Chapter 18

Preparation and Assay of Simple *Light off* Biosensor Based on Immobilized Bioluminescent Bacteria for General Toxicity Assays

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Abstract

We describe a simple *light off* bioluminescent biosensor for general environmental toxicity assays based on immobilized bioluminescent bacteria engineered with beetle luciferases.

Key words Bioluminescent biosensor, Toxicity biosensor, pH-sensitive firefly luciferase

1 Introduction

Biosensors, analytical devices that combine the recognition of a biological substance of interest and the transduction of the response in a measurable signal, are widely used for environmental applications, allowing a quantitative and specific analysis [1–4]. They are useful due to their easy preparation and storage, minimum quantity of sample required, relatively low cost, good reproducibility, selectivity and sensitivity, fast response time, and, in addition they are useful to analyze bioavailability [1, 3].

Among the different kinds of biosensors, bioluminescent biosensors are very fast and sensitive and are gaining in popularity, because they rely on luciferases or photoproteins (enzymes that require oxygen to oxidize luciferin, producing light in a quantitative fashion). They can be divided into enzymatic and whole cell biosensors, in which the whole cell biosensor can be further divided into *light on* and *light off*. In the *light off* biosensors, the luminescence is inhibited by specific agents that affect the metabolism, reducing the intensity of the light signal, which can be monitored by photodetecting devices such as photometers, luminometers, and CCD cameras [5–7].

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A *light off* biosensor is built by cell transformation with an expression vector containing the reporter gene expressing luciferase [8] and the cells can be used in liquid cultures or immobilized to detect the toxicity of liquid samples and chemicals based on the decrease of bioluminescence intensity upon contact with toxic factors [9], such as heavy metals [5, 6, 10–12]. Here we describe the preparation and use of a simple *light off* biosensor to measure general toxicity of water samples and bioprospection of extracts, based on the transformation of *E. coli* with two beetle luciferases (*Macrolampis* sp2 firefly and *Pyrearinus termitilluminans* click beetle) [13]. Each luciferase has its own peculiarities, allowing their use for specific applications.

2 Materials

2.1 Buffers and Solutions

1. 0.1 mg/mL Ampicillin.
2. 40 mM ATP in 80 mM $MgSO_4$.
3. 10 mM d-Luciferin.
4. 10 mM d-Luciferin in 50 mM sodium citrate buffer pH 5.0.
5. 1 M dithiothreitol (DTT).
6. 1 M isopropyl-β-d-1-thiogalactopyranoside (IPTG).
7. 100 mM Tris–HCl pH 5.0 and pH 8.0.
8. 1.0 mg/mL 2,3,5-triphenyl tetrazolium chloride (TTC).

2.2 Media

1. Luria-Bertani (LB) Miller medium.
2. Agar pH 7.5: 10.0 g/L Casein enzymic hydrolysate, 5.0 g/L Yeast extract, 10.0 g/L Sodium chloride, 15.0 g/L Agar.
3. Broth pH 7.5: 10.0 g/L Casein enzymic hydrolysate, 5.0 g/L Yeast extract, 10.0 g/L Sodium chloride.
4. Top agar: LB broth with 0.7% (w/v) agarose.

2.3 Vectors and Bacterial Strains

1. *Escherichia coli* BL21 (DE3).
2. pPro vector (plasmid: Invitrogen).

2.4 Beetle Luciferases cDNA

1. *Macrolampis* sp2 firefly.
2. *Pyrearinus termitilluminans* larval click beetle.

2.5 Metal Solutions at 5.0 μg/mL

1. 0.0294 mM $AgNO_3$.
2. 0.02 mM $CuSO_4$.
3. 0.0184 mM $HgCl_2$.
4. 0.019 mM $NiSO_4$.

5. 0.0174 mM $PbCl_2$.
6. 0.0173 mM $ZnSO_4$.

2.6 Disinfectant and Sanitizing Reagents

1. 0.0831 mM 1-Propanol.
2. 3% Boric acid.
3. 70% Ethanol.
4. 1% Phenol.
5. 3% Hydrogen peroxide.
6. 2% Iodine.
7. 0.0471 mM Sodium carbonate.

2.7 Equipments

1. Charge-coupled device (CCD) camera Light Capture II (ATTO, Japan).
2. Centrifuge 5810 R (Eppendorf).
3. Ice water bath.
4. Incubator (37 °C) (EletroLab, Brazil).
5. Luminometer AB-2200 (ATTO, Japan).
6. Sonicator Ultrassonic Processor XL (Misonix, USA).
7. Spectrofluorometer F-4500 (Hitachi, Japan).
8. Water bath preset at 42 °C (Stern 6, Sieger, LojaLab, Brazil).

3 Methods

This method consists in preparing immobilized bioluminescent *E. coli*, by transformation of *E. coli* with pPro-Mac or pPro-Pte luciferase plasmids, followed by IPTG induction, immobilization in agarose matrix and, finally, assaying the in vivo luminescence in the absence (control) and presence of toxicants. Inhibition of bacterial luminescence intensity in relation to the respective controls is taken as evidence of general toxicity due to inhibition of ATP production in the cells. The luminescence assay can be performed using liquid cultures, or more conveniently using immobilized bacteria.

3.1 Bacterial Transformation

E. coli are transformed with recombinant plasmids pPro-Mac and pPro-Pte, containing *Macrolampis* sp2 firefly and *Pyrearinus termitilluminans* click beetle luciferase genes, respectively.

1. Add 1 μL of the luciferase plasmids (~10–50 ng) to 50 μL of competent *E. coli* cells. Gently swirl the microtubes several times to mix their contents. Place the tubes on ice for 30 min.
2. Immediately incubate the microtubes in a preheated water bath at 42 °C during 45 s. Do not shake the tubes.

3. Rapidly transfer the microtubes to ice. Allow the cells to cool down for 2 min.
4. Add 200 μL of LB broth medium to each microtube. Incubate the microtubes in a shaking incubator at 37 °C at 225 cycles/min during 1 h to allow the bacteria to recover and express the antibiotic resistance marker encoded by the plasmid.
5. Transfer 100 μL of the transformed cells into LB agar medium Petri dishes containing the appropriate antibiotic (in our case we use ampicillin) and spread with the aid of the handle Drigalski spatula.
6. Invert the plates and incubate them at 37 °C O/N. After 12–16 h the transformed colonies should appear in the plates.

3.2 Induction of Heterologous Luciferase Expression in Bacteria

1. Select one transformed colony with the aid of the Henle loop and inoculate it in Falcon tube filled with 5 mL of LB broth and the appropriate antibiotic (in our case we use ampicillin). Transfer the Falcon tubes to a shaking incubator at 37 °C at 225 cycles/min to grow up the cells overnight or until Abs_{600} ~1.0.
2. Transfer 1 mL of the cultured cells to a sterile Erlenmeyer containing 100 mL of LB broth, and place it in a shaking incubator at 37 °C at 225 cycles/min, monitoring the cell growth until $Abs_{600} = 0.40$.
3. Discontinue the growth by transferring the Erlenmeyer to a refrigerator for approximately 10–15 min.
4. Add 40 μL of 1 M IPTG to the cultured cells in the Erlenmeyer and induce protein expression by incubating in a shaking incubator at 20 °C at 225 cycles/min until $Abs_{600} = 1.5$.
5. After induction verify the luciferase expression by in vivo bioluminescence activity (*see* **Note 1**) using a luminometer (*see* **Note 2**).

3.3 In Vivo Luminometric Assay of Bioluminescence in Liquid Cultures

The induced bacteria in liquid cultures can be directly incubated with toxic agents as follows (*see* **Note 3**):

1. Mix 500 μL of the liquid cultures (of the **step 5** in Subheading 3.2) with 50 μL of toxic samples in water (metals at the concentration of 5.0 μg/mL or sanitizers). Incubate the treated cultures during 1 h at room temperature (~22 °C).
2. Mix 90 μL of the treated suspension with 10 μL of 10 mM d-Luciferin at pH 5.0 in a luminometer tube and measure luminescence intensity in *cps* in the luminometer.

3.4 Luminescence Assay Using Immobilized Bacteria and CCD Imaging

In order to assay several samples at the same time, an 96-well plate with immobilized bioluminescent bacteria can be prepared and used to simultaneously screen several samples in a more convenient, fast, and sensitive format.

3.4.1 Preparation of Immobilized Bacteria

The immobilized bacteria are prepared as follows:

1. Mix in 9 mL of melted Top Agar cooled down to ~40 °C in a water bath with 1 mL of the induced liquid cultures (of the **step 5** in Subheading 3.2), 10 μL of 1 M IPTG and 10 μL of the appropriate antibiotic (in our case we use ampicillin).
2. With the aid of a micropipette, quickly transfer 50–100 μL of this mixture in each well of the 96-well microplate.
3. Allow the bacterial/agarose medium to solidify during 1 h, and leave the immobilized bacteria during 12 h at room temperature (~22 °C).
4. After that, store the Elisa plate at 4 °C for up to 30 days (*see* **Note 4**).
5. Whenever the immobilized bacteria are to be used, remove the Elisa from the refrigerator, allow to warm up at room temperature (~22 °C) for at least 2 h, and use them for the toxicity assays.

3.4.2 Luminescence Assay of Immobilized Bacteria Using CCD Imaging

When the immobilized bacteria are ready to be used, proceed as follows to do the luminescence assay using CCD imaging:

1. Add 10 μL of toxicant sample to each well of the Elisa plate (*see* **Note 5**).
2. Allow the toxicant sample to penetrate the immobilized bacteria in each well by incubation at room temperature (~22 °C) during 2 h.
3. As a control use water or the solvent were the samples are diluted.
4. Add 10 μL of 10 mM d-Luciferin pH 5.0 to each well. Allow luciferin to penetrate the agarose gel during 30 min at room temperature.
5. Analyze the bioluminescence using a CCD photodetection system or in a plate reader.
6. The luminescence intensity of the samples and controls can be quantified by densitometry using the proper algorithm provided by the instrument maker (*see* Fig. 1).

3.5 Tetrazolium Colorimetric Assay of Cell Viability

In order to check for cell viability after treatment of the bacterial cultures, the tetrazolium salt colorimetric assay is employed as follows:

1. Mix 90 μL of the liquid or immobilized cultures with 10 μL of toxic samples (for example, metals at 5.0 μg/mL or sanitizers) and 10 μL of 2, 3, 5-triphenyltetrazolium chloride (TTC) at 1 mg/mL. Incubate at 37 °C for 1 h and check for the appearance of red color as an indication of reducing bioactivity (*see* **Note 6** and Fig. 2).

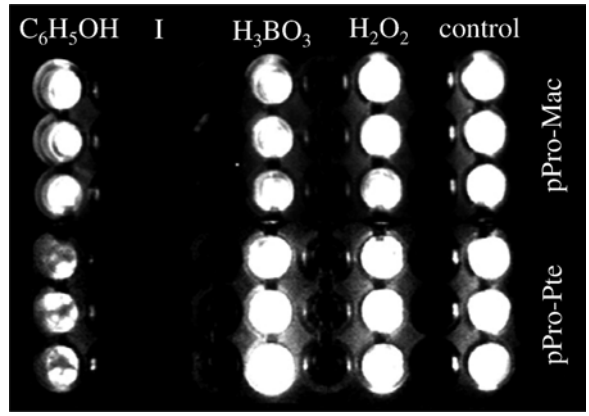

Fig. 1 CCD bioluminescence imaging of bioluminescent *E. coli* transformed with pPro-Mac and pPro-Pte after exposure to sanitizing agents 1 % phenol (C_6H_5OH), 2 % iodine (I), and 3 % boric acid (H_3BO_3) for 1 h at 4 °C (Adapted from Gabriel et al. [13])

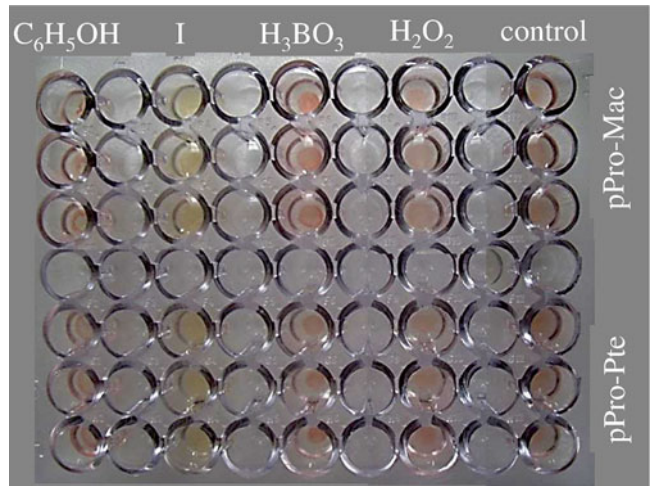

Fig. 2 Tetrazolium colorimetric assay of *E. coli* transformed with pPro-Mac and pPro-Pte after exposure to sanitizing agents 1 % phenol (C_6H_5OH), 2 % iodine (I), and 3 % boric acid (H_3BO_3) and incubation for 1 h at 4 °C (Adapted from Gabriel et al. [13])

4 Notes

1. The bioluminescent activity depends on the enzyme utilized. In our assay, *Macrolampis* sp2 luciferase can be used for fast assays using a more sensitive detection system, due to the rapid flash-like kinetics, whereas *Pyrearinus termitilluminans* click beetle luciferase can be used in cases where a less sensitive detection system is used due to its higher activity and slower kinetics, allowing integration for longer time. For *Macrolampis* sp2 the expected luciferase activity is around 15×10^7 cps/mL

and for *P. termitilluminans* the expected luciferase activity is around 20×10^7 cps/mL [13].

2. Use the induced cells of the liquid culture to do the in vivo assays (*see* Subheading 3.2) and to prepare immobilized the bacteria in Top Agar (*see* Subheading 3.4.1).
3. It must be emphasized that this cell biosensor assay, does not give any clue about the specific compound or group of toxic compounds present in the assayed sample, it just gives a fast and general information about the toxicity of the sample.
4. The plate can be stored at 4 °C until 3 weeks with high luminescence activity.
5. This *light off* biosensor can be adapted to be used for bioprospection of antibiotics and other bactericidal samples.
6. The tetrazolium reagent has no color. When reduced it gives a bright red color, indicating the presence of reducing power of live bacteria.

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