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DEVELOPMENT AND APPLICATION OF BIOLUMINESCENT YEAST-REPORTER SYSTEMS FOR SCREENING CHEMICALS FOR ESTROGENIC AND ANDROGENIC EFFECTS

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ABSTRACT: The Endocrine Disruptor Screening and Testing Advisory Committee (EDSTAC) mandated the Environmental Protection Agency (EPA) to develop rapid, high throughput screening systems to assess a chemical compound's effects on hormonal systems. In response, two yeast strains, *S. cerevisiae* BLYES and *S. cerevisiae* BLYAS, were developed that produce a rapid, measurable bioluminescent signal in response to either an estrogenic or androgenic compound, respectively. To validate the utility of each strain, approximately 70 chemicals with known estrogenic and androgenic responses were tested. The BLYES assay produced a 17β-estradiol standard curve with an EC_{50} value of $7.3 \pm 3.1 \times 10^{-10}$ M (n = 20) and lower and upper limits of detection of 4.5×10^{-11} to 2.8×10^{-9} M. In the BLYAS assay using dihydrotestosterone as a standard, the lower limit of detection and the EC_{50} values were 2.5×10^{-9} M and $(9.7 \pm 4.6) \times 10^{-9}$ M (n = 17), respectively. In both assays, bioluminescence was observed in as little as 1 hour and data was collected in 3 – 4 hours. A third strain, *S. cerevisiae* BLYR which produces constant bioluminescence has also been developed to measure toxicity of each chemical, demonstrated by a decrease in bioluminescence.

In the laboratory, the assays can be used in a microtiter plate format to screen an array of chemicals or water samples. The primary advantages of these bioluminescent assays over other reporter assays include ease of use, efficiency of gathering data, and potential for use of multiple assays per microtiter plate. Further, when combined with integrated circuit-based photodetection technology, they may be used for rapid, remote monitoring of industrial and municipal waste effluents carrying hormonal active agents. Prototype circuits and instruments have been developed for evaluating this purpose. Potential users of these assays include (i) EPA for screening chemicals, (ii) EPA, United States Geological Survey, state, and local municipalities for monitoring water quality, and (iii) industry for screening new pharmaceuticals and personal care products.
KEYWORDS: endocrine disruptors; detection; bioluminescent bioreporters; 17β-estradiol; dihydrotestosterone

INTRODUCTION

Evidence suggests a wide variety of chemical compounds including pesticides, plasticizers, synthetic hormones and naturally occurring chemicals, possess steroid-like activity that may lead to the disruption of endocrine systems in vertebrates (Cooper and Kavlock, 1997; Fang *et al*., 2000; Folmar *et al*., 2002; Kavlock *et al*., 1996; Tyler *et al*., 1998). In response to public health concerns, the United States Congress, as part of the Food Quality Protection Act (FQPA) of 1996 and the Safe Drinking Water Act of 1996, contained provisions for determining whether pesticides or chemical substances found in or on food or in drinking water may have estrogenic or other endocrine effects. The FQPA directed the Environmental Protection Agency (EPA) to develop a screening program for evaluating the potential of pesticides and other substances to induce hormone-related health effects. This screening approach is enormous in scope, with the EPA estimating that 87,000 existing and new chemicals require testing (EDSTAC, 1998).

To accomplish this task, the EPA proposed a tiered screening mechanism to prioritize chemicals for in-depth testing. To accomplish this, a high throughput pre-screening (HTPS) mechanism is required. Preferably, a HTPS would rapidly determine if a chemical has endocrine disrupting properties that require further evaluation. Yeast bioassays have the potential for high throughput chemical screening for endocrine disruption. Two widely used receptor/reporter assays for detecting estrogenic and androgenic compounds are the Yeast Estrogen Screen (YES) (Routledge and Sumpter, 1996) and the Yeast Androgen Screen (YAS) (Purvis *et al*., 1991). Theses *Saccharomyces cerevisiae* strains contain the human estrogen receptor (hER-α) or the human androgen receptor (hAR) integrated into the chromosome. The reporter plasmid contains the estrogen response element (ERE)-*lacZ* reporter fusion or the androgen response element (ARE)-*lacZ* reporter fusion. When a chemical with estrogen-like or androgen-like properties binds to the receptor protein, it in turn, binds to the response element inducing transcription of *lacZ*. The β-galactosidase transforms the chromogenic substrate chlorophenol red-β-D-galactopyranoside (CPRG) to a red product measured by absorbance at 540 nm. These assays have been used extensively to measure endocrine responses to polychlorinated biphenyls (PCBs) and hydroxylated derivatives (Schultz *et al*., 1998; Layton *et al*., 2000), pesticides (Sohoni *et al*., 2001) as well as detection of estrogens/androgens in environmental waterways (Thomas *et al*., 2002), aquifers (Conroy *et al*., 2005), wastewater treatment systems (Layton *et al*., 2000) and dairy manure (Raman *et al*., 2004).

Although proven effective for the *in vitro* determination of endocrine activity, the colorimetric YES/YAS assay's incubation time of 3 – 5 days is impractical when considering the 87,000 chemicals requiring Tier I screening. Further, the colorimetric assay is not suitable for on-line automated detection of endocrine disruptors present in the environment. The long-range goal of this project is to develop and utilize yeast-based bioluminescent bioreporters for the rapid detection of endocrine disruptors in the environment. The specific objective of the present research is to validate each bioreporter's response to known estrogenic and androgenic chemicals.

MATERIALS AND METHODS

Strains and Growth Media

Three *S. cerevisiae* strains incorporating the bacterial *lux* operon were genetically engineered to respond to estrogens (*S. cerevisiae* BLYES; Sanseverino *et al*., 2005), androgens (*S. cerevisiae* BLYAS; Eldridge *et al*., submitted), and constitutive bioluminescence production for toxicity measurements (*S. cerevisiae* BLYR). In the conceptual model shown in Figure 1, the gene for the human estrogen receptor (hER) is incorporated into the yeast chromosome. This receptor binds with an estrogen forming a complex which in turn binds specific estrogen response elements (EREs). When the complex is bound to the EREs, transcription and translation of the bioreporter genes occur thus generating a measurable bioluminescent response. For specific chemical assays, strains BLYES, BLYAS and BLYR were grown in yeast minimal medium (YMM; leu^-, ura^-) overnight at 30°C and 200 rpm shaking to an OD_{600} of ~1.0.

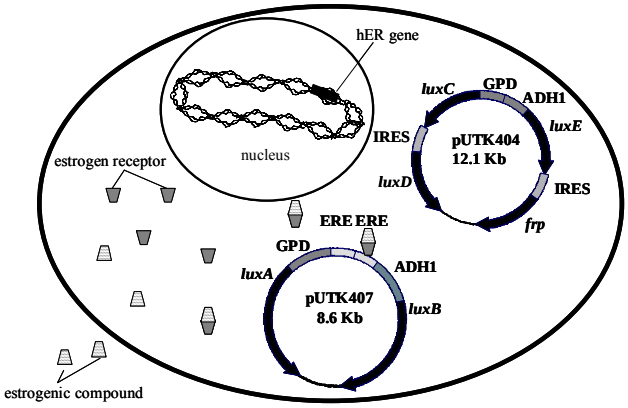

Figure 1. Schematic representation of *S. cerevisiae* BLYES. Estrogenic compounds cross the cell membrane and bind to the estrogen receptor. This complex interacts with the ERE initiating transcription of *luxA* and *luxB*. The gene for the human estrogen receptor is located on the chromosome. The four remaining genes required for autonomous bioluminescence production (*luxC, luxD, luxE* and *frp)* are co-located on a second plasmid under the control of two constitutive promoters.

Bioluminescence Assay

The assay is conducted in a 96-well microtiter plate format. Typically, test chemicals are diluted in methanol to 1 mM, 0.5 mM and 0.25 mM, and then placed on the Beckman F/X Automated Liquid Handling System platform. The robotic system performs 1:10 serial dilutions, placing 20 µl of each solution into the appropriate wells of multiple black 96-well Microfluor microtiter plates (Dynex Technologies, Chantilly, VA) (Fig. 2). Residual methanol is removed by evaporation. Two hundred µl of BLYES or BLYAS is placed into each well of the 96-well plate. For each test assay, a duplicate plate is created using the toxicity control strain BLYR. Bioluminescence is measured every 60 min for 12 h in a Perkin-Elmer Victor2 Multilabel Counter with an integration time of 1 s/well.

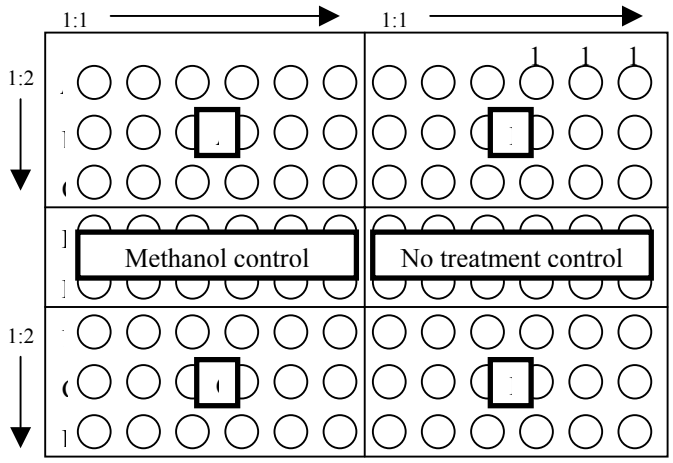

Figure 2. Current plate setup in the high throughput system of chemical screening. A series of dilutions of one chemical in methanol is performed with the robot, across 18 wells of a 96-well plate. Dilutions for *S. cerevisiae* BLYES begin with 1 mM and end with 2.5 nM, while initial tests with *S. cerevisiae* BLYAS have begun with 1 μM and ended with 2.5 pM. Methanol only controls consist of wells to which methanol (without chemical) was added to wells. Standards (either 17β-estradiol or dihydrotestosterone) are used on every plate at this time.

RESULTS AND DISCUSSION

This research has standardized two bioluminescent yeast assays for screening estrogen- and androgen-mimicking compounds. To date, approximately 70 chemicals with known estrogenic and androgenic responses have been tested with the BLYES and BLYAS assays, respectively. The BLYES assay produced a 17β-estradiol standard curve with an EC_{50} value of $7.3 \pm 3.1 \times 10^{-10}$ M (n = 21) (Fig. 3). For the BLYAS assay, the average EC_{50} value for DHT was $1.07 \pm 0.47 \times 10^{-8}$ (n = 13) (Fig. 4). The results from these assays compared favorably with the colorimetric assays (Sanseverino *et al.*, 2005; Eldridge *et al*., submitted). These data are being used to develop a set of rules for data acceptance which will lead to developing a computer algorithm for automating data output.

In its current configuration, the assay may be employed in a properly equipped laboratory to screen multiple water samples simultaneously. Using a microtiter plate reader to record bioluminescence over time and appropriate software, the data output can be recorded and interpreted for the end user. While the assay does not identify each chemical, it preempts usage of expensive and time-consuming analytical methodology for routine analysis. If an assay records a response, then a more rigid analysis can be performed to identify the endocrine disruptor(s).

The long-range goal is to combine each bioluminescent bioreporter with appropriate photodetection technology for use as remote, near real-time monitoring of our nation's waterways for endocrine disrupting activity (Bolton *et al*., 2002; Nivens *et al*., 2004). Integrated circuits equipped with a photodetector are under development (Fig. 5). Conceptually, these devices will have bioreporters immobilized onto the photodetector, then sealed in a water permeable membrane. The integrated circuits can be equipped with wireless communications and GPS to transmit data and position of the device.

Potential users of these assays include (i) the Environmental Protection Agency (EPA) for screening chemicals, (ii) EPA, the United States Geological Survey (USGS), state, and local municipalities for monitoring water quality, and (iii) industry for screening new pharmaceuticals and personal care products. In addition, these strains are available to the broader scientific community (national as well as international) for research.

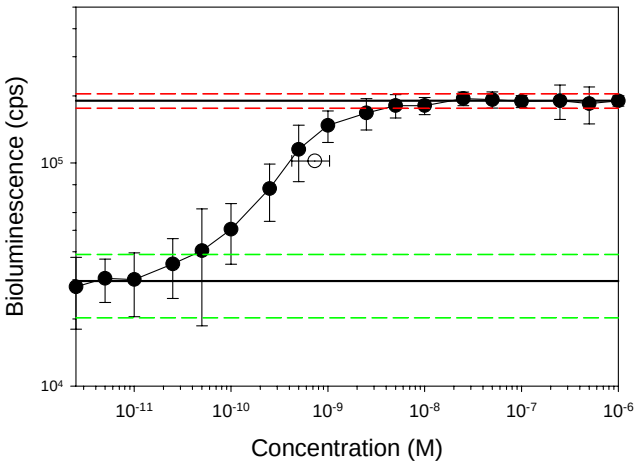

Figure 3. This curve was compiled from 21 individual BLYES assays. Error bars represent the standard deviation for each data point. The upper and lower solid horizontal lines represent the maximum and minimum bioluminescence produced with dashed lines representing one standard deviation. The open circle represents the calculated EC_{50} value for 17β-estradiol (7.3 x 10^{-10} M) with the error bars representing one standard deviation (± 3.1 x 10^{-10} M).

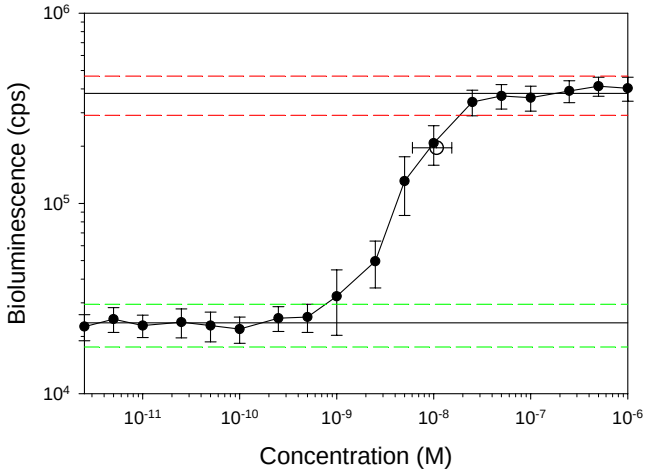

Figure 4. Average standard curve (n = 13) to dihydroxytestosterone using *S. cerevisiae* BLYAS. Error bars represent the standard deviation for each data point. The upper and lower solid horizontal lines represent the maximum and minimum bioluminescence produced with dashed lines representing one standard deviation. The open circle represents the calculated EC_{50} value for DHT (1.07 x 10^{-8} M) with the error bars representing one standard deviation (± 4.7 x 10^{-9} M).

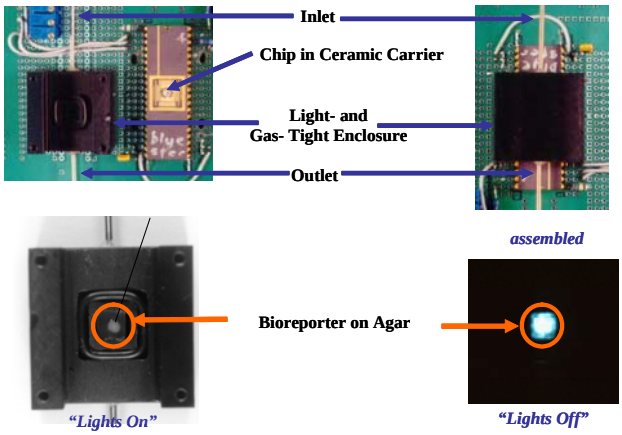

Figure 5. Bioluminescent bioreporter integrated circuit. In this configuration, the bioreporters are immobilized in agar over the photodetector. This chip is covered with a light-tight enclosure. Fluid is circulated through this enclosure and over the immobilized cells. Data can be stored on the chip or downloaded directly to a computer for analysis.

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