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Protocols for a Rapid Clean-up/Extraction Procedure and an Improved P450RGS Dioxin Screening Assay for Sediments

PURPOSE: The purpose of this technical note is to describe protocols for performance of an improved biomarker-based screening assay for dioxin toxic equivalents (TCDD TEQs) in sediments and other environmental samples using a recombinant human hepatoma cell line.

BACKGROUND: For background refer to McFarland, McCant, and Inouye (1998) in which the use of cultured mammalian cells as the basis of assays for dioxins in environmental samples is described. There are several reasons why using cultured animal cells in assays for these contaminants is attractive. First, cell-based assays cost far less to perform and are done far more quickly than, for example, gas chromatograph/mass spectrometer (GC/MS) analysis of dioxins; the difference in cost is at least an order of magnitude, and may be more depending on the number of samples done in a batch, the number of replicates of each sample, and other factors. Additionally, the sensitivity of the cell-based assays can be made to be of the same order as that of the GC/MS. For these reasons, the cells can provide screening tests of sediment samples that permit the prioritization of sites to receive definitive chemical analysis when analyses are required for regulatory or other purposes. When cleanup and remediation involving soils or sediments contaminated with polyaromatic hydrocarbons (PAHs), polychlorinated biphenyls (PCBs), or polychlorinated dibenzodioxins/furans (PCDDs/ PCDFs) are issues, the cells offer a less expensive alternative to chemistry for monitoring the effectiveness of treatments. Since the costs are relatively much lower, many more samples can be assayed for the same resource expenditure, allowing better characterization of the extent of contamination at sites.

A second type of advantage involves the requirements of risk assessment. The mechanism by which cell-based assays detect and measure the activity of dioxin and dioxinlike chemicals employs the same cellular machinery responsible for the toxicity of these chemicals in whole organisms. While the cell line responds to all dioxins/furans containing chlorines at the 2-, 3-, 7-, and 8-ring positions, coplanar PCBs, and many of the PAHs, the most potent compound is 2,3,7,8- tetrachlorodibenzo-*p*-dioxin (TCDD). Other dioxins and furans give lesser responses (from about 0.5 to only about 0.001 the response of TCDD), and the response to PCBs is even lower (0.008 to 0.000001 the response of TCDD). The PAHs have the least potency as individuals, but because they are often far more abundant than dioxins or PCBs, their contribution to the TCDD TEQ measured by the cells can be significant. If both planar chlorinated hydrocarbons (dioxins, etc.) and PAHs are present, the PAHs are easily removed during sample preparation. Unlike chemical analysis, cell-based assays possess the ability to integrate the toxicological potency of complex mixtures of chemicals that have similar modes of action but differ in potency as individuals.

Since the Reuber H4IIE rat hepatoma cell line was introduced in an assay for screening planar chlorinated hydrocarbons in environmental samples (Bradlaw and Casterline 1979), a variety of cell lines have been used in biomarker tests of exposure to chemicals and in identifying toxic substances in environmental samples according to the primary physiological effect they produce. The most

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common application has been testing samples such as birds' eggs or the livers of fish for the induction of detoxifying enzymes in response to environmental PCB, PAH, or PCDD/PCDF exposure. Recently, cell-based assays have been used to test contaminated sediments. Hoke et al. (1994) reported using H4IIE cells in a battery of biomarker tests on organic extracts of sediments. Murk et al. (1996) also tested sediment extracts and found that a recombinant cell line (CALUX) based on H4IIE and transfected with the firefly luciferase gene as a reporter gave improved results over the wild-type H4IIE. The P450 Reporter Gene System (P450RGS) based on human HepG2 cells similarly uses the luciferase gene as a reporter. P450RGS has been used in several studies to identify and measure PAHs and/or planar chlorinated hydrocarbons in sediment extracts (Anderson et al. 1995, 1996; Anderson, Zeng, and Jones 1999; Kim et al. 1997; McFarland et al. 1999). In McFarland et al. (1999), P450RGS was shown to have advantages for this purpose compared with the wild-type rat hepatoma H4IIE cell line. The nonlabor costs incurred in testing the same number of sediment samples were similar for the two assays, but P450RGS was considerably less time consuming. Since the publication of McFarland et al. (1999), the P450RGS assay has been adapted to a 96-well microtiter plate format, providing an even more rapid screening assay at lower cost.

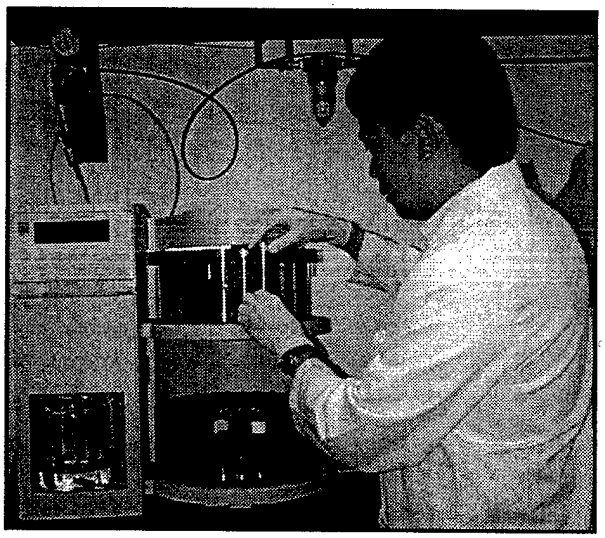

Figure 1. Dionex accelerated solvent extraction system

Additional modifications to sample preparation have also reduced the time and cost of sediment extraction. Previously the sediment samples were extracted according to U.S. Environmental Protection Agency (USEPA) Method 3540 (USEPA 1986) using Soxhlet apparatus. Sediment extraction was later accomplished with the more rapid Dionex Accelerated Solvent Extraction (ASE™) apparatus (Figure 1) using methods that complied with USEPA Method 3545 (USEPA 1986) guidelines for soils and sediments. The extracts were cleaned on sulfuric acid/silica gel (SA/SG) in a separate step, then solvent exchanged to iso-octane for use in the cell-based assays. The latest ASE method now combines the cleanup step with the extraction step.

MATERIALS AND METHODS: The ASE one-step extraction/cleanup procedure was performed as described in McCant, Inouye, and McFarland (1999). The detailed laboratory protocols are included as Appendix I. Briefly, a 33-mL extraction cell was packed with 2 g of neutral silica gel followed by 8 g of SA/SG. The column was conditioned with hexane, after which a 2.5-g aliquot of freeze-dried sediment thoroughly mixed with diatomaceous earth (DE) in a 4:1 weight-to-weight ratio (sediment:DE) was added. The cell was then extracted on the Dionex ASE using the following parameters: 1,500 psi, 100 °C, with a static and heat time of 5 min, a flushing volume of 60 mL, 60-sec purging, and a 60 percent flushing volume for two cycles, using 10 percent dichloromethane (DCM) in hexane. Clean extracts were concentrated to approximately 1 mL in a Zymark® TurboVap II and solvent exchanged to iso-octane (0.1 to 1.0 mL final volume). Extracted samples were stored at -20 °C until assayed.

Assays were performed using the 96-well plate format of the P450RGS screening assay[1] with 2,3,7,8-TCDD as a standard (Figure 2) (Appendix II); results are expressed as TCDD TEQs. Results for standard curves and sediment extracts for the 96-well plate format and the standard 6-well plate format (McFarland et al. 1999) were compared. The limit of detection (LOD) was determined with the same methods used in standard chemical analysis, i.e., the LOD is calculated as three times the average replicate standard deviation above background level. The LOD will vary from day to day depending on numerous random variables; the LODs reported in Table 1 are averages from three separate assays.

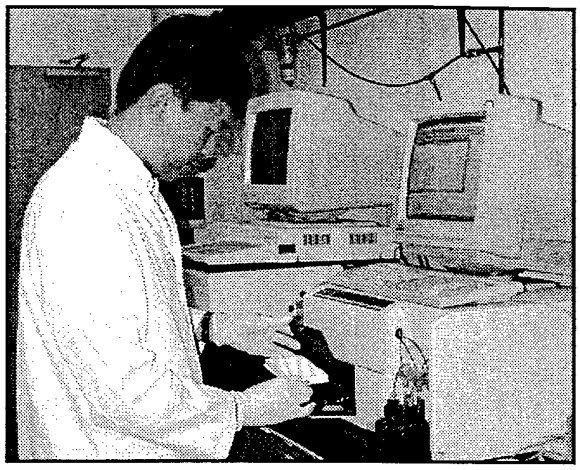

Figure 2. The P450RGS assay: 96-well plate luminescence reader

Table 1
Time, Cost, and Performance Comparison of Cell-Based Dioxin Assays

Factor	P450RGS 96-well format	P450RGS 6-well format	H4IIE 96-well format
Expense Item (Not Including Sample Preparation)[1]			
Cost of supplies, $	18	52	65
Labor time, hr	3.5	4.5	11
Sensitivity			
Lowest detectable response,[2] pg TCDD/well	0.2	2.0	0.08
LOD,[3] pg TCDD TEQ/g sediment	7 ± 2	22 ± 8	5 ± 2

[1] Time and cost of materials based on testing two samples of six replicates each.
[2] Lowest standard used.
[3] LOD based on 3σ + background response (see methods) ± standard deviation.

RESULTS AND DISCUSSION: The P450RGS assay has been shown to have good reproducibility between laboratories and within a laboratory (McFarland et al. 1999). In the first version of the assay used at the U.S. Army Engineer Research and Development Center, Environmental Laboratory (EL), Vicksburg, MS, the cell exposures were conducted in 6-well plates, after which the cells were lysed and transferred to microcentrifuge tubes. After centrifugation the lysate supernatants were transferred to a 96-well plate to be read. The protocol for the P450RGS assay given in Appendix II incorporates advances developed at EL in which all operations are carried out on the same 96-well plate (Figure 3). These changes reduce the time required for the assay by about

1 101L cells are available from Dr. Jack Anderson (*jaanderson@kelso.caslab.com*), Columbia Analytical Services, Inc., 1185 Park Center Drive, Suite A, Vista, CA 92083, under Waterways Experiment Station Cooperative Research and Development Agreement No. WES-97-GL-0X.

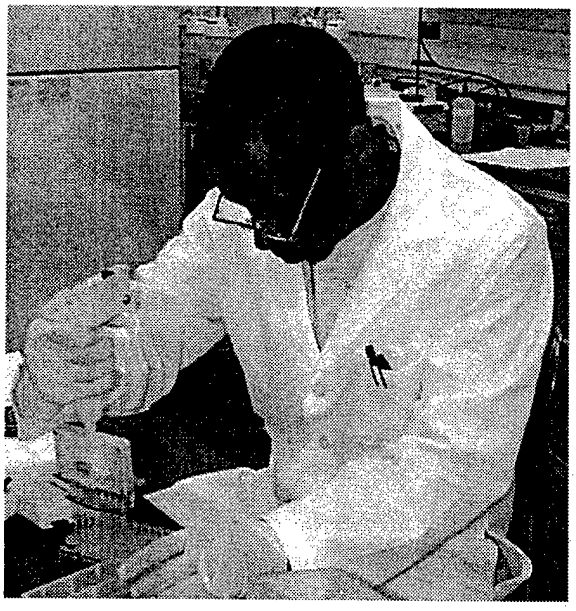

Figure 3. Adding reagents to a 96-well plate using a multichannel pipet

25 percent and the expendable supplies cost by about 65 percent compared with the 6-well plate method, and produce a slight increase in sensitivity (Table 1).

Significant additional savings in time and cost were gained by modifications to the extraction method. Combining the SA/SG cleanup step with extraction reduced sample preparation time from 17 hr/sample to 2.5 hr/sample with no loss in extraction efficiency. The extraction efficiencies were determined for both ASE and ASE one-step methods using ^{3}H-TCDD and were found to be similar: 87.2 ± 1.8 percent recovery for the standard ASE, and 80 ± 6.2 percent for the ASE one-step method. An additional advantage of the ASE one-step method is that the amount of waste solvent requiring disposal is reduced from 270 mL/sample for ASE with separate cleanup to 130 mL/sample for ASE one-step. The efficacy of SA/SG removal of PAHs for the two methods was tested by spiking a clean sediment with a PAH standard prior to analysis with the H4IIE cell line. No response due to PAH addition was detected after cleanup with either method (Table 2).

To test whether samples containing high levels of PAHs could overload the SA/SG column and allow PAH breakthrough, a harbor sediment highly contaminated with PAH was extracted with the ASE standard method, resulting in an extremely high TEQ (15,125 ± 2,850 pg TCDD TEQ/g dry sediment, n = 5), which dropped to below detection limits following SA/SG column cleanup. Spiking the extract with PAHs prior to column cleanup did not affect the results, indicating that the SA/SG column was not overloaded. Extracts of the same sample prepared by the ASE one-step method did not produce any detectable TCDD TEQ as measured by the H4IIE cell-based assay, indicating that this method is as effective in removing PAHs as the more laborious column cleanup.

Table 2
H4IIE Maximal Responses of PAH-Spiked Sediments Extracted by ASE™

Method	Response, ng resorufin/μg protein
ASE, no cleanup	540
ASE, with SA/SG cleanup	Below LOD
ASE one-step	Below LOD

CONCLUSIONS: The ASE one-step extraction/cleanup procedure in combination with the 96-well plate adaptation of the P450RGS assay provides a very cost-effective and rapid alternative to conventional analytical chemistry for screening sediments for dioxin and dioxinlike activity. By scheduling the separate operations properly, it is possible for one person to extract and test three replicates of 24 sediment samples in two workweeks. PAHs in sediments may also be assayed using these methods. These methods are currently being applied in a field demonstration in which a Federal channel with a historic point source of creosote contamination is being dredged, and samples

of the sediments at a series of reaches away from the point source will be analyzed by both chemical analysis and cell-based assays. The results of this study will be reported in a future DOER Technical Note.

POINT OF CONTACT: For additional information, contact Dr. Laura S. Inouye (601-634-2910, *inouyel@wes.army.mil*), Dr. Victor A. McFarland (601-634-3721, *mcfarlv@wes.army.mil*), or the Manager of the Dredging Operations Environmental Research Program, Dr. Robert M. Engler (601-634-3624, *englerr@wes.army.mil*).

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APPENDIX I
LABORATORY PROTOCOLS FOR SAMPLE PREPARATION AND ACCELERATED SOLVENT EXTRACTION ONE-STEP CLEANUP

Protocol I. Sediment Drying Methods.

I. MATERIALS AND EQUIPMENT.

Aluminum weigh pans.
Drying oven.
Aluminum foil.
60-mL precleaned jars.
Hexane.
Mortar and pestle.
Freeze-dry system.

II. SAMPLE PREPARATION.

A. SAMPLE INFORMATION. Upon arrival, sediments are logged describing their physical appearance, quantity/container size, and label information, and stored at 4 °C until ready for use. Samples are mixed thoroughly to obtain homogeneity before removing subsamples for the following methods.

B. PERCENT SOLIDS DETERMINATION.

1) Record the weight of a hexane-rinsed aluminum pan, then tare the pan.

2) Weigh 2-4 g of wet sediment sample onto the tared pan, recording the exact weight of the sediment, then place in 100 °C drying oven overnight.

3) Reweigh the pan and dry sediment immediately upon removal from the oven to limit the amount of moisture taken up by sediments from the atmosphere.

4) Obtain true dry sediment weights by subtracting the initial pan weight. The dry sediment weight divided by wet sediment weight multiplied by 100 equals the percent solids. This value is used to calculate the amount of wet sediment required to obtain the necessary amount of dry sediment for extraction based on the percent solids value. For example, if 8 g dry soil are needed and the percent solids was calculated at 60 percent, (8 g/0.60) = 13.3 g wet weight would be needed.

C. FREEZE-DRYING.

1) Appropriate portions of wet sediments (approximately 20-30 g) are placed in labeled 60-mL precleaned jars. The sample should be spread evenly inside the jar or at an angle to increase surface area before freezing the sediment in -80 °C freezer for a minimum of 2 hours.

2) Freeze-dry following instructions provided by the manufacturer. Sediments are left on the freeze-dryer for at least 24 hours. The vacuum should reach less than 500×10^{-3} mb for best results.

Protocol II. Accelerated Solvent Extraction One-Step Cleanup.

I. MATERIALS AND EQUIPMENT.

Dionex Accelerated Solvent Extraction (ASE) 200.
Zymark TurboVap II (Automatic sample evaporator).
MiniVap Evaporator/Concentrator (Supelco # 22971).
33-mL cells for ASE 200.
60-mL collecting vial with Teflon septa.
130 °C oven.
Bottle roller.
Balance.
6-in. by 1/2-in.-dowel.
Dionex cellulose filters (1.91 cm).
Funnel.
Freeze-dried environmental sample (2.5 g preweighed).
4-mL amber vial with Teflon-lined polypropylene cap.
Conical inserts and springs for vial (Supelco #60840-639 and #60842A-2).
Safety glasses.
Gloves.
Solvent-resistant gloves.
Dust mask.

II. REAGENTS.

20-30 Mesh Ottawa Sand Standard.
10 percent dichloromethane (DCM) in hexane.
Hexane (pesticide grade).
Silica gel 60-200 mesh.
Diatomaceous earth.
Concentrated sulfuric acid.
Sulfuric Acid/Silica Gel (SA/SG) (See following preparation instructions).

III. SA/SG PREPARATION.

Caution! Always wear dust mask, safety glasses, and gloves when preparing SA/SG.

A. Activate silica gel by oven drying for at least 24 hr at 130 °C.

B. Tare a 1-L glass bottle. Fill the bottle approximately two-thirds full with oven-activated silica gel.

C. Calculate two-thirds of the weight of silica gel; place this amount of concentrated sulfuric acid in the bottle.

D. Cap with a Teflon-lined screw cap and shake vigorously until there are no lumps and the contents exhibit a dry, powdery appearance.

E. Tumble on a bottle roller for a minimum of 2 hr.

F. Label the bottle with date prepared and store at room temperature. The maximum shelf life of SA/SG is 6 months, after which the unused portion should be discarded.

IV. ACCELERATED SOLVENT EXTRACTION ONE-STEP CLEANUP.

Accelerated Solvent Extraction utilizes heat and high pressure to extract compounds such as polychlorinated biphenyls, polycyclic aromatic hydrocarbons (PAHs), organochlorine pesticides, organophosphorus pesticides, polychlorinated dibenzo-*p*-dioxins, polychlorinated dibenzofurans, and other common contaminants from environmental samples. However, to obtain a response in cell-based assays based solely on the dioxin and dioxinlike compounds, PAHs and polar compounds present in the crude extract must be removed. This is typically accomplished by passing the crude extracts through a separate SA/SG column after extraction by ASE, Soxhlet, or sonication techniques. In the one-step ASE method, the ASE extracting vessels/cells are packed with neutral silica gel (SG) and SA/SG before the contaminated sediment is placed in the cells. By extracting the sediment and cleaning the extract in one step, this method significantly reduces time and solvent-related expenses without sacrificing extraction or cleanup efficiency.

A. PACKING AND CONDITIONING THE CELL COLUMN.

Caution! Always wear dust mask, safety glasses, and gloves while working in ventilated hood.

1) Place one end cap onto the ASE extraction cell opposite the end with the serial number, tightening by hand (do not overtighten). It is important to place the cap on the proper side in order to keep track of how the cell was loaded; if the cell is placed on the ASE in the wrong direction, the extract will not pass over the SA/SG and will not be cleaned.

2) Place one cellulose filter in the open end of the cell, and using the dowel, push the filter snugly against the cell cap.

3) Add 2 g Ottawa Sand to cover the filter.

4) Add 2 g neutral SG followed by 8 grams SA/SG and tap with a small rubber mallet to pack the column tightly.

5) Firmly clamp the cells in a lab stand situated in a ventilated hood. Place a beaker under each cell to collect waste solvent. Carefully remove the end cap (the cellulose filter will keep the packed column in place) and add 80 mL hexane per

cell in ~15-mL increments, being careful not to let column dry out. Recap bottom end.

B. ADDING SEDIMENT TO CELLS/COLUMNS.

1) Mix preweighed freeze-dried sediment with diatomaceous earth (1:4 by weight). The diatomaceous earth adsorbs any water associated with the sample that would cause inefficient extraction.

2) Transfer the mixture to the conditioned packed cell using a small funnel with short 1/2-in. stem. Record cell serial numbers with weights of sediment. It is important to track the cells via their serial numbers. Labeling with tape will not work, since the cells will be exposed to high temperature. Markers are not desirable because even permanent ink can be lost upon exposure to solvents.

3) Fill the remaining space in the cell with Ottawa Sand, leaving just enough room for a cellulose filter.

4) Install the cellulose filter and seat snugly with the dowel.

5) Screw on the end cap by hand (do not overtighten).

6) Repeat these procedures for all samples.

7) Turn on the ASE 200, press the menu button, set up the following parameters, and save under a method number:

Parameter	Setting	Parameter	Setting
Temperature	100 °C	Flush volume	60 mL
Pressure	1,500 psi	Cycles	2
Static Time	5 min	Purge	60 sec
Heat	5 min	Flush percent	60 vol.

8) Select the method/schedule button and the method number under which the program was saved. Type in the appropriate starting vial/cell number.

9) Place the cells in the tray, with the serial numbers on top (if samples are put in the tray in the wrong direction, the extract will not pass over the SA/SG and will not be cleaned).

10) Label the 60-mL collecting vials with the corresponding serial number on the cells and place the vials in the correct position in the carousel.

11) Press Start. Extraction should take about 30 minutes per sample.

12) After all samples have been extracted, pour the contents of the vials into numbered, solvent-rinsed, graduated Zymark tubes, rinsing each vial once with approximately 2-3 mL of DCM.

C. SOLVENT EXCHANGE.

1) Concentrate the extract in the graduated Zymark tube to 1.5 mL.

2) Add 1.5 to 2.0 mL iso-octane and concentrate the extract again to 0.2 mL.

3) Transfer the sample to a 0.3-mL conical insert marked at the 100-µL level.
4) Rinse the Zymark tube with 80 µL iso-octane and transfer rinse to the conical insert.
5) Using the MiniVap (nitrogen evaporator), gently blow down the extract to the 100-µL mark.
6) Place the conical insert into a 4-mL amber glass vial with Teflon-lined cap for storage in a -4 °C freezer. The conical insert minimizes evaporative loss by reducing both the head space over the sample in the storage vial and the surface area exposed when the vial is opened.

APPENDIX II

LABORATORY PROTOCOLS FOR P450RGS DIOXIN 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.
Ultrapure water.
Sterile filters (0.1 µm), VacuCap 90 (Gelman Sciences #09730265B).
Sterile, disposable 5- and 10-mL and pasteur pipets (9 in., autoclaved).
Sterile T-75 (75-cm^2 growth surface area) culture flasks.
Culture tubes (10 × 75, FisherBrand #14-961-25).
Sterile syringes and 0.22-µm syringe filters.
Squirt bottle of 70 percent reagent alcohol.
Parafilm.
Waterbath at 37 °C.
Alcohol bath.
Aspirator.
Rechargeable portable multichannel pipettors (8-channel) and tips.
Inverted microscope.
Isopropanol freezing chamber .
Liquid nitrogen Dewar, -195 °C.
101L human hepatoma cells

II. REAGENTS.

A. PRODUCT INFORMATION.

Minimum Essential Medium (MEM), GIBCO BRL #51200-038.
Versene (1x, 0.2 g EDTA.4Na), GIBCO BRL #15040-066.
Fetal Bovine Serum (FBS), GIBCO BRL #16000-044.
L-Glutamine (200 mM, 29.2 mg/mL), GIBCO BRL #25030-081.
Geneticin (G418), GIBCO BRL #11811-031.
Phosphate Buffered Saline (PBS), GIBCO BRL #14190-144.
Sodium Pyruvate (11.0 mg/mL), SIGMA #S-8636.
Trypsin (with phenol red, 2.5 mg/mL), SIGMA #T-4424.

Trypan Blue (0.4 percent), SIGMA #T-8154.
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 P450RGS assay (DAY 4 (ASSAY TAKEDOWN), Protocol II, "Performance of the Assay") need not be sterile.

The reagents described in the next section 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 (100x), sodium pyruvate (100x), and FBS; stored refrigerated.

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

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

   c) When warmed, wipe the outside of the containers with a dry paper towel followed with a paper towel moistened with 70 percent alcohol.

   d) Under a sterile flow hood, remove 65 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 MEM bottle:

Component	Volume, mL	Percent
MEM	435	87
FBS	50	10
L-Glutamine (100x)	10	2
Sodium pyruvate (100x)	5	1

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

   f) ***IMPORTANT: Because L-Glutamine breaks down in the medium, it should be replenished at 2 percent by volume of the remaining medium in the bottle every 3 weeks.***

2) **Sterile-filtered, ultrapure water,** stored refrigerated.

   a) Place a 500-mL beaker of ultrapure 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 ultrapure water.

d) Turn on the 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, wipe the outside of the container with a dry paper towel followed with a paper towel moistened with 70 percent alcohol.

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

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

4) **Trypsin, 1x,** stored frozen.

a) Remove 1x trypsin stock (100 mL) from -20 °C freezer and place in 37 °C water bath.

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

c) Aseptically transfer 6-mL aliquots of 1x trypsin into 15-mL sterile centrifuge tubes.

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

5) **Geneticin (G418)**, 40 mg/mL or 100x solution, stored frozen.

a) Dissolve 1 g of Geneticin (G418) disulfate salt in 25 mL of sterile-filtered, ultrapure water.

b) Transfer 1.5-mL aliquots in sterile cryovials.

c) Store in the -20 °C freezer.

d) ***NOTE: The Geneticin concentration needed to maintain selection pressure on these recombinant cells is 0.4 mg/mL.*** In a T-75 flask containing 15 mL of medium, add 150 µl (or 10 percent of medium volume) of the 40 mg/mL Geneticin stock. Geneticin is added to the medium in every cell passage.

6) **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 culture tube. The solution prepared provides 27 aliquots.

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

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

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

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

a) Aseptically transfer these reagents in a sterile 15 mL centrifuge tube:

i. 3.30 mL unsupplemented MEM (66 percent).

ii. 1.25 mL FBS (25 percent).

iii. 0.40 mL DMSO (8 percent).

iv. 0.05 mL Penicillin/Streptomycin (1 percent).

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

c) Filter sterilize the 5 mL medium through a 0.22-µm syringe filter. Keep the filtered medium at 4 °C prior to use.

III. CELL CULTURE.

The 101L is a transgenic human cell line obtained from Columbia Analytical Services (Vista, CA) for the P450RGS luciferase assay. These cells are shipped in cryovials frozen on dry ice and are immediately placed in liquid nitrogen upon receipt. Whenever these cells are thawed, they are cataloged as "passage 0." Subsamples from the original shipment have been frozen for later use. Cells that have undergone 25 or more passages or consistently showed 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, wipe the outside of the container with a dry paper towel followed with a 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 101L cells from the liquid nitrogen storage Dewar.

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

6) Thaw cryovial contents quickly (within a minute) in a 70 percent alcohol solution bath.

7) Aseptically transfer all the thawed cell suspension into the 12 mL of medium. Mix the cell suspension thoroughly with a sterile pipet to dilute the DMSO in the freezing medium.

8) Centrifuge the cell suspension at 1,000 rpm for 5 minutes.

9) Siphon off the supernatant medium using a pasteur pipet attached to an aspirator to remove any traces of DMSO.

10) Resuspend the cell pellet with 20 mL of fresh medium.

11) Transfer the cell suspension into a new T-75 flask.

12) Add 200 µL of 40 mg/mL Geneticin solution into the flask.

13) Place flask in the CO_2 incubator to allow the cells to attach overnight.

14) Siphon off the medium and transfer 15 mL of fresh medium and 150 µL of Geneticin the next day. Incubate cells.

15) 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, and versene (EDTA) in 37 °C water bath. Thaw out a cryovial of Geneticin in the alcohol bath.

2) Siphon off old medium from the T-75 flask. Transfer 2 mL of versene into the flask; this volume should just cover the surface of cells. Wait for about 2 minutes before pipetting off the versene.

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

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

5) ***CAUTION: These cells <u>should not</u> stay in trypsin for more than 15 minutes.***

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

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

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

9) Centrifuge the 13 mL of cell suspension at 1,000 rpm for 5 minutes.

10) 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.

11) 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.

12) Pipet 150 µL of 40 mg/mL Geneticin solution into the flask.

13) 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 five sections on each side of the hemacytometer.

4) Total the number of live and the number 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, multiply the number of dead cells by 10^4 to obtain the number of dead cells/mL. The viability of the cell suspension is the total number of live cells divided by the total (live and dead) number of cells.

6) ***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 Section II.B, "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.

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

8) 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 0, preferably a Monday or Friday, cells are plated in two 96-well microtiter plates and incubated for 3 days. On Day 3, preferably the later part of the afternoon, the cells are dosed with serial dilutions of the 2,3,7,8-tetrachlorodibenzo-*p*-dioxin (TCDD) standard and with the environmental extracts. The cells are again incubated for another 16 hours to allow for enzyme (luciferase) induction. On Day 4, the cells are lysed and the luciferase production is quantified using a luminometer.

Two 96-well microtiter plates are used for testing up to 24 environmental samples with 3 replicates each. One-half of a 96-well plate is allocated for the TCDD standards while the rest of the wells are used for the environmental samples. The second 96-well plate will not be necessary if testing fewer than five samples.

The time required to complete each daily task using the 96-well plates is described in the following tabulation:

Task	Time, hr
Day 0	1.0
Day 3	0.5
Day 4	1.0
Solution preparation/equipment setup	1.0
Total	3.5

II. DAY 0 (PLATING CELLS).

A. MATERIALS AND EQUIPMENT.

MEM.
Versene (1x, 0.2 g EDTA.4Na).
Geneticin (G418) (40 mg/mL) -1 aliquot.
Trypsin (1x) - 1 aliquot.
Trypan Blue (0.05 percent) - one aliquot.
Multichannel motorized pipettor (8 channels, 250 µL).
96-well microtiter plate, flat bottom, sterile (Corning #25860).
Sterile pipet tips (250 µL).
Pasteur pipets (9 in., 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 minutes for equilibration.

3) Turn on 37 °C water bath.

4) Place supplemented MEM, versene, and one aliquot each of Trypan Blue and trypsin in the water bath for about 30 minutes.

5) Trypsinize one confluent (T-75) flask of 101L cells with 3 mL trypsin each as described in Section III.B, "SUBCULTURING CELLS" of Protocol I. A confluent flask contains approximately 10 to 13 × 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 for 5 min.

8) Count the cells using a hemacytometer to determine the number of cells/mL of cultured stock (described in Section III.C, "COUNTING CELLS" of Protocol I).

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. Add 420 µL of Geneticin to the cell suspension. This volume of cell suspension will be sufficient for seeding two 96-well plates.

11) **Example.**

    a) 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.

    b) 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.

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

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

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

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

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

III. DAY 3 (DOSING THE CELLS).

The cells are dosed with 2,3,7,8-TCDD standards and environmental samples prepared in iso-octane on this day. The following setup is for testing 24 environmental samples with 3 replicates each.

A. MATERIALS AND EQUIPMENT.

2,3,7,8-TCDD stock (1 µg/mL or 1,000,000 pg/mL) in iso-octane, 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.

IMPORTANT: 2,3,7,8-TCDD is highly toxic. Wear latex gloves and lab coat while handling TCDD.

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 minutes for equilibration.
3) Turn on 37 °C water bath.
4) Place supplemented MEM in the water bath for about 30 minutes.
5) Remove TCDD standard and environmental samples (both stored in 4-mL amber vials) from the freezer. Vortex the standards and samples and let vials warm to room temperature.
6) 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.
7) Replenish the wells with 200 µL fresh medium. This precaution is taken to ensure that the volume of medium is consistent throughout the wells. There may be some loss in medium in the peripheral wells during the 3-day incubation.

8) Place eight 500-µL microcentrifuge tubes on a rack and number them from 1 to 8. Pipet about 1 mL of iso-octane into a 1.5-mL microcentrifuge tube and place it on the rack.

9) Prepare serial dilutions of TCDD standards using positive displacement pipettors as follows:

Tube No.	Tube Concentration,* pg/mL	Well Concentraton, pg/mL	Makeup
TCDD Stock	1,000,000		
1	100,000		10 µL stock + 90 µL of iso-octane
2	10,000	100	10 µL tube 1 + 90 µL of iso-octane
3	5,000	50	25 µL tube 2 + 25 µL of iso-octane
4	2,500	25	10 µL tube 2 + 30 µL of iso-octane
5	1,000	10	10 µL tube 2 + 90 µL of iso-octane
6	500	5	25 µL tube 5 + 25 µL of iso-octane
7	250	2.5	10 µL tube 5 + 30 µL of iso-octane
8	100	1	10 µL tube 5 + 90 µL of iso-octane

* The TCDD standards are made 100x since the cells are dosed with 2 µL of the standard into 200-µL cell medium.

10) Dose the cells with 2 µL of iso-octane (serve as control background), TCDD standards, and environmental samples using a positive displacement pipettor in Plate 1, as shown on the following page

11) Dose the remaining 16 environmental samples in Plate 2 similar to that in Plate 1.

12) Write down the time and date of dosing on the lids of the plates. Dosing in the late afternoon is preferable since the cells will be exposed to the TCDD standards and environmental samples for 16 hours. The assay can be taken down in the morning of the following day.

IV. DAY 4 (ASSAY TAKEDOWN)

The exposure to the TCDD standard and environmental samples is terminated at 16 hours. The cell medium is removed, and the cells washed and lysed. The lysates are then transferred into an opaque luminometer plate and read. The luminometer is programmed to inject two substrates (A and B) into each well simultaneously and integrate the luminescence reading over 2 seconds.

A. MATERIALS AND EQUIPMENT.

Multichannel motorized pipettor (250 µL).

PBS, GIBCO BRL #14190-144.

Substrate A, PHARMINGEN #556868 - 1 aliquot (reconstituted).

PLATE 1: Iso-octane and TCDD Standards and Environmental Samples (Eight Samples, Three Replicate Extracts per Sample, Two Wells per Extract)

Iso-octane	Iso-octane	Iso-octane	Iso-octane	Iso-octane	Iso-octane	Sample 1 Extract 1	Sample 1 Extract 1	Sample 1 Extract 2	Sample 1 Extract 2	Sample 1 Extract 3	Sample 1 Extract 3
1 pg/mL	1 pg/mL	1 pg/mL	1 pg/mL	1 pg/mL	1 pg/mL	Sample 2 Extract 1	Sample 2 Extract 1	Sample 2 Extract 2	Sample 2 Extract 2	Sample 2 Extract 3	Sample 2 Extract 3
2.5 pg/mL	2.5 pg/mL	2.5 pg/mL	2.5 pg/mL	2.5 pg/mL	2.5 pg/mL	Sample 3 Extract 1	Sample 3 Extract 1	Sample 3 Extract 2	Sample 3 Extract 2	Sample 3 Extract 3	Sample 3 Extract 3
5 pg/mL	5 pg/mL	5 pg/mL	5 pg/mL	5 pg/mL	5 pg/mL	Sample 4 Extract 1	Sample 4 Extract 1	Sample 4 Extract 2	Sample 4 Extract 2	Sample 4 Extract 3	Sample 4 Extract 3
10 pg/mL	10 pg/mL	10 pg/mL	10 pg/mL	10 pg/mL	10 pg/mL	Sample 5 Extract 1	Sample 5 Extract 1	Sample 5 Extract 2	Sample 5 Extract 2	Sample 5 Extract 3	Sample 5 Extract 3
25 pg/mL	25 pg/mL	25 pg/mL	25 pg/mL	25 pg/mL	25 pg/mL	Sample 6 Extract 1	Sample 6 Extract 1	Sample 6 Extract 2	Sample 6 Extract 2	Sample 6 Extract 3	Sample 6 Extract 3
50 pg/mL	50 pg/mL	50 pg/mL	50 pg/mL	50 pg/mL	50 pg/mL	Sample 7 Extract 1	Sample 7 Extract 1	Sample 7 Extract 2	Sample 7 Extract 2	Sample 7 Extract 3	Sample 7 Extract 3
100 pg/mL	100 pg/mL	100 pg/mL	100 pg/mL	100 pg/mL	100 pg/mL	Sample 8 Extract 1	Sample 8 Extract 1	Sample 8 Extract 2	Sample 8 Extract 2	Sample 8 Extract 3	Sample 8 Extract 3

PLATE 2: Remaining Environmental Samples (Sixteen Samples, Three Replicate Extracts per Sample, Two Wells per Extract)

Sample 9 Extract 1	Sample 9 Extract 1	Sample 9 Extract 2	Sample 9 Extract 2	Sample 9 Extract 3	Sample 9 Extract 3	Sample 17 Extract 1	Sample 17 Extract 1	Sample 17 Extract 2	Sample 17 Extract 2	Sample 17 Extract 3	Sample 17 Extract 3
Sample 10 Extract 1	Sample 10 Extract 1	Sample 10 Extract 2	Sample 10 Extract 2	Sample 10 Extract 3	Sample 10 Extract 3	Sample 18 Extract 1	Sample 18 Extract 1	Sample 18 Extract 2	Sample 18 Extract 2	Sample 18 Extract 3	Sample 18 Extract 3
Sample 11 Extract 1	Sample 11 Extract 1	Sample 11 Extract 2	Sample 11 Extract 2	Sample 11 Extract 3	Sample 11 Extract 3	Sample 19 Extract 1	Sample 19 Extract 1	Sample 19 Extract 2	Sample 19 Extract 2	Sample 19 Extract 3	Sample 19 Extract 3
Sample 12 Extract 1	Sample 12 Extract 1	Sample 12 Extract 2	Sample 12 Extract 2	Sample 12 Extract 3	Sample 12 Extract 3	Sample 20 Extract 1	Sample 20 Extract 1	Sample 20 Extract 2	Sample 20 Extract 2	Sample 20 Extract 3	Sample 20 Extract 3
Sample 13 Extract 1	Sample 13 Extract 1	Sample 13 Extract 2	Sample 13 Extract 2	Sample 13 Extract 3	Sample 13 Extract 3	Sample 21 Extract 1	Sample 21 Extract 1	Sample 21 Extract 2	Sample 21 Extract 2	Sample 21 Extract 3	Sample 21 Extract 3
Sample 14 Extract 1	Sample 14 Extract 1	Sample 14 Extract 2	Sample 14 Extract 2	Sample 14 Extract 3	Sample 14 Extract 3	Sample 22 Extract 1	Sample 22 Extract 1	Sample 22 Extract 2	Sample 22 Extract 2	Sample 22 Extract 3	Sample 22 Extract 3
Sample 15 Extract 1	Sample 15 Extract 1	Sample 15 Extract 2	Sample 15 Extract 2	Sample 15 Extract 3	Sample 15 Extract 3	Sample 23 Extract 1	Sample 23 Extract 1	Sample 23 Extract 2	Sample 23 Extract 2	Sample 23 Extract 3	Sample 23 Extract 3
Sample 16 Extract 1	Sample 16 Extract 1	Sample 16 Extract 2	Sample 16 Extract 2	Sample 16 Extract 3	Sample 16 Extract 3	Sample 24 Extract 1	Sample 24 Extract 1	Sample 24 Extract 2	Sample 24 Extract 2	Sample 24 Extract 3	Sample 24 Extract 3

Substrate B, PHARMINGEN #556870 - 1 aliquot (reconstituted).

Cell Lysis Buffer, PHARMINGEN #556871 - 1 aliquot (1x) .

50-mL solution basins, nonsterile.

Vortex (with microplate adaptor holder).

Inverted microscope.

Countertop Centrifuge (with microplate adaptor holder).

Luminometer (Dynex MLX 1000; DYNEX Technologies, Chantilly, VA).

96-well luminometer plate, opaque, nonsterile (Microlite 2.0, Dynex # 7417).

B. REAGENT PREPARATION.

The reagents for the P450RGS assay can be prepared ahead of time and stored in aliquots in the -80 °C freezer. Contents and date of preparation should be properly labeled and initialed on these reagent containers.

1) **Substrate A.**

a) Reconstitute lyophilized Substrate A with 50 mL of ultrapure water.

b) Transfer 12.5-mL aliquot of Substrate A solution into each 15-mL tube.

c) Store tubes in -80 °C freezer.

2) **Substrate B** ***(Light Sensitive)***.

a) Reconstitute lyophilized Substrate B with 50 mL of ultrapure water.

b) Transfer 12.5-mL aliquot of Substrate B solution into each 15-mL tube.

c) Wrap each tube with aluminum foil. ***Substrate B is light sensitive.***

d) Store tubes in -80 °C freezer.

3) **Cell Lysis Buffer** (1x).

a) Pipet 100 mL of ultrapure 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.

C. SETTING UP THE LUMINOMETER (Dynex MLX 1000 Luminometer).

1) Turning on and initializing the luminometer.

a) Turn on the power switches of the luminometer (bottom-right corner of the luminometer) and monitor. The luminometer will run a self test. The results of the test will be displayed on the self-test window. The Revelation window will then be displayed.

b) Click on the **Utility** menu, then the **Dispensers** option, followed by the **Initialize** option.

2) Washing/Purging the injectors A and C.

   a) Click on **Wash/Purge** option and then both **Injector A** and **C**.

   b) Click on **Utility** menu and then **Plate Out** option.

   c) Place the drain plate in the mask door and close the mask door. Click on **Plate In** option.

   d) The dispenser will draw water (ultrapure water) from the dispenser tubes three times to wash the injectors. After the injectors are washed, pour off the contents of the drain plate and place the plate back into the mask door.

3) Priming the injectors.

   a) Transfer Substrate A into a dispenser bottle (amber) and Substrate B into another.

   b) Connect dispenser A to the Substrate A bottle and dispenser C to the Substrate B bottle.

   c) Click on **Prime Injector**, followed by the **Injector A**. The injector will draw Substrate A through three times. Injector A is now primed.

   d) Pour off the contents of the drain plate and return the plate to the mask door.

   e) Prime Injector C similarly for Substrate B.

   f) Remove drain plate and click on **Done**.

   g) The luminometer is now primed.

4) Retrieving the assay template

   a) Click on the **File** menu.

   b) Open a previously prepared file for the assay template, designed to read an entire luminometer plate comprising 1 control background, 7 TCDD standards, and 24 samples in triplicate wells as matching the plate dosing template (see Section III, "DAY 3, DOSING THE CELLS").

   c) Click on **Settings** and choose the **Template** option. (Take out the wells that were preset in the assay template if fewer samples are tested for the day.)

   d) Click on the **Assay Option** from the **Settings** menu. The numbers should correspond with the numbers in the assay template. However, if fewer samples are being tested for the day, the numbers can be changed accordingly. These numbers should correspond to the number of wells included in the **Template** option.

5 Reading the plate and printing the results.

   a) Click on **Run A Plate** (the icon with a person facing the monitor) and take off the **Search for Sample ID**.

b) Click on **Plate ID** and type the filename as **datergs** (e.g., August 1 as **213rgs**) for the day's experiment. If a second plate is run on the same day, type **datergs2**, or for a third plate, then **datergs3**, and so on.

c) ***NOTE: The results will be automatically saved on the computer and can be called up when required.***

d) Click on **Start**. The luminometer will start reading. The injectors will inject 100 µL of Substrate A and Substrate B into each well simultaneously.

e) Click on the **Continue** button to instruct the luminometer to read from one well to another. There is a 2-second delay between reading the wells.

f) When all the wells are read, the Printer Menu will appear at the end of the run. Click on **Cancel** (the entire report is very long) to avoid printing all the flash data report.

g) Click on **View Data Matrix** and print the raw data in relative luminescence unit (RLU).

h) Click on **View Area Statistics**. Print the page(s) as needed, usually 1 page long. This report will show the average and other simple statistics for every sample.

6) Recovering Substrate A and B from the injectors.

a) Click on **Utility** menu.

b) Place drain plate in the mask door.

c) Click on **Dispensers** and then **Recover Injectors** option.

d) Transfer any remaining Substrate A and Substrate B into their respective storage tubes. Wrap the tube containing Substrate B with aluminum foil.

e) Store the substrates immediately in the -80 °C freezer for later use.

f) Go through the wash/purge steps to clean the injector with ultrapure water.

7) Closing the template file.

a) Click on **File** followed by the **Close** button twice to close the View Area Statistics and Data Matrix reports.

b) Click on **File** and **Close** again to close the assay template. If some changes were made on the template, a screen will appear. It is not necessary to save any changes made on the template.

8) Exiting the program.

a) Click on **File** and then on **Exit** to exit the Revelation program.

b) Click on **File** and then on **Exit** again to exit the Windows program. Click on **OK** to get into the C:> prompt.

c) Turn off the luminometer switch.

D. PROCEDURE (nonsterile techniques) - at the end of the 16-hour exposure.

1) Turn on the luminometer to warm up and run its self-test program. The injectors of the luminometer will be primed during the 15-minute interval when the cells are being lysed. Program the luminometer to inject 100 µL each of Substrate A and Substrate B simultaneously and to integrate luminescence readings over 2 seconds for each well.

2) Place an aliquot each of Substrate A, Substrate B, and Cell Lysis Buffer (1x) in the water bath (room temperature).

3) Take out the two plates from the incubator to the hood.

4) Pipet off the cell medium in both plates 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.

5) Pipet 200 µL of PBS (1x) into each well to wash the cells. Pipet off the PBS. The cell medium and PBS rinse are treated as TCDD waste material and are transferred to an appropriate container for later treatment and disposal.

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

7) Add 40 µL of cell lysis buffer to each well.

8) Place each 96-well plate onto the microplate adaptor holder of a vortex.

9) Set to vortex at number 4. Turn on switch and vortex for 15 minutes. In the meantime, the luminometer can be primed (see Section IV.C, "SETTING UP THE LUMINOMETER").

10) 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.

11) Centrifuge plates at 5,000 rpm for 5 minutes. This step is taken to spin down the cell debris (which can interfere with the luminometer reading) to the bottom of the wells.

12) With a multichannel pipettor, transfer 25 µL of the lysate in the first column of cell lysate into the corresponding first column of wells of a 96-well luminometer plate (opaque, nonsterile).

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

14) Transfer the second 25 µL of lysate from the second column of cell lysate to the first column of cells in the 96-well luminometer plate.

15) ***IMPORTANT: Remember always to combine the 25-µL lysates from two consecutive wells into the 96-well luminometer plate. The lysates from the two 96-well plates should finally be transferred into one luminometer plate. See following diagram for example.***

Example for transferring samples from dosing plate to reading plate

DOSING PLATE 1

	Column 1	Column 2	Column 3	Column 4	Column 5	Column 6	Column 7	Column 8	Column 9	Column 10	Column 11	Column 12
A	Iso-octane	Iso-octane	Iso-octane	Iso-octane	Iso-octane	Iso-octane	Sample 1 Extract 1	Sample 1 Extract 1	Sample 1 Extract 2	Sample 1 Extract 2	Sample 1 Extract 3	Sample 1 Extract 3
B	1 pg/mL	1 pg/mL	1 pg/mL	1 pg/mL	1 pg/mL	1 pg/mL	Sample 2 Extract 1	Sample 2 Extract 1	Sample 2 Extract 2	Sample 2 Extract 2	Sample 2 Extract 3	Sample 2 Extract 3
C	2.5 pg/mL	2.5 pg/mL	2.5 pg/mL	2.5 pg/mL	2.5 pg/mL	2.5 pg/mL	Sample 3 Extract 1	Sample 3 Extract 1	Sample 3 Extract 2	Sample 3 Extract 2	Sample 3 Extract 3	Sample 3 Extract 3
D	5 pg/mL	5 pg/mL	5 pg/mL	5 pg/mL	5 pg/mL	5 pg/mL	Sample 4 Extract 1	Sample 4 Extract 1	Sample 4 Extract 2	Sample 4 Extract 2	Sample 4 Extract 3	Sample 4 Extract 3
E	10 pg/mL	10 pg/mL	10 pg/mL	10 pg/mL	10 pg/mL	10 pg/mL	Sample 5 Extract 1	Sample 5 Extract 1	Sample 5 Extract 2	Sample 5 Extract 2	Sample 5 Extract 3	Sample 5 Extract 3
F	25 pg/mL	25 pg/mL	25 pg/mL	25 pg/mL	25 pg/mL	25 pg/mL	Sample 6 Extract 1	Sample 6 Extract 1	Sample 6 Extract 2	Sample 6 Extract 2	Sample 6 Extract 3	Sample 6 Extract 3
G	50 pg/mL	50 pg/mL	50 pg/mL	50 pg/mL	50 pg/mL	50 pg/mL	Sample 7 Extract 1	Sample 7 Extract 1	Sample 7 Extract 2	Sample 7 Extract 2	Sample 7 Extract 3	Sample 7 Extract 3
H	100 pg/mL	100 pg/mL	100 pg/mL	100 pg/mL	100 pg/mL	100 pg/mL	Sample 8 Extract 1	Sample 8 Extract 1	Sample 8 Extract 2	Sample 8 Extract 2	Sample 8 Extract 3	Sample 8 Extract 3

Combine 25 µL each from row A, columns 1 and 2, of dosing plate into reading plate row A, column 1. Repeat for rows B-H.

Combine 25 µL each from row A, columns 3 and 4, of dosing plate into reading plate row A, column 2. Repeat for rows B-H.

Combine 25 µL each from row A, columns 5 and 6, of dosing plate into reading plate row A, column 3. Repeat for rows B-H.

Combine 25 µL each from row A, columns 7 and 8, of dosing plate into reading plate row A, column 4. Repeat for rows B-H.

Combine 25 µL each from row A, columns 9 and 10, of dosing plate into reading plate row A, column 5. Repeat for rows B-H.

Combine 25 µL each from row A, columns 11 and 12, of dosing plate into reading plate row A, column 6. Repeat for rows B-H.

READING PLATE

The second half of the reading plate will be used for samples from the second dosing plate

	Column 1	Column 2	Column 3	Column 4	Column 5	Column 6	Column 7	Column 8	Column 9	Column 10	Column 11	Column 12
A	Iso-octane	Iso-octane	Iso-octane	Sample 1 Extract 1	Sample 1 Extract 2	Sample 1 Extract 3						
B	1 pg/mL	1 pg/mL	1 pg/mL	Sample 2 Extract 1	Sample 2 Extract 2	Sample 2 Extract 3						
C	2.5 pg/mL	2.5 pg/mL	2.5 pg/mL	Sample 3 Extract 1	Sample 3 Extract 2	Sample 3 Extract 3						
D	5 pg/mL	5 pg/mL	5 pg/mL	Sample 4 Extract 1	Sample 4 Extract 2	Sample 4 Extract 3						
E	10 pg/mL	10 pg/mL	10 pg/mL	Sample 5 Extract 1	Sample 5 Extract 2	Sample 5 Extract 3						
F	25 pg/mL	25 pg/mL	25 pg/mL	Sample 6 Extract 1	Sample 6 Extract 2	Sample 6 Extract 3						
G	50 pg/mL	50 pg/mL	50 pg/mL	Sample 7 Extract 1	Sample 7 Extract 2	Sample 7 Extract 3						
H	100 pg/mL	100 pg/mL	100 pg/mL	Sample 8 Extract 1	Sample 8 Extract 2	Sample 8 Extract 3						

16) Continue with the transfer of the cell lysate into the 96-well luminometer plate as described.

17) Read the plates on the luminometer. It takes only 5 minutes to read a plate.

18) Print out raw data report in RLU and save data files.

V. DATA PROCESSING.

A. DATA ANALYSIS.

1) Calculate the fold inductions (with standard deviations) for the various concentrations of TCDD (= TCDD RLU/iso-octane RLU) and test samples (= sample RLU/iso-octane RLU). The fold induction of iso-octane should be 1 (= iso-octane RLU/iso-octane RLU).

2) Average all the TCDD standard deviations (average σ).

3) Calculate the Limit Of Detection (LOD) by adding 3 times the average standard deviations to the fold induction of iso-octane (= [3* average σ] + 1).

4) Generate a linear TCDD standard curve (fold induction versus TCDD concentration (pg/mL)) utilizing only points that are linear. The linear correlation of TCDD standards below 50 pg/mL is usually better than 0.95.

5) Calculate the TCDD equivalents (pg/mL) for the test samples based on the linear TCDD standard curve.

6) Convert the sample concentration to obtain pg TCDD equivalents/g sediment using the information on the volume of extract and dilutions tested, and the amount of sediment (g) used.

7) Average the values for the different replicates tested for the final value.

8) Any sample tested below the LOD is recorded as below limit of detection (BLD). Any sample tested above the highest standard used to generate the standard curve should be diluted appropriately and retested.

B. FILE LABELING FORMAT. CODE: 000XXX11.dat

1) 000 = Day of year, e.g., 001 is 1 Jan and 233 is 21 Aug.

2) XXX = Project ID, a three-letter designation recorded in the lab notebook.

3) 11 = Plate number analyzed on the day.

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