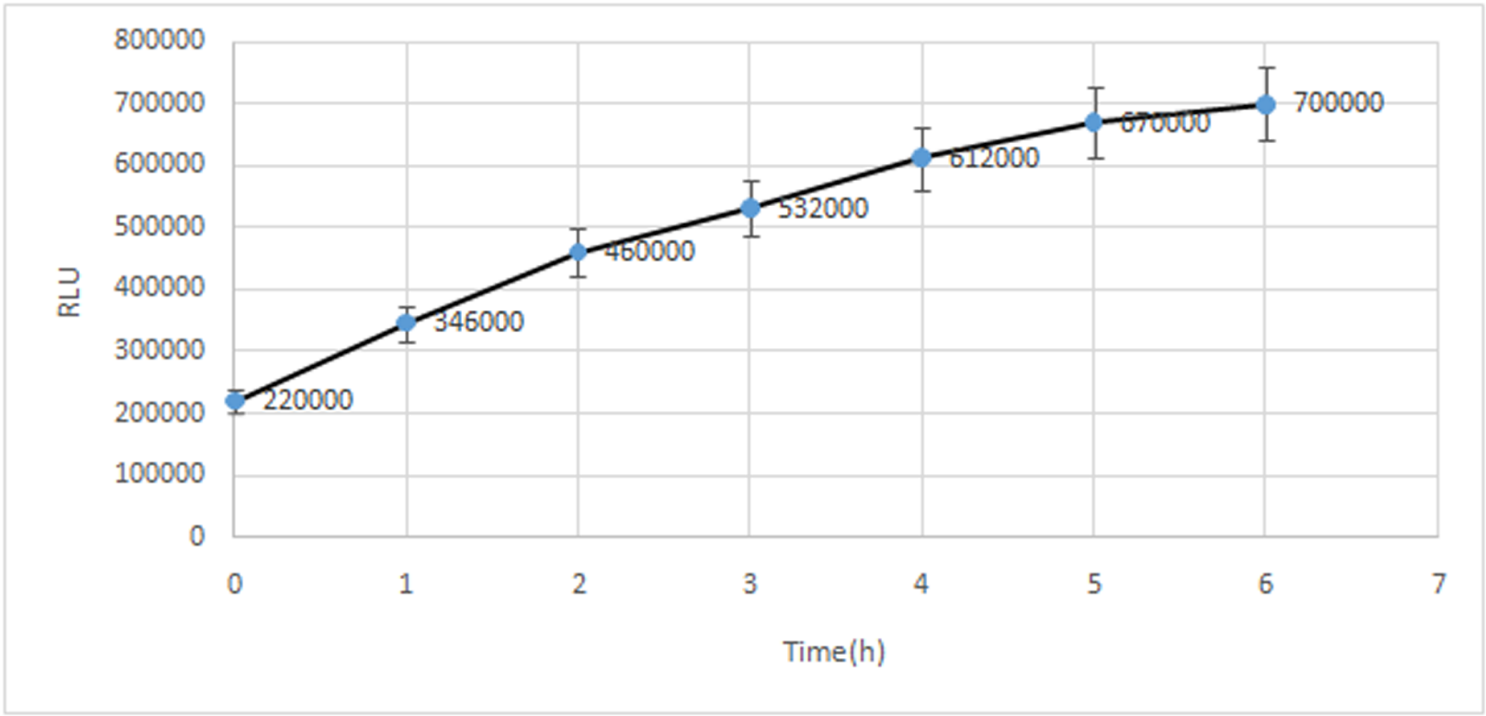

Figure 11

The expression of luciferase at different times at 1 μM lead concentration. During 2h, the amount of expression is high enough to measure Luciferase, in biological sensors; the pollution is measured at low rates. Relative luminescence units (RLU).