ERDC TN-DOER-C30
February 2003

A New Transgenic Cell Line for Detection of Invertebrate Endocrine Disrupters: Laboratory Protocol for its Use

PURPOSE: The purpose of this technical note is to report the development of a cell-based assay for the detection of endocrine disrupting activity related to crustacean and insect molting and to provide a detailed laboratory protocol for its conduct.

BACKGROUND: The advent of transgenic technology and the ability to insert foreign reporter genes into the genomes of standard culturable cells has led to the improvement of cell-based assay techniques. For example, mammalian cell cultures have been used for many years in assays for dioxins and related compounds. The basis for their use is the presence in these cells of the aryl hydrocarbon receptor (AhR) system that binds certain planar uncharged organic chemicals, and mediates their toxicities. The assays have been improved by inserting genes of bacterial or insect origin into the nuclear DNA that act as "reporters." When upstream dioxin recognition sequences are activated by binding of AhR-dioxin complexes, the reporter genes express gene products that result in signals that can be measured instrumentally (Inouye and McFarland 2000).

The P450RGS assay for polychlorinated dibenzodioxins/dibenzofurans (PCDD/Fs), polychlorinated biphenyls (PCBs), and polycyclic aromatic hydrocarbons (PAHs) is based on the transgenic 101L cell line, a modification of human liver cancer HepG2 cells developed at the University of California, Berkeley. In 101L cells the human CYP1A1 gene promoter sequence has been fused with the insect firefly luciferase gene downstream as a reporter. Hence, the designation "P450" for the cytochrome P450 family of monoxygenases of which the CYP1A1 gene encodes isozymes responsive to dioxin and related compounds, and "RGS," which refers to Reporter Gene System. When 101L cells are exposed in the assay to chemicals with dioxinlike activity, light is produced in proportion to the intensity of the exposure and is measured in a luminometer. The assay has relevance to biota that possess the CYP1A1 gene or genes that are homologous with it. These include the teleost fishes, birds, amphibians, reptiles, and mammals; the CYP1A1 gene is absent or less inducible in lower organisms. Consequently, although chemicals such as dioxins and related compounds are extremely toxic to higher organisms possessing the CYP1A1 gene, they are either nontoxic to lower organisms, or if toxic, their effects are manifested through mechanisms that do not involve CYP1A1. Presently there are no methods similar to the P450RGS assay that are capable of discriminating the potential for chronic toxicity to invertebrate organisms in environmental samples.

INTRODUCTION: This Technical Note describes a newly developed transgene system designed to detect and measure the activity of environmental contaminants that are chronically toxic to lower organisms lacking the CYP1A1 gene but which are susceptible to contaminant toxicity through other mechanisms. The assay based on these new cells detects chemicals that disrupt the invertebrate endocrine system responsible for ecdysis (molting). Molting is a multistage process of shedding the exoskeleton and replacing it with a larger one, and its successful completion is essential for the survival, growth, and reproduction of arthropods, the invertebrate phylum that includes crustaceans and insects. In both classes of organisms molting is under the control of ecdysteroid hormones.

These hormones function similarly to other steroidal endocrine hormones such as the estrogens or corticoids in higher organisms. A cascade of events is initiated by binding of the hormones with receptors within the cytoplasm of cells. When bound, the receptor-hormone complexes are translocated across the nuclear membrane. Activation of DNA by these complexes results in the synthesis of proteins that regulate molting. Disruption of the molting process by chemicals has been observed. For example, the PCB mixture Aroclor 1242 (A1242), a common environmental contaminant, has been shown to drastically inhibit the molting of fiddler crabs (*Uca pugilator*) (Fingerman and Fingerman 1977). In the same study octachlorodibenzofuran-exposed crabs molted at about the same rate as did the controls. Both A1242 and octachlorodibenzofuran bind the AhR and are active in the P450RGS assay. Aroclor 1242 is a mixture of about 100 PCB congeners, seven of which are coplanar and bind the AhR. It is these seven that apparently account for all of the A1242 activity in the P450RGS assay. Considering the lack of molt inhibition observed for octachlorodibenzofuran – which is also coplanar and binds the AhR – it appears that the molt-inhibiting potency of A1242 must reside with the non-coplanar PCB congeners.

In a recent investigation, exposure to a single PCB congener, 2,4,6-trichlorobiphenyl (PCB30), disrupted molting in crawfish (Jones et al. 2000). This particular congener was chosen because it was demonstrated to have potent estrogenic effects in juvenile fish (Westerlund et al. 2000), and there are structural and functional similarities between the estrogen receptor in fish and the ecdysteroid receptor in arthropods. It was hypothesized that the probable toxic mode of action of molt disruption by such chemicals involves mimicking ecdysteroids or blocking their normal receptor binding. PCB30 is not coplanar and does not bind the AhR. Consequently, PCB30 shows no activity in the P450RGS assay. The implication of this is that environmental PCBs likely have multiple modes of toxicity, and represent different kinds of hazard to different taxa. For completeness in ecological risk assessments, tests are required with endpoints specific for those different taxa and hazards.

Sediments contaminated with complex mixtures high in PAHs and metals have been shown to have severe effects on molting and survival of crustaceans. Peddicord and McFarland (1976) exposed 3- to 4-cm juvenile crabs (*Cancer magister*) to high concentrations of suspended Oakland Inner Harbor sediments over a period of 25 days. High mortality resulted, and 92 percent of the deaths occurred during the molting process. The molt period was abnormally extended, and crabs were trapped within the old exoskeleton and died. Those that were not killed during molting were severely deformed and would not likely have survived in the wild. Survivors that were removed from exposure and maintained in clean water resumed normal molt activity. These results indicate that environmental contaminants that are characteristic of ship channel sediments can affect the molting process and by so doing can have serious consequences for survival, growth, and reproduction of invertebrate organisms.

CONSTRUCTION OF THE HepG2-EcR CELL LINE: Initial attempts to develop a cell-based assay for molt-disrupting chemicals were based on a commercially available ecdysone receptor-based system, the Ecdysone-Inducible Mammalian Expression System® (Invitrogen). Functioning of the system is described at the Invitrogen Tech-Online website (*http://www.invitrogen.com/*). The system can be used to study any gene of interest and its products by transiently transfecting it into isolated human embryonic kidney cells (EcR-293 cell line, Invitrogen) where gene expression can be triggered by the addition of a foreign agonist, in this case an ecdysteroid. The system was derived

from the fruitfly (*Drosophila melanogaster*) but modified to function in mammalian cells that do not ordinarily respond to ecdysone or other ecdysteroids. Two plasmids, pVgRXR and pIND, form the basis of the system. The pVgRXR plasmid expresses the ecdysteroid receptor and pIND contains the receptor-ligand response element E/GRE. When ecdysone or another ligand having ecdysteroid activity is present, binding of the receptor-ligand complex to the response element results in transcription of the protein of interest (Figure 1). The bacterial β-galactosidase gene is contained downstream from the response element and serves as a reporter. The enzyme β-galactosidase is expressed and cleaves a substrate in response to binding of the receptor-ligand complex with the response element. The yellow product that results from the cleavage can be monitored colorimetrically. This mechanism provides the principle on which an assay for contaminants affecting molting at the level of receptor binding can be based. In the assay, Ponasterone A, a high-potency analog of the molting hormone, ecdysone, is added to the cells prepared on a microtiter plate. Used alone, Ponasterone A will produce an ecdysteroid response in proportion to its molar concentration. If xenobiotic chemicals are present that also bind the ecdysone receptor, the production of β-galactosidase and the resultant amount of colored product will be affected; i.e., either increased or decreased. An increase signals the presence of a xenobiotic agonist of the receptor, and a decrease signals the presence of an antagonist. Both types of effect indicate the potential for disruption of the molting process due to ecdysone receptor-active foreign chemicals in the assay.

Several problems were encountered with using Invitrogen's human embryonic kidney EcR-293 cell line during preliminary assays. The cells are semi-adherent, resulting in high variability between replicates as cell numbers varied due to losses in transfection and rinsing steps. Additionally, the transient transfection with the second plasmid was time and reagent consuming, and required the use of a marker plasmid to correct for transfection efficiency. It was decided to address these problems by developing a stably transfected cell line in which the Invitrogen ecdysone receptor plasmids are inserted into the genome of the adherent HepG2 cell line (human liver hepatoma origin) also used in the P450RGS assay. This transfection was successful and resulted in permanent cultures of the new cell line, HepG2-EcR.

Studies are now ongoing to characterize the responses of HepG2-EcR to a series of model ecdysteroids, environmental chemicals having the potential for interaction with ecdysteroid receptors, and extracts of contaminated sediments and surface water. Since A1242 has been previously shown to inhibit molting, it was selected as a model contaminant to compare the responses of the new cell line with molting inhibition in an experimental organism, the crawfish (*Procambarus clarkii*). The laboratory protocol for performance of the assay is included as Appendix I. HepG2-EcR culture samples are available by request for research purposes only.

METHODS AND MATERIALS

Generation of the New Cell Line. Optimized electroporation methods were used to transfect HepG2 cells with the pVgRXR plasmid. Cells were allowed to recover for 3 days before treatment with Zeocin to select for cells containing the plasmid. Colonies were then generated from single cells and screened for activity (the second plasmid, pIND, was transiently inserted using the cationic transfection reagent, LipoFectamine,® then tested for β-galactosidase activity after exposure to the EcR agonist, Ponasterone A). Cells from the colony with the highest activity were further transfected with the second plasmid (pIND) and allowed to recover for 3 days before treatment with

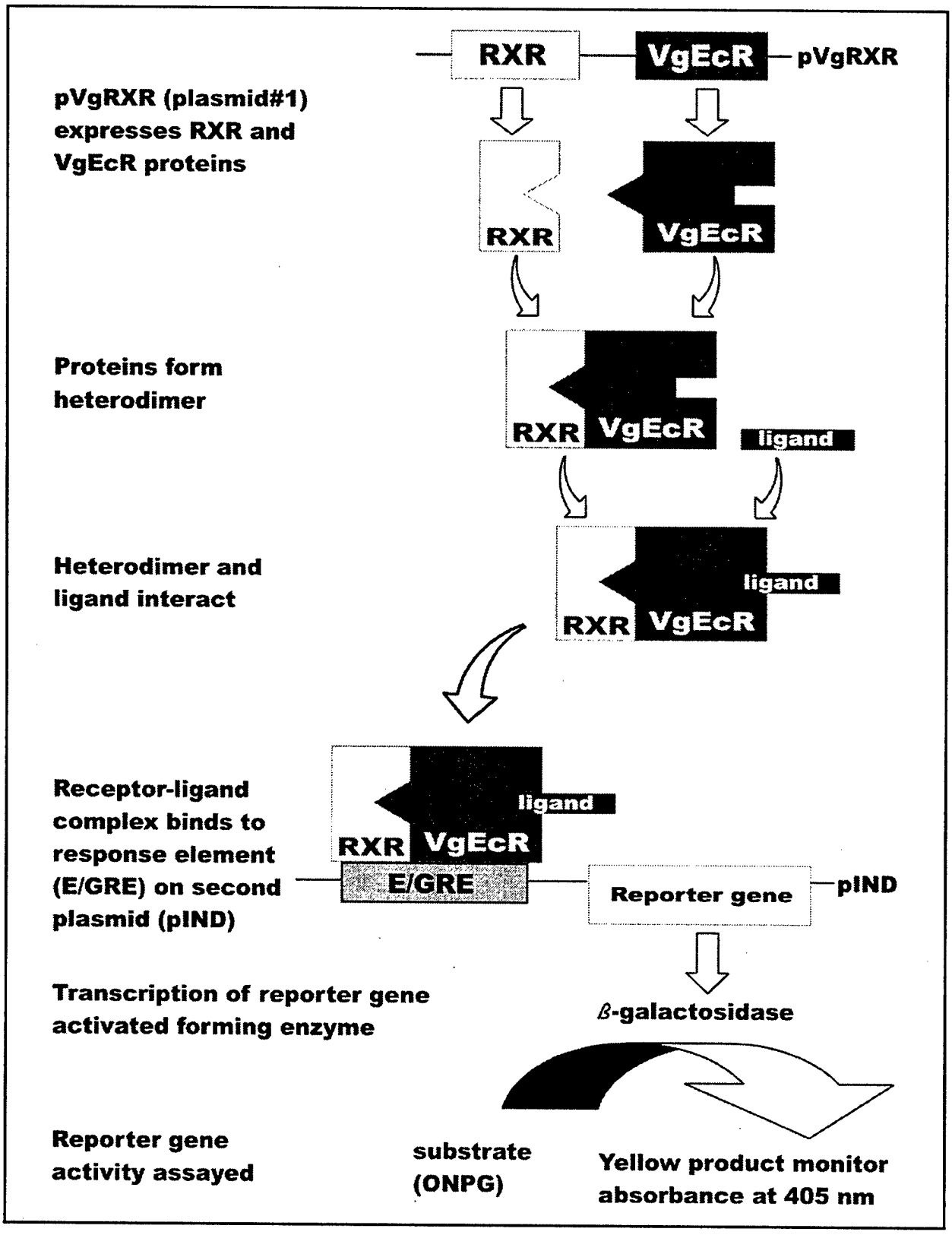

Figure 1. Invitrogen's Ecdysone-Inducible Mammalian Expression System

Hygromycin to select for cells carrying the pIND plasmid. Colonies were again generated from single cells, and screened to isolate the one with the highest activity, which was then designated HepG2-EcR.

HepG2-EcR Assay. For details, see Appendix I. Briefly, cells were plated in 96-well plates at 40K cells per well. After the cells were allowed to attach overnight, cells were treated with A1242 (1, 10, 100 and 500 ppb) in the presence of 0, 1, 3, 10, 30, or 100 μM Ponasterone A. That is, six series of dose combinations were prepared in which each of the A1242 concentrations was combined with one of the Ponasterone A mass concentrations. Additionally, one set of cells was treated with Ponasterone A alone at the five 1-100 μM mass concentrations. After 48 hr, cells were lysed in 100 μL of lysis buffer and the lysate analyzed for β-galactosidase activity.

β-Galactosidase Assay. The lysate (50 μl) was transferred to a 96-well plate, and Buffer A added to each sample (110 μL; 100 mM NaH_2PO_4, pH 7.5, 10 mM KCl, 1 mM $MgSO_4$, 50 mM β-mercaptoethanol). After a 5-min pre-incubation at 37 °C, 50 μL of substrate was added (4 mg/mL of *o*-nitrophenyl-*p*-galactopyranoside (ONPG) to each well. Immediately after addition of the substrate, the absorbance was measured at 405-420 nm every minute for 15 min in a temperature-controlled (37 °C) plate reader.

Data Analysis. Data from five separate experimental runs were combined by normalizing the activity to the maximal activity achieved with Ponasterone A alone. Data were plotted as log-dose versus response to determine whether there were shifts in the effective doses for either 50 percent maximal activity (EC_{50}), or for maximal activity (EC_{max}) in the presence of A1242. Data from 10-μM Ponasterone A co-exposures were compared to 10 μM Ponasterone A to determine whether this single endpoint is a viable alternative to running complete dose response curves.

Crawfish Molting Assay. In separate experiments, juvenile crawfish (<1.5 cm) were held individually in 22-mL vials, with water changes twice daily. They were fed once daily with crushed catfish food pellets. Animals were held until molting was observed, at which time exposures were begun. Exposures included nondosed controls, solvent controls (DMSO), and 100, 500, or 1,250 ppb A1242; exposures continued until a second molting was observed.

RESULTS AND DISCUSSION

HepG2-EcR Assay. Enzymatic activity as changes in optical density (mOD/min) at five Ponasterone A mass concentrations (1, 3, 10, 30, and 100 μM) is shown in Figure 2. The data are normalized to the activity of 100 μM Ponasterone A alone (maximal induced activity). No significant changes were observed in the effective dose for EC_{50} in the cells, but a depression in the EC_{max} activity was observed with the 500-ppb A1242 dose in the presence of 10-100 μM Ponasterone A, indicating that A1242 disrupts the normal ligand-receptor interaction. In an effort to streamline the testing protocols, the A1242 dilutions in the 10-μM Ponasterone A doses were compared to determine whether responses at that single dose are indicative of responses observed in the full dose-response curve. Activity was significantly suppressed in both the 500- and 100-ppb A1242 exposures when compared to Ponasterone A alone (Figure 3), and the suppression appeared to be dose-related ($r^2 = 0.80$ for log-dose versus response regression).

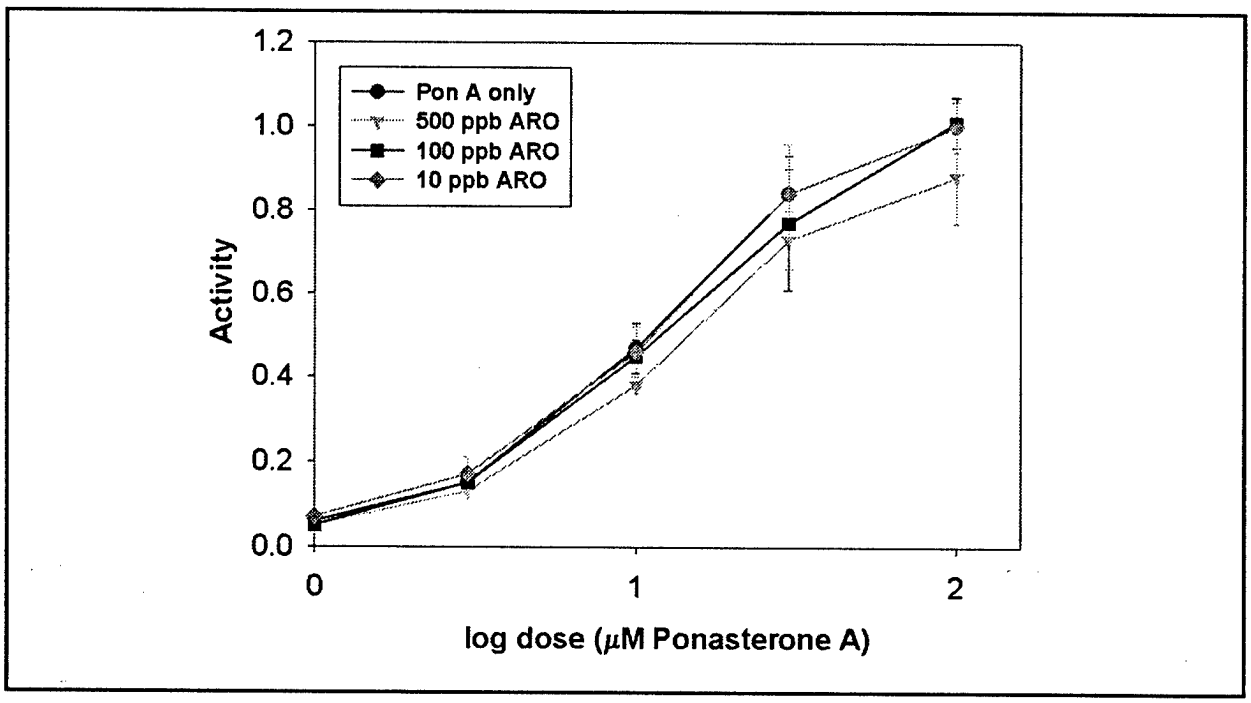

Figure 2. HepG2-EcR cell line dose-response. Data from co-exposure with Ponasterone A and A1242. Activity (mOD/min) in Aroclor treatments is normalized to the maximal induction of Ponasterone A alone to allow comparison between experiments. Error bars represent standard deviation of the measurements for the five experiments used to generate the data

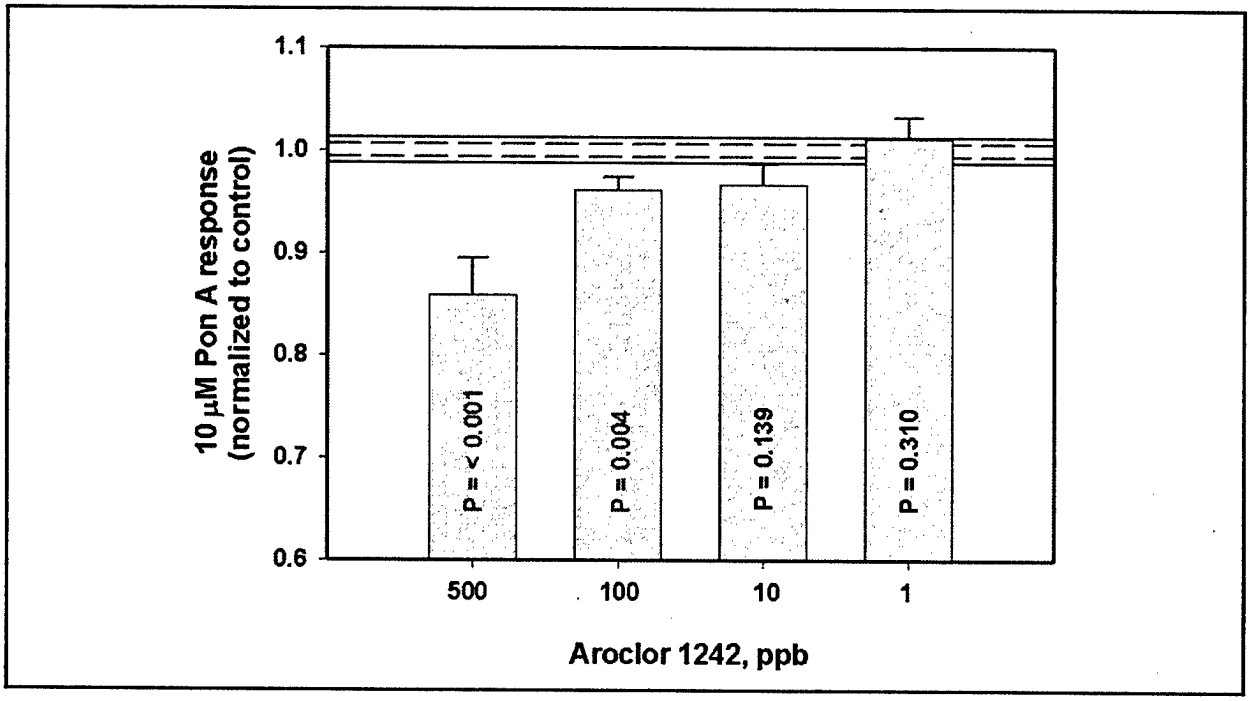

Figure 3. Effect of co-administration of A1242 on 10-mM Ponasterone A response of HepG2-EcR cells. Data from five exposures, each normalized to their respective responses to 10-μM Ponasterone A alone. Horizontal lines are Ponasterone A alone 95 percent confidence limits, dashed lines are Ponasterone A alone standard error, and error bars are standard error. Treatments are significant or not significant at the probabilities indicated

Crawfish Molting Assay. Molting was significantly inhibited at 500 ppb and 1,250 ppb A1242 (Figure 4). Although a direct connection with the observed molting inhibition cannot be definitively linked to EcR receptor disruption without more studies (i.e., it may be a nonreceptor-related effect because molting involves the interactions of a number of physiological processes), the crawfish and the cell-based assays both indicated that A1242 is capable of inhibiting molting in juvenile crawfish, as in fiddler crabs (Fingerman and Fingerman 1977).

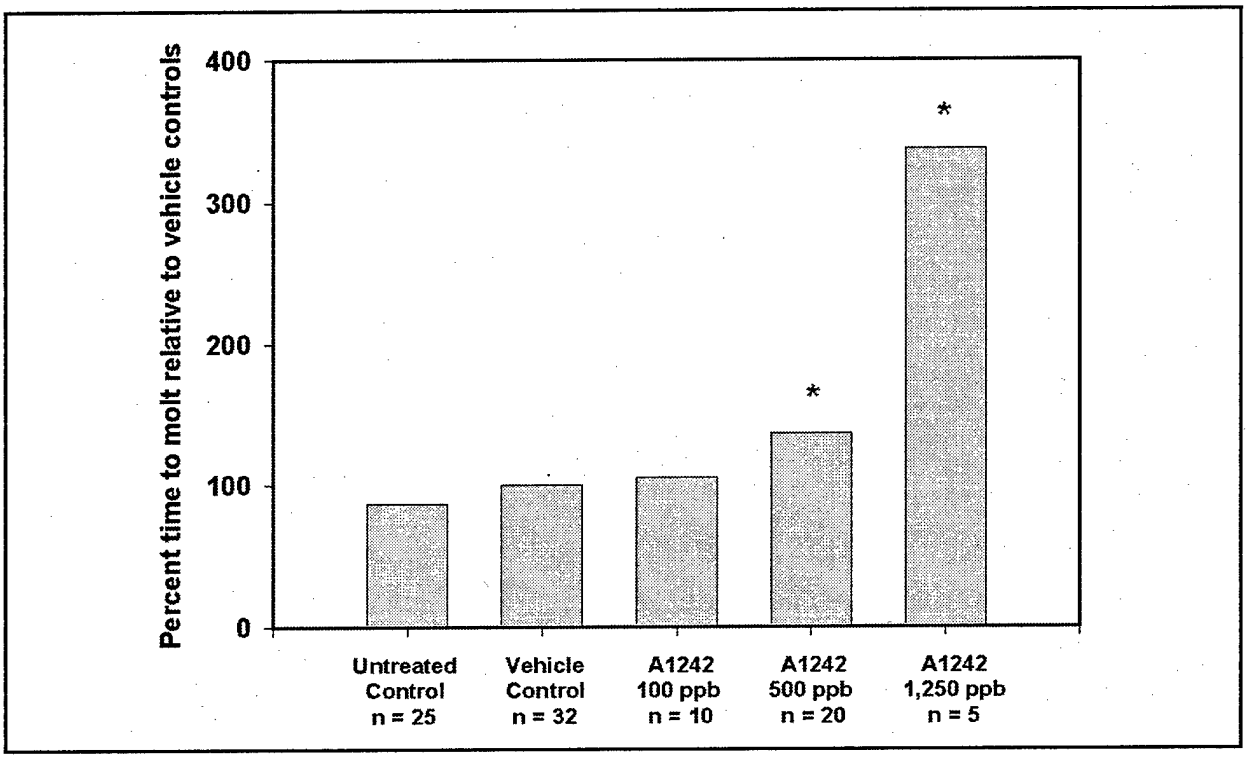

Figure 4. Aroclor 1242-induced molt inhibition in juvenile crawfish as median percentage of exposure duration relative to vehicle (DMSO) controls. Asterisks indicate treatment results are significantly different from vehicle control by one-way Analyses of Variance (ANOVA) on ranks followed by Dunn's test, $P < 0.05$. Data of Jones, Ang, and Inouye (2002)

CONCLUSIONS AND FUTURE DIRECTIONS: These initial results indicate that the HepG2-EcR assay has the potential to become a useful rapid screening test to detect contaminants that may adversely affect arthropods. Highly sensitive cell-based assays are presently available to test environmental samples for contaminants whose toxic effects are mediated through the Ah receptor. Ah receptor mediation has high relevance to vertebrate organisms, including humans, but little relevance to invertebrate organisms that lack the Ah receptor. At the present time there are no testing methods that are sensitive to xenobiotic toxicants that specifically threaten these invertebrate organisms. Ecological risk assessment methods are being developed for which the information provided by such tests could prove highly useful. Ongoing work with the U.S. Geological Survey is being conducted on environmental extracts to determine whether the HepG2-EcR cell-based assay correlates with observed invertebrate community degradation in watersheds due to urban runoff. A positive correlation would warrant further development of the assay.

Cultures are available for research purposes only from:

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> McFarland, V. A., Inouye, L. S., and Ang, C. Y. (2003). "A new transgenic cell line for detection of invertebrate endocrine disrupters: Laboratory protocol for its use," *DOER Technical Notes Collection* (ERDC TN-DOER-C30), U.S. Army Engineer Research and Development Center, Vicksburg, MS. *www.wes.army.mil/el/dots/doer*

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Peddicord, R. K., and McFarland, V. A. (1976). "Effects of suspended dredged material on the commercial crab, *Cancer magister*." *Dredging and its Environmental Effects: Proceedings of the Specialty Conference*, Mobile, Alabama, January 26-28, American Society of Civil Engineers, New York, 633-644.

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Appendix I
LABORATORY PROTOCOLS
FOR HepG2-EcR SCREENING ASSAY

Protocol I. Cell Culture and Reagent Preparation

I. MATERIALS AND EQUIPMENT

Vertical laminar flow hood
37 °C CO_2 incubator
Countertop Centrifuge
Autoclave
Ultra-pure water
Sterile filters (0.1 µm), VacuCap 90 (Gelman Sciences #09730265B)
Sterile, disposable 5, 10 mL, and pasteur pipets (9", autoclaved)
Sterile T-75 (75 cm^2 growth surface area) culture flasks
Sterile syringes and 0.22-µm syringe filters
Squirt bottle of 70 percent reagent alcohol
Parafilm
Water bath @ 37 °C
Alcohol bath
Aspirator
Rechargeable portable pipettors and tips
Inverted microscope
Isopropanol freezing chamber
Liquid nitrogen Dewar @ -195 °C
HepG2-EcR (recombinant human hepatoma) cells

II. REAGENTS

A. PRODUCT INFORMATION

Minimum Essential Medium (MEM), GIBCO BRL #51200-038
Fetal Bovine Serum (FBS), GIBCO BRL #16000-044
L-Glutamine (200 mM, 29.2 mg/ml), GIBCO BRL #25030-081
Hygromycin B (100 mg/ml), InVitrogen #55-0280
Zeocin (100 mg/ml), InVitrogen #R250-01
Phosphate Buffered Saline (PBS), GIBCO BRL #14190-144
Trypsin-EDTA (0.5-percent Trypsin, 0.53-mM EDTA), 10x, GIBCO BRL #15400-054
Trypan Blue (0.4 percent), GIBCO BRL# 15250-061
Dimethyl Sulfoxide (DMSO), SIGMA #D-2650

Cell culture handling and reagent preparation throughout the assay should be carried out only under a sterilized laminar hood using aseptic techniques. Only the last part of the HepG2-EcR assay ("Day 4 (Assay Takedown)" in the "Performance of the Assay" section) need not be sterile.

The following reagents are prepared in bulk ahead of time and stored in aliquots in the refrigerator or freezer. Contents and date of preparation should be properly labeled and initialed on these reagent containers.

B. REAGENT PREPARATION

1) **Minimum Essential Medium (MEM)** - supplemented with L-glutamine and FBS; stored refrigerated.

   a) Remove L-glutamine (100x) and FBS from refrigerator or freezer and place in 37 °C water bath.

   IMPORTANT: As a precaution to avoid cell contamination, the water bath should be cleaned and the water changed at least once a month.

   b) When warmed, dry outside of containers with paper towel and wipe with paper towel moistened with 70 percent alcohol.

   c) Under a sterile flow hood, remove 55 mL of MEM from a new bottle. This unsupplemented medium can be saved for later use (such as in the making of the cell freezing medium). Aseptically, transfer the following components into the 500-mL bottle.

Component	Volume, mL	Percent
MEM	445	89
FBS	50	10
L-Glutamine (100x)	5	1

   d) Refrigerate the fully supplemented medium when not in use.

   IMPORTANT: L-glutamine should be replenished at 1 percent by volume of the remaining medium in the bottle every 3 weeks.

2) **Sterile-filtered, ultra-pure water**, stored refrigerated.

   a) Place a 500-mL beaker of ultra-pure water (not sterile) in the hood.

   b) Attach the bottle-top (0.1-µm VacuCap90) filter to a sterile, autoclaved (500-mL) bottle.

   c) Connect tubings on the filter to the aspirator and to the beaker containing ultra-pure water.

   d) Turn on aspirator.

   e) When the bottle is filled, turn off the aspirator and disconnect the tubings.

   f) This filtered water can be stored in the refrigerator until further use.

3) **Fetal Bovine Serum (FBS)**, stored frozen.

   a) Remove FBS from freezer and place in 37 °C water bath.

b) When thawed, dry outside of the container with paper towel and wipe with paper towel moistened with 70 percent alcohol.

c) Aseptically transfer 50-mL aliquots of FBS into sterile centrifuge tubes.

d) Cap tubes and store in -20 °C freezer until further use.

4) **Trypsin-EDTA (1x)**, stored frozen.

a) Remove 10x trypsin-EDTA stock (100 ml) from -20 °C freezer and place in 37 °C water bath.

b) When thawed, dry outside of container with paper towel and wipe with paper towel moistened with 70-percent alcohol.

c) Aseptically transfer the 10x trypsin-EDTA into a sterile 1-L bottle containing 900 mL of sterile-filtered water and mix contents well.

d) Transfer 12-mL aliquots of 1x trypsin-EDTA into 15-mL sterile centrifuge tubes.

e) Cap tubes and store in -20 °C freezer.

5) **Hygromycin B**, 100-mg/mL or 500x solution, stored refrigerated.

a) Transfer 1.5-mL aliquots in sterile cryovials.

b) Store in the refrigerator.

6) **Zeocin**, 100-mg/mL or 500x solution, stored frozen.

a) This antibiotic comes in 8 sterile aliquots of 1.25 mL.

b) Store in the -20°C freezer.

NOTE: The Hygromycin B and Zeocin concentration needed to maintain selection pressure on these recombinant cells is 0.2 mg/mL. In a T-75 flask containing 15 mL of medium, add 30 µL (or 0.2 percent of medium volume) each of the 100-mg/mL Hygromycin B and 100-mg/mL Zeocin stock. Both Hygromycin B and Zeocin are added to the medium in every cell passage.

7) **Trypan Blue**, 0.05 percent, stored refrigerated, nonsterile.

a) Transfer 6.3 mL of 0.4 percent Trypan Blue solution (stored at room temperature) into 43.7 mL of PBS solution in a beaker and mix well.

b) Pipet 1.800 mL of the 0.05 percent Trypan Blue solution with a micropipettor into each of the 5-mL culture tubes.

CAUTION: Trypan Blue is a suspected mutagen. Wear gloves at all times when handling this reagent.

c) Cover the neck of each tube with a piece of parafilm.

d) Place tubes in a rack and store in refrigerator.

8) **Freezing Cell Medium**, made fresh each time.

a) Aseptically transfer these reagents in a sterile 15-mL centrifuge tube:
9.0-mL supplemented MEM (90 percent)
1.0-mL DMSO (10 percent)

NOTE: For a larger volume of freezing medium, these ingredients can be increased proportionally.

b) Filter sterilize the 10-mL medium through a 0.22-µm syringe filter. Keep the filtered medium cool at 4 °C prior to use.

III. CELL CULTURE

Subcultures of the HepG2-EcR cells are frozen in liquid nitrogen as a secondary source while a working culture is kept for routine testing. Whenever the cells are thawed, they are cataloged as "passage 0." Cells that undergo 25 or more passages or consistently show poor viability are terminated.

A. STARTING THE CULTURE FROM FROZEN PERMANENTS

1) Place supplemented MEM in 37 °C water bath before thawing cells.
2) When warmed, dry outside of container with paper towel and wipe with paper towel moistened with 70-percent alcohol.
3) Transfer about 12 mL of MEM into a 15-mL sterile centrifuge tube.
4) Remove a cryovial of HepG2-EcR cells from the liquid nitrogen storage Dewar.

CAUTION: Always wear a protective full-face mask, cryogenic gloves, and lab coat during the retrieval or storage of cryovials from liquid nitrogen Dewar.

5) Thaw cryovial contents quickly (within a minute) in a 70 percent alcohol solution bath.
6) Aseptically transfer all the thawed cell suspension into the 12 mL of medium. Mix the cell suspension thoroughly to dilute the DMSO in the freezing medium.
7) Centrifuge the cell suspension at 1,000 RPM for 5 min.
8) Siphon off the supernatant medium using a Pasteur pipet attached to an aspirator. This step is taken to remove any traces of DMSO.
9) Resuspend the cell pellet with 20 mL of fresh medium.
10) Transfer the cell suspension into a new T-75 flask.
11) Add 30 µl each of 100-mg/mL Hygromycin B and Zeocin solution into the flask.
12) Place flask in the CO_2 incubator to allow the cells to attach overnight.
13) Siphon off the medium and transfer 15 mL of fresh medium and 30 µL each of Hygromycin B and Zeocin the next day. Incubate cells.
14) Check cell growth daily. A successful thawing of frozen permanents will show confluent growth of cells within a week.

B. SUBCULTURING CELLS (for subculturing one confluent flask of cells)

1) Warm supplemented MEM, trypsin-EDTA, and PBS in 37 °C water bath. Place an aliquot each of Hygromycin B and Zeocin in the alcohol bath.

2) Siphon off old medium from the T-75 flask. Transfer 10 mL of PBS into the flask. Wait for about 2 min before pipetting off the PBS.

3) Add 3 mL of freshly warmed trypsin-EDTA and place the flask in the incubator for 5-10 min.

4) Tap sides of the flask sharply to loosen cells (incubate the flask for another 3-5 min if the cells are not detached from the flask).

CAUTION: These cells <u>should not</u> stay in trypsin for more than 15 min.

5) Add 10 mL of MEM. Triturate cell suspension to break up any cell clumps.

6) Place the cell suspension into a sterile 15-mL centrifuge tube.

7) Pipet 200 µL of cell suspension into the 1.800 mL of Trypan Blue solution. Count cells (see "COUNTING CELLS").

8) Centrifuge the 13 mL of cell suspension at 1,000 rpm's for 5 min.

9) Siphon off supernatant medium, being very careful not to remove any of the cell pellet. Resuspend cell pellet in 10 mL of fresh medium, or any volume appropriate for an assay.

10) If the cells are not used for an assay, transfer the required volume of cell suspension based on the number of cells counted into a new T-75 flask. Seed about 2 to 3 × 10^6 cells per T-75 flask (typically, a flask seeded with 3 × 10^6 cells will be confluent in 4 to 5 days). Add fresh medium to give a total of 15 mL.

11) Pipet 30 µL each of 100-mg/mL Hygromycin B and Zeocin solution into the flask.

12) Incubate flask.

C. COUNTING CELLS

1) Transfer 200 µL of cell suspension that has been triturated into the test tube containing 1.800 mL of freshly warmed 0.05 percent Trypan Blue solution.

2) Mix the suspension thoroughly and apply about 10 µL to each side of the hemacytometer under the cover glass.

3) Count cell numbers in 5 sections on both sides of the hemacytometer. The total number of sections counted should be 10.

4) Add up the number of live and of dead cells for all 10 sections.

5) Multiply the total number of live cells by a factor of 10^4 to obtain the number of live cells/mL in the cell suspension. Likewise, the dead cells/mL is obtained by multiplying by the same 10^4 factor. The viability of the cell suspension is the total number of live cells divided by the total (live and dead) number of cells.

IMPORTANT: A culture with a viability of <95 percent should not be used for an assay.

D. FREEZING CELLS

1) Trypsinize, count, and centrifuge cells to form a pellet as described previously.

2) Siphon off supernatant medium.

3) Resuspend cell pellet in an appropriate volume of freezing medium (see "REAGENT PREPARATION"). The ideal cell concentration is 3 to 5 × 10^6 cells/mL.

4) Transfer 1.0-mL cell suspension into prelabeled sterile cryovials. The label should include the name of cell line, date, cell number, viability, and technician's initials.

5) Place cryogenic vials into the isopropanol freezing chamber and place the freezing chamber into the -80 °C freezer to cool at a rate of 1 °C/min overnight.

6) Transfer cryovials from the freezing chamber into a labeled cryobox. Place cryobox into the liquid nitrogen storage Dewar.

CAUTION: Always wear a protective full-face mask, cryogenic gloves, and lab coat during the retrieval or storage of cryovials from liquid nitrogen Dewar.

7) Record the number of cryovials, name of cell line, date, technician's initials, and rack and holder number into the Dewar record book.

Protocol II. Performance of the Assay

I. Preliminary

Four days are required for performance of the assay. On Day 1, preferably a Monday, cells are plated into two 96-well microtiter plates and incubated for a day. On Day 2, the cells are dosed with Ponasterone A standards and environmental extracts. The cells are again incubated for another 48 hours to allow for enzyme (β-galactosidase) induction. On Day 4, the cells are lysed and the β-galactosidase induction is quantitated using a spectrophotometer.

Two 96-well microtiter plates can be set up to test up to a total of 14 environmental samples with 3 replicates each. One-quarter of a 96-well plate is allocated for a complete Ponasterone A dose-response, while the rest of the wells are assigned to test the environmental samples in combination with Ponasterone A (4 concentrations). The second 96-well plate will not be necessary if testing 6 samples or less.

The following times are required to complete each daily task using the 96-well plates:

Day	Time, hr
1	1.0
2	1.0
4	1.0
Solution preparation/equipment setup	1.0
Total	4.0

II. Day 1 (PLATING CELLS)

A. MATERIALS AND EQUIPMENT

MEM
PBS
Trypsin-EDTA (1x) - 1 aliquot
Trypan Blue (0.05 percent) - one aliquot
Multichannel motorized pipettor (250 µL)
Sterile pipet tips (250 µL)
Pasteur pipets (9", autoclaved)
Hemacytometer and cover slip
Inverted microscope
50-mL sterile centrifuge tube
50-mL sterile solution basins
Water bath set at 37 °C

B. PROCEDURE (use sterile technique)

1) Wipe down the working surfaces of the laminar flow with 70 percent alcohol.

2) Turn on the ultraviolet light and blower of the laminar flow hood. Allow 15 min for equilibration.

3) Turn on 37 °C water bath.

4) Place supplemented MEM and one aliquot each of Trypan Blue and trypsin-EDTA in the water bath for about 30 min.

5) Trypsinize one confluent (T-75) flask of HepG2-EcR cells with 3-mL trypsin-EDTA each as described in "SUBCULTURING CELLS." A confluent flask contains approximately 10 to 13 $\times$ 10^6 cells. Thus, one confluent flask of cells should be sufficient for seeding two 96-well plates at 40,000 cells/200 µL/well.

6) Transfer the cell suspension (approximately 13 mL) from the T-75 flask into a sterile 50-mL centrifuge tube.

7) Pipet 200 µL of cell suspension into the 1.800 mL of Trypan Blue solution. Centrifuge the remaining cell suspension at 1,000 rpm's for 5 min.

8) Count the cells using a hemacytometer to determine the number of cells/mL of cultured stock (described in "COUNTING CELLS").

9) Siphon off supernatant medium, being very careful not to remove any of the cell pellet. Resuspend in 10 ml of fresh medium.

10) Prepare 42 mL of 200,000 cells/mL suspension from the cultured stock. This volume of cell suspension will be sufficient for seeding two 96-well plates.

Example
If 100 viable cells were counted in the 13-mL stock suspension, add 13 mL of fresh medium to the cell pellet to make a 1,000,000-cells/mL suspension.

Then to make 42 mL of a 200,000-cells/mL suspension, transfer 8.4 mL of the 1,000,000-cell/mL suspension into a sterile 50-mL centrifuge tube containing 33.6 mL fresh medium.

11) Transfer the 42 mL of cell suspension into a sterile 50-mL solution basin.

12) Set the multichannel pipettor to pipet 200 µL and seed the two 96-well plates.

IMPORTANT: To ensure that the cells are evenly distributed, mix the cell suspension thoroughly before pipetting each plate.

13) Write the time and date on the lids of the plates.

14) Incubate plates in the CO_2 incubator for 3 days.

III. Day 2 (DOSING THE CELLS)

The cells are dosed with Ponasterone A standards (prepared in DMSO) and environmental samples (prepared in iso-octane) on this day. The following setup is for testing 14 environmental samples with 3 replicates each.

A. MATERIALS AND EQUIPMENT

Ponasterone A (10 mM) stock in DMSO, stored in -20 °C freezer
Environmental samples in iso-octane, stored in -20 °C freezer
Iso-octane (pesticide grade)
MEM
Positive displacement micropipettor (10 µL and 100 µL)
Multichannel motorized pipettor (250 µL)
Sterile pipet tips (250 µL)
50-mL sterile solution basins
1.5-mL microcentrifuge tube (1) - autoclaved
500-µL microcentrifuge tubes (8) – autoclaved

B. PROCEDURE

1) Wipe down the working surfaces of the laminar flow with 70 percent alcohol.

2) Turn on the ultraviolet light and blower of the laminar flow hood. Allow 15 min for equilibration.

3) Turn on 37 °C water bath.

4) Place supplemented MEM in the water bath for about 30 min.

5) Remove Ponasterone A standard and environmental samples (stored in 4-mL amber vials) from the freezer. Vortex the standard and samples, and let vials warm to room temperature.

6) Prepare serial dilutions of Ponasterone A standards in DMSO using positive displacement pipettors as follows:

Tube Number	Tube Concentration μM*	Well Concentration μM	Make-Up
1	10,000	100	Ponasterone A Stock
2	3,000	30	120 µL tube 1 + 280 µL of DMSO
3	1,000	10	40 µL tube 1 + 360 µL of DMSO
4	300	3	120 µL tube 3 + 280 µL of DMSO
5	100	1	40 µL tube 3 + 360 µL of DMSO
6	30	0.3	120 µL tube 5 + 280 µL of DMSO
7	10	0.1	40 µL tube 5 + 360 µL of DMSO
* The Ponasterone A standards are made 100x.			

7) Prepare serial dilutions of Ponasterone A standards in MEM as follows:

Tube Number	Well Concentration μM	Make-Up
1	100	50 µL Ponasterone A tube 1 + 5,000 µL of MEM
2	30	10 µL Ponasterone A tube 2 + 1,000 µL of MEM
3	10	50 µL Ponasterone A tube 3 + 5,000 µL of MEM
4	3	10 µL Ponasterone A tube 4 + 1,000 µL of MEM
5	1	50 µL Ponasterone A tube 5 + 5,000 µL of MEM
6	0.3	10 µL Ponasterone A tube 6 + 1,000 µL of MEM
7	0.1	50 µL Ponasterone A tube 7 + 5,000 µL of MEM
8	0	10 µL DMSO + 1,000 µL of MEM

8) Pipet off the cell medium from the two 96-well plates with a multichannel pipettor. The plates can be tilted at a 30-deg angle so that the sharp ends of the pipet tips can reach the medium at the bottom of the wells.

9) Pipet 200 µL of the Ponasterone A medium (prepared in step 7) into each well.

10) Dose each well in the first 3 columns with 1 µL of iso-octane, and 1 µL of the environmental samples (in iso-octane) in triplicate wells over 4 Ponasterone A concentrations using a positive displacement pipettor in Plate 1 as follows:

PLATE 1

Ponasterone A + Iso-octane			Ponasterone A (4 concentrations) + Environmental samples (6 samples, 3 replicate extracts per sample)								
➨ 0			➨ 0.1 μM			➨ 0.1 μM			➨ 0.1 μM		
➨ 0.1 μM			➨ 1			➨ 1			➨ 1		
➨ 0.3			➨ 10			➨ 10			➨ 10		
➨ 1			➨ 100			➨ 1-0			➨ 100		
➨ 3			➨ 0.1 μM			➨ 0.1 μM			➨ 0.1 μM		
➨ 10			➨ 1			➨ 1			➨ 1		
➨ 30			➨ 10			➨ 10			➨ 10		
➨ 100			➨ 100			➨ 100			➨ 100		

11) Dose the remaining 8 environmental samples in Plate 2 similar to Plate 1, replacing the Ponasterone dose-response series (first three columns) with environmental samples.

12) Write down the time and date of dosing on the lids of the plates.

IV. Day 4 (ASSAY TAKEDOWN)

The exposure to the Ponasterone A standard and environmental samples is terminated at 48 hr. The cell medium is removed, and the cells washed and lysed. The lysates are then transferred into the 96-well plate and read. The spectrophotometer is programmed to monitor the formation of the end product at 405-420 nm every minute for 15 min at 37 °C.

A. MATERIALS AND EQUIPMENT

Multichannel motorized pipettor (250 μL)
PBS, 1x, nonsterile
o-Nitrophenyl-Galactopyranoside (ONPG) substrate (see "REAGENT PREPARATION" section)
Buffer A (see "REAGENT PREPARATION" section)
β-mercaptoethanol
Cell lysis buffer (1x), PHARMINGEN #556871 - 2 aliquots
50-mL solution basins, nonsterile
Vortex (with microplate adaptor holder)
Inverted microscope
Countertop centrifuge (with microplate adaptor holder) spectrophotometer (with temperature control and kinetics package)
96-well plate, clear flat-bottomed, nonsterile

B. REAGENT PREPARATION

1) **Cell lysis buffer (1x)**

a) Pipet 100 mL of ultra-pure water into 50 mL of the 3x cell lysis buffer in a beaker.

b) Transfer 12.5 mL of the 1x cell lysis buffer in 15-mL tubes.

c) Store tubes in -80 °C freezer.

2) **ONPG substrate**

a) Make a stock solution of 100-mM NaH_2PO_4, pH 7.5.

i) Weight 6 g of anhydrous NaH_2PO_4 into a 500-mL volumetric flask.

ii) Add ultra-pure water to dissolve the crystals, leaving room to adjust the pH with NaOH (5 M).

iii) Adjust the pH to 7.5 using a 5-M solution of NaOH.

iv) Bring the final volume up to 500 mL.

v) Store in a refrigerator until needed.

b) Weigh out 0.4 g of ONPG into a 100-mL volumetric flask, then bring up to 100 mL with the 100-mM NaH_2PO_4 solution prepared in step 2a.

c) Aliquot into 5.5-mL portions and store in a -20 °C freezer until needed. This aliquot is sufficient for one 96-well plate.

d) On the day the assay is to be conducted, remove one aliquot per assay plate and place in a 37 °C water bath.

3) **Buffer A**

a) Make a stock solution of 100-mM NaH_2PO_4, pH 7.5, with 10-mM KCl and 1-mM $MgSO_4$.

i) Weigh out 0.373 g of anhydrous KCl and 0.123 g of $MgSO_4$* 7 H_2O into a 500-ml volumetric flask.

ii) Bring the volume to 500 mL with the 100-mM NaH_2PO_4 solution prepared in step 2a.

b) Store in the refrigerator until needed.

c) On the day of the assay, add 70 µL of β-mercaptoethanol to a 20-mL aliquot of buffer A, and place the mixture in a 37 °C water bath. This aliquot is sufficient for one 96-well plate.

C. SETTING UP THE SPECTROPHOTOMETER

The spectrophotometer should be a 96-well format, temperature-controlled system with the ability to run kinetic analysis in the 405- to 420-nm range (yellow). The following directions are for a Dynex MRX temperature-controlled plate reader with a 405-nm filter and Revelation software for kinetics analysis.

1) Connect the Dynex MRX plate reader to the computer and turn the reader and the computer on.

2) Open the Revelation program, and open the assay file. The assay file should use the following parameters:
   Filter = 405 nm
   Temperature = 37 °C
   Read at 1-min intervals for 15 min

3) Start a trial blank run to be sure cable connections and assay setup are correct.

D. PROCEDURE (nonsterile techniques) - at the end of the 48th-hour exposure time point.

1) Place an appropriate amount of ONPG substrate and Buffer A (with β-mercaptoethanol) in a 37 °C water bath, along with an aliquot of cell lysis buffer (1x).

2) Take out the plate from the incubator to the hood.

3) Pipet off the cell medium in plate using a multichannel pipettor, being very careful not to remove any cells. The plates can be tilted at a 30-deg angle so that the sharp ends of the pipet tips can reach the medium at the bottom of the wells.

4) Pipet 200 µL of PBS (1x) into each well to wash the cells. Pipet off the PBS.

IMPORTANT: Check each well to make sure that the PBS medium is completely removed.

5) Add 100 µL of cell lysis buffer to each well.

6) Place the plate onto the microplate adaptor holder of a vortex.

7) Shake plates for 10 min at setting number 2. In the meantime, the spectrophotometer can be set up.

8) Check the wells under the microscope to see if the cells have been lysed. The cell debris will clump up and float in the cell lysate.

9) Centrifuge plates at 5,000 rpm's for 5 min. This is done to spin down the cell debris (which can interfere with the spectrophotometer reading) to the bottom of the wells.

10) With a multichannel pipettor, transfer 50 µL of the lysate into the corresponding wells of a clean 96-well plate.

11) Rinse the pipet tips with PBS twice before proceeding with the next column of wells.

Be sure that no bubbles are generated during the sample transfer; bubbles will interfere with the spectrophotometric measurements.

12) When all lysates have been transferred, add 110 µL of the prewarmed Buffer A with β-mercaptoethanol to each well.

13) Cover the plate and place in the incubator for 5 min at 37 °C.

14) Add 50 µL of ONPG to each well and place plate in the temperature-controlled plate reader.

15) Monitor the reaction in the 405- to 420-nm range, taking readings every minute for at least 15 min.

Check the kinetics of each well to be sure that the reaction remained linear over the time course; if the reaction proceeds at too fast a rate, the rate will plateau and the overall average mOD/min will be lower than the maximal rate during the linear phase.

16) Print out the data report (should give a rate of reaction for each well, in mOD per minute) and save the data files.

17) Remove the plate, exit the program, then shut down the computer and plate reader.

V. DATA PROCESSING

The data generated from the plate reader (mOD/min) is used to generate a log-dose response curve for both the Ponasterone A alone and the Ponasterone A with the sample. Using a graphics package such as SigmaPlot, a sigmoidal regression can be generated and an EC_{50} and maximal rate determined for each curve. Significant shifts in either parameter indicate that the environmental sample is interacting with the receptor-ligand complex (EcR receptor-Ponasterone A complex).