Designation: E 1853M – 98

AMERICAN SOCIETY FOR TESTING AND MATERIALS
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Standard Guide for Measuring the Presence of Planar Organic Compounds Which Induce CYP1A, Using Reporter Gene Test Systems[1]

This standard is issued under the fixed designation E 1853M; the number immediately following the designation indicates the year of original adoption or, in the case of revision, the year of last revision. A number in parentheses indicates the year of last reapproval. A superscript epsilon (ε) indicates an editorial change since the last revision or reapproval.

1. Scope

1.1 This guide covers the recommended guidelines for performing a test for presence of organic compounds that bind to the Ah Receptor and induce the CYP1A locus on the vertebrate chromosome. Under appropriate test conditions, induction of CYP1A is evidence that the cells have been exposed to one or more of these xenobiotic organic compounds, including dioxins, furans, coplanar PCBs, and several polycyclic aromatic hydrocarbons (PAHs). Detection of induction has been made simple and rapid by the stable integration of the firefly plasmid such that Ah-receptor binding results in the production of luciferase. Luciferase production is a function of both the potency of the compound(s) and the concentration. This type of Reporter Gene System (RGS) has shown concentration-response relationships using 2,3,7,8-TCDD, 5 coplanar PCBs, and several polycyclic aromatic hydrocarbons (PAHs) **(1,2)**.[2] This guide describes test conditions under which solvent extracts of environmental samples (water, tissue, soil, or sediments) may be tested for the presence of CYP1A-inducing organic compounds.

1.2 The test procedures presented in this guide have been published previously **(1-3)**. These references should be consulted to obtain details regarding the construction and maintenance of the cell line, and the response of the cells to various organic substances.

1.3 All laboratory health and safety procedures should be followed. This includes the use of glasses, gloves, and other protective clothing, when handling the reagents. Information on toxicity, handling procedures and waste procedures should be reviewed prior to use of all chemicals.

1.4 *This standard does not purport to address all of the safety concerns, if any, associated with its use. It is the responsibility of the user of this standard to establish appropriate safety and health practices and determine the applicability of regulatory limitations prior to use.*

1.5 This guide is arranged as follows:

2. Referenced Documents

2.1 *ASTM Standards:*

D 3976 Practice for Preparation of Sediment Samples for Chemical Analysis[3]

E 1023 Guide for Assessing the Hazard of a Material to Aquatic Organisms and Their Uses[4]

E 1391 Guide for Collection, Storage, Characterization, and Manipulation of Sediments for Toxicological Testing[4]

3. Terminology

3.1 *Definitions:*

3.1.1 The words "must,"" should," "may,"" can," and "might" have very specific meanings in this guide. " Must" is used to express the strongest possible recommendation, just short of an absolute requirement. "Must" is only used in connection with factors that relate directly to the acceptability of the test." Should" is used to state that the specific condition is recommended and ought to be met if possible. Although violation of one "should" is rarely a serious matter, the violation of several will often render the results questionable.

[1] This guide is under the jurisdiction of ASTM Committee E-47 on Biological Effects and Environmental Fateand is the direct responsibility of Subcommittee E47.09on Biomarkers.

Current edition approved March 10, 1998. Published September 1998. Originally published as E 1853 - 96. Last previous edition E 1853 - 96.

[2] The boldface numbers in parentheses refer to a list of references at the end of this guide.

[3] *Annual Book of ASTM Standards*, Vol 11.02.

[4] *Annual Book of ASTM Standards*, Vol 11.05.

Terms such as “is desirable,” “is often desirable,” and “might be desirable” are used in connection with less important factors. “May” is used to mean “is (are) allowed to,” “can” is used to mean “is (are) able to,” and “might” is used to mean “could possibly.” Thus the classic distinction between “may” and “can” is preserved, and “might” is never used as a synonym for either“ may” or “can.”

3.2 *Definitions of Terms Specific to This Standard:*

3.2.1 *AhR-aryl hydrocarbon receptor—in the cell cytoplasm that binds to planar compounds*, receptors forming an AhR-ligand complex, which is translocated to the nucleus where the complex activates transcription of the CYP1A gene.

3.2.2 *B(a)P-benzo(a)pyrene*—a model PAH and one of the most toxic and carcinogenic PAHs.

3.2.3 *Coplanar PCBs-polychlorinated biphenyls*—biphenyls that possess a flat (planar) configuration, such as 3,3′,4,4′,5-pentachlorobiphenyl (PCB No. 126).

3.2.4 *CYP1A*—gene coding for a group of P450 metabolic enzymes that are induced by planar organic compounds (ligand), such as dioxin (2,3,7,8-tetrachlorodibenzo-p-dioxin; TCDD), through the Ah-receptor mediated process.

3.2.5 *DCM-dichloromethane (methylene chloride)*—a solvent used to extract organic contaminants from tissue or sediments.

3.2.6 *DMSO-dimethylsulfoxide*—a solvent frequently used as a carrier to apply compounds that are relatively insoluble in water.

3.2.7 *PAHs-polycyclic aromatic hydrocarbons*—hydrocarbons commonly found in crude oils, petroleum products, and combustion products.

3.2.8 *Reporter Gene System (RGS)*—a plasmid from the firefly that has been linked to CYP1A promoter sequences, such that induction of CYP1A results in the production of luciferase.

3.2.9 *3MC-3-Methylcholanthene*—a compound known to induce the CYP1A gene, resulting in the increased production of the enzyme P450 1A.

4. Application

4.1 These tests are designed as rapid, specific, sensitive, and inexpensive screening approaches for determining if an environmental sample contains significant amounts of planar organic compounds, including dioxins, furans, coplanar PCBs, and polycyclic aromatic hydrocarbons. Responses obtained may be compared to an acceptable baseline, or those samples found to most strongly induce the CYP1A test system might be selected for comprehensive chemical characterization. An example illustrating the test sensitivity of one cell line is shown in Annex A1.

5. Summary of Guide

5.1 The test systems are based on transgenic cell lines developed and tested previously **(1-7)**. The 101L (human hepatoma) cells have a stably integrated plasmid that contains the human CYP1A1 promoter and the 5′-flanking sequences, fused to the firefly luciferase gene (Reporter Gene). Induction at the CYP1A1 site in this cell line results in the production of luciferase. Solvent (DCM) extracts of environmental samples (see Practice D 3976) are added to individual wells of 6-well culture plates, containing approximately one million cells, and the exposure time is 16 h. Volumes of solvent successfully tested are 2 to 20 µL, which produce a low background (blank) induction when applied to the 2 mL of culture medium. After exposure, the cells are rinsed, and then lysed. Luminescence is either determined in the exposure well (96-well plate luminometer) or the contents of each well of a 6-well plate are removed and placed in a microcentrifuge tube and centrifuged. In the latter case, the pellet is discarded, and aliquots of the supernatant are used to determine the content of luciferase (single or 96-well luminometer) and protein. The luminescence (in relative light units, RLU) of the cell extract from each well is determined. With each batch of samples (test run), luminescence is also measured in cells exposed to a solvent control, and reference inducers (3-methylcholanthrene, TCDD, benzo[a]pyrene, etc.). The mean RLUs of the control wells is set equal to unity. Mean RLUs of samples are converted to Fold Induction by dividing by the mean RLUs of the solvent (control). This biochemical response represents the integrated induction from all planar organic compounds present in the extract, that bind to the Ah-receptor in the same manner as dioxin **(6)**. Final results may be expressed as fold induction, or by use of the reference toxicant; results can be expressed as equivalents of TCDD or benzo(a)pyrene. For best comparisons between samples, the initial dry weight (determined on a separate subsample) of the extracted sample, the final volume of the solvent containing the extracted material (1 or 2 mL), and the amount applied (2 to 20 µL) to the cells are all recorded. Induction may then be expressed as nanograms of dioxin (or another reference inducer; for example, benzo[a]pyrene) equivalents per gram dry weight or per litre.

6. Significance and Use

6.1 The compounds that bind to the Ah-receptor and induce CYP1A have often been shown to be either more toxic or carcinogenic, or both, than other organic compounds. Dioxins and PCBs have been shown to bioconcentrate in exposed organisms and biomagnify in the food web (see Guide E 1023). Testing with birds, mammals, and fish species has shown that exposure to these compounds can produce physiological, reproductive and histopathological effects **(8-10)**. Concern for the possible contamination of water, food, wildlife, soil, and aquatic sediment from these compounds has led to the requirement for analytical chemical analyses of a great many environmental samples. Use of a screening tool such as these Reporter Gene Systems (RGS) will allow identification of significantly contaminated samples. These methods will aid in the cost-effective separation of high priority samples from those that do not require further expensive chemical characterization.

7. Interferences

7.1 The general nature of contamination in environmental samples is a mixture of organics, possibly including polycyclic aromatic hydrocarbons (PAHs), and chlorinated hydrocarbons (pesticides, PCBs, dioxins and furans). The total response from the RGS assay gives an integrated response to the mixture of planar organic compounds. The response is often additive (multiple PAHs and PAHs plus a coplanar PCB), but not all

combinations have been tested. Antagonistic interaction between specific polychlorinated biphenyl (PCB) congeners has been observed **(5)**. Since extracts are applied, there is little chance for metals to interfere with the response. Tests with extracts of sediment, highly contaminated with a range of toxic metals (cadmium, copper, lead, zinc, etc.), have shown strong induction and thus no indication of interference from sediment metals. Some studies have indicated that high levels of tributyl-tin (TBT) may inhibit the induction of CYP1A **(11)**.

8. Apparatus

8.1 *Instruments*:

8.1.1 *Sonic Probe*, as Braun-Sonic 1510.

8.1.2 *Microcentrifuge*.

8.1.3 *Luminometer*, as Dynatech ML1000 or ML2251.

8.1.4 *Laminar Flow Hood*.

8.1.5 *Incubator* with CO_2 regulation.

8.1.6 *Microcentrifuge*.

8.2 *Reagents*:

8.2.1 *3MC*.

8.2.2 *DCM*.

8.2.3 *DMSO*.

8.2.4 *B(a)P*.

8.2.5 *TCDD*.

8.2.6 *Luciferase Assay Kit* (with Luciferin).

8.2.7 *Luciferase Standard*.

8.3 *Supplies*:

8.3.1 *Centrifuge Tubes*.

8.3.2 *Sterile 6-well Culture Plates*, with covers.

8.3.3 *Tissue Culture Flasks*, 250 mL canted neck, sterile, polystyrene.

8.3.4 *Human Hepatoma Cell Culture Media*, as Dulbecco's modified Eagle medium, with 10 % fetal calf serum, and 0.4 mg G418/mL.

8.3.5 *96 Microwell Luminometer Plates*.

8.3.6 *Cell Scraper*.

9. Sample Extraction

9.1 While other unique methods may be developed, tested and used to extract organic contaminants from water, tissue, sediments, or soil samples, the preferred procedures are the EPA Methods 3540 and 3550, using dichloromethane (DCM) extraction. Samples of approximately 40 g of tissue, sediment (see Guide E 1391), or soil and 1 L of water are appropriate. Extraction and evaporation will result in a small volume of DCM (1 or 2 mL) in a tightly sealed vial. These samples may readily be shipped from an extraction laboratory to the testing laboratory, if these are not the same. If the test sediments are highly contaminated, it may be possible to test the pore water for CYP1A induction. Sediments should be centrifuged to produce about 0.5 mL of porewater, which may be applied after filtration (for sterilization) through 0.45-µ or 0.22-µ filters to the cells in the wells in volumes up to 200 µL.

9.2 *Volume Selection*:

9.2.1 Experience has shown that the shade of the extract (brown) is an indication of the quantity of petroleum hydrocarbons, including PAHs. Therefore, a smaller aliquot of a dark extract (2 to 5 µL) is often appropriate, avoiding the possibility of introducing a toxic level of compounds or saturating the test system. Fold induction as high as 900 times control has been observed, so the range of response is quite broad. Light colored (clear or yellow) samples are often tested with volumes of 10 or 20 µL of extract. Even 20 µL of extract only represents 0.1 % of the medium bathing the cells. Volumes up to 200 µL of interstitial or pore water may be applied, after filtration through 0.45-µ or 0.22-µ filters to remove bacteria. There is no chance that the color of the extract will quench the luminescence of a sample, since the exposure medium is rinsed away before the cells are lysed in 200 µL of buffered solution.

9.3 *Controls*:

9.3.1 If more than one volume of test sample is added to the test wells, then it is necessary to use the same volumes of blank solvent, for measuring control luminescence (in RLUs). The control of the same volume as the test sample should be used to determine fold induction, by dividing by the RLUs exhibited by the solvent (blank).

9.4 *Reference Toxicants*:

9.4.1 Reference toxicants and concentrations that are appropriate are 1.0 ng/mL (3.1 nM) 2,3,7,8-TCDD, 10 µM 3-methylcholanthrene, and 0.5 µg/mL benzo(a)pyrene. These concentrations all represent the final concentration in the exposure wells. The use of one or more reference toxicants or concentrations with each set of samples provides a quality control check on the performance of the cells, and also allows conversion of the data to equivalents of the reference toxicant. It is also recommended that one of these reference toxicants be applied as a matrix spike to duplicate samples of approximately 10 % of the extracts of environmental samples. This would test for the possible reduction in RGS response to a toxicant caused by the matrix of the sample. A matrix effect is unlikely, since the primary matrix is solvent (DCM or DMSO), and RGS response to the solvents is covered by control samples. A high level of PAH in the sample matrix may somewhat reduce the RGS response to chlorinated organics (antagonism). When testing for the presence of planar chlorinated organics, it is best to spike duplicate samples with TCDD.

10. Treatment of Data

10.1 *Interpretation of Data*:

10.1.1 Data from the luminometer should be entered on a standard computer spreadsheet (example attached, Table 1). The test solutions listed in Table 1 are solvents and two inducing reference compounds. From the spreadsheet (formulas embedded in sheet), the mean RLUs for each control, reference toxicant, and sample may be determined. Setting the control response equal to unity, it is then possible to calculate the mean fold induction (with standard deviations) for each reference toxicant and test sample. If protein is determined for each sample, the data may be expressed as fold induction per milligram of protein. The data may also be expressed as reference toxicant equivalents, per gram (wet or dry) of sample.

10.2 *Acceptability of Data*:

10.2.1 Responses to solvent blanks and reference toxicants should be compared to previous data by use of control charts to determine if the test was valid. If the response to reference toxicants was greater than two standard deviations from the mean, the test may not be acceptable. Replicate plates may be

TABLE 1 Example of Spreadsheet Used in P450 RGS Assay

File Name:															
		TV101L P2, 1.5 × 10 |AX 6/plate													
	16 h	Harvest 03/30/94													
	Protein Assay 04/04/94							Luciferase Assay 03/30/94							
		Protein													
	Inducer (Final Concentration)	Volume used	Conc(1) µm/µL	Conc(2) µg/µL	Avg Conc µg/µL	Volume used	µg used	RLU(1) U	RLU(2) U	Avg U	RLU/µg U/µg	Avg U/µg protein	Avg Fold Induc-tion	Indivi dual Fold Induction	Stan dard Devia-tion
1	DMSO	2 µL	3.32	3.37	3.34	10 µL	33.4	1114	1137	1126	32				
2	DMSO	2 µL	3.67	3.55	3.61	10 µL	36.1	1279	1253	1266	34				
3	DMSO	2 µL	3.61	3.81	3.71	10 µL	37.1	1285	1290	1288	33	33.0	1.0	1.00	0.64
4	3MC 10 µM (final)	2 µL	3.28	3.31	3.29	10 µL	32.9	69895	72020	70958	2153.55			65.19	
5	3MC 10 µM (final)	2 µL	3.21	3.19	3.20	10 µL	32.0	71975	73775	72875	2277.03			68.93	
6	3MC 10 µM (final)	2 µL	3.39	3.61	3.50	10 µL	35.0	80208	79770	79989	2284.92	2238.5	67.8	69.17	1.82
7	TCDD 10 nM (final)	2 µL	3.95	4.26	4.11	10 µL	41.1	230918	232136	231527	5638.87			170.7	
8	TCDD 10 nM (final)	2 µL	3.74	3.98	3.86	10 µL	38.6	216539	218486	217513	5636.54			170.6	
9	TCDD 10 nM (final)	2 µL	3.75	3.95	3.85	10 µL	38.5	217182	219488	218335	5670.29	5648.6	171.0	171.7	0.46

used to assess the viability of cells, by adding the vital stain trypan blue (5 %). Normal viability is 70 to 90 %, and a decrease to less than 60 % is unacceptable. When protein is measured, a reduction in protein is also a measure of cell viability. Dead cells do not adhere to the plates, and are washed away during rinsing, before the centrifugation step. Loss of cells would be observed as low protein values, and the response (RLUs) would be normalized to milligrams of protein.

11. Reporting Data

11.1 The report should include the dates of cell transfer and testing, sample descriptions, sample weights or volumes, and the responses of the cells (RLUs). Findings should be reported as the fold induction produced by the samples, with the dry weight of each sample, the volume of the final extract, and the volume applied to the cells. If the reference toxicant results were within acceptable limits, this should be noted. The responses of the cells may also be reported on a basis of equivalents of the reference toxicant (2,3,7,8-dioxin or benzo[a]pyrene) per gram dry weight of sample. The spreadsheet in Table 1 provides an example of a report data sheet.

12. Keywords

12.1 Ah-receptor; biomarker; carcinogenic; coplanar organic compound; CYP1A; induction; P450 1A; reporter gene system; toxic

ANNEX

(Mandatory Information)

A1. THE P450 REPORTER GENE SYSTEM, USING 101L CELLS

A1.1 Procedure

A1.1.1 *Overview*—This Annex describes the use of a transgenic cell line (101L)[5] derived from the human hepatoma cell line, HepG2 **(1)**. Fig. A1.1 provides an overview of the protocol used in testing the P450 RGS response to either organic extracts or water (including porewater) samples. This assay requires proper training on cell culture (sterile techniques), the correct use of all instruments, and spiking techniques. Any personnel with adequate training in these techniques will be able to perform the assay. Sterile techniques should be employed when handling cells. It is very important that all equipment is handled properly so that no contaminants are introduced. Proper record keeping is required so that all reagents are labeled, all samples logged in, and all cell culture material is properly stored.

A1.1.1.1 In the latter part of 1996 and in 1997, five publications have appeared in the literature, which regard the use of this test system. Two methods papers were published **(12, 13)**, and the results of analyses of environmental samples from San Diego Bay **(14)**, a coastal region of Korea **(15)**, and elsewhere **(16)** were described.

A1.1.2 *Culture Maintenance*—The maintenance of the cell

[5] The sole source of supply of these reagents known to the committee at this time is Columbia Analytical Services, 1185 Park Center Dr., Suite A, Vista, CA 92083. If you are aware of alternative suppliers, please provide this information to ASTM Headquarters. Your comments will receive careful consideration at a meeting of the responsible technical committee,[1] which you may attend.

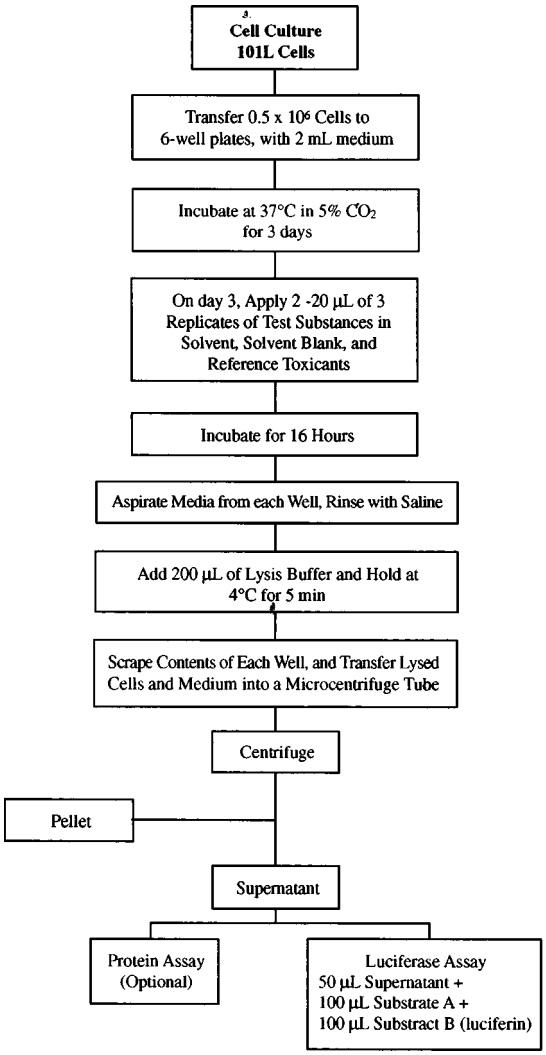


FIG. A1.1 Flow-chart for the P450 Reporter Gene System Assay

line requires biweekly changes of media. The cells are incubated at 37°C in an atmosphere of 5 % CO_2. The disulfate salt antibiotic Geneticin (G418) is added to media at a concentration of 0.4 mg/mL with each change of media, to eliminate those cells that do not possess the stably integrated plasmid.

A1.1.3 *Instrument Calibration*—The luminometer should be calibrated within two days of the analysis of a set of samples. The calibration curve produced should be compared to previous curves to demonstrate suitable sensitivity and linearity of the instrument. Using the Luciferase Standardization Kit,[6] it should be possible to detect one picogram of luciferase.

A1.1.4 *Testing Protocol*:

A1.1.4.1 Transfer 0.25×10^6 cells to each well of a 6-well culture plate in 2 mL media (1.5×10^6 cells per plate).

A1.1.4.2 Incubate for three days to allow for an increase in cell numbers and to ensure adhesion to the plastic well.

A1.1.4.3 Apply 2 µL of 10 mM 3MC to 2 mL in three wells, producing a 10 µM exposure.

A1.1.4.4 Apply 2 µL of 1.0 ng/µL solution of 2,3,7,8-TCDD to three wells containing 2 mL of medium, producing a 1.0 ng/mL exposure in the wells.

A1.1.4.5 Apply up to 20 µL of the solvent DMSO or 10 µL of the solvent DCM, or up 300 µL of water with microsyrinnge or positive displacement micropipettor to three replicate wells and record the volumes.

A1.1.4.6 Incubate for 16 h at 37°C and 5 % CO_2.

A1.1.4.7 After 16 h, aspirate media and rinse cells with a saline solution (PBS w/o Mg^{++}).

A1.1.4.8 Add 200 µL of lysis buffer to each well and incubate for 15 min at 4°C.

A1.1.4.9 Scrape contents of each well and transfer the suspension to a microcentrifuge tube.

A1.1.4.10 Spin cells for 10 s at about 10 000 r/min to separate the cellular debris.

A1.1.4.11 Remove the supernatant and discard the pellet.

A1.1.4.12 Add 50 µL of the supernatant from each test well to one or two replicate microwells of the 96-well plate, designed for the specific luminometer. A single sample of cell extract and a single well luminometer may also be used.

A1.1.4.13 Inject 100 µL of Substrate A (from Luciferase assay kit) into each well and then inject 100 µL of Substrate B (Luciferin from kit) within 10 min. The time between adding Substrate B and measurement of luminescence should be as short as possible (no longer than 5 min) and consistent from plate to plate.

A1.1.4.14 Measure luminescence in each sample with the luminometer, and record the RLUs.

A1.1.4.15 Record all information on the weights of samples, the volume of extract produced from the extraction, any dilution, the volume applied to the cells in the 2 mL of media, and the duration of exposure.

A1.1.4.16 The information should be recorded on a bench sheet and later entered onto the final spreadsheet. Luminometer readings should either be captured on print-out or on disk, if available on the instrument.

A1.1.4.17 To normalize the fold induction values to protein content, a subsample of the supernatant of each sample may be analyzed for protein **(17)**.

A1.2 Sensitivity

A1.2.1 Table A1.1 and Table A1.2 list the concentrations of specific organic compounds that were found to produce a ten-fold induction of CYP1A, using the P450 RGS assay. A ten-fold induction is clearly a significant response to toxicants. The range of CYP1A1 induction has been from two- to three-fold for very low levels of inducing compounds, or from weak inducers to as much as 900-fold induction from an extract of highly contaminated marine sediment. However, induction responses greater than 100 may indicate saturation of the test system. Those values listed in Table 1 are therefore somewhat higher than detection limits. Concentrations are listed in terms of nanograms per millilitre in the exposure well with the cells, and the concentration of a soil or sediment sample that would produce the same response. The conversion of the data from the exposure well to a soil sample (factor of 2.5) is based on the

[6] The sole source of supply of these reagents known to the committee at this time is Pharmingen, Inc., San Diego, CA. If you are aware of alternative suppliers, please provide this information to ASTM Headquarters. Your comments will receive careful consideration at a meeting of the responsible technical committee,[1] which you may attend.

TABLE A1.1 Relative Responses of the P450 RGS Assay to Specific PAHs Concentrations which Produce a 10-Fold Induction (10 times background)

PAH Compound	Concentration		
	Medium (ng/mL)	Soil (ng/g)	Water (ng/L)
PAH Mixture	150	750	30
Benzo(k) fluoranthene	8	40	1.6
DiBenz(a,h)anthracene	50	2.50	10
Benzo(b)fluoranthene	100	500	20
Indeno(1,2,3-cd)Pyrene	100	500	20
Benzo(a)pyrene	200	1000	40
Benzo(a)anthracene	500	2500	100
Chrysene	500	2500	100
Benzo(a)fluorene	500	2500	100
Benzo(ghi)perylene	10 000	50 000	2000
Acenaphthene	>10 000	>50 000	>2000
Acenaphtylene	>10 000	>50 000	>2000
Anthracene	>10 000	>50 000	>2000
Fluorene	>10 000	>50 000	>2000
Naphthalene	>10 000	>50 000	>2000
Phenanthrene	>10 000	>50 000	>2000

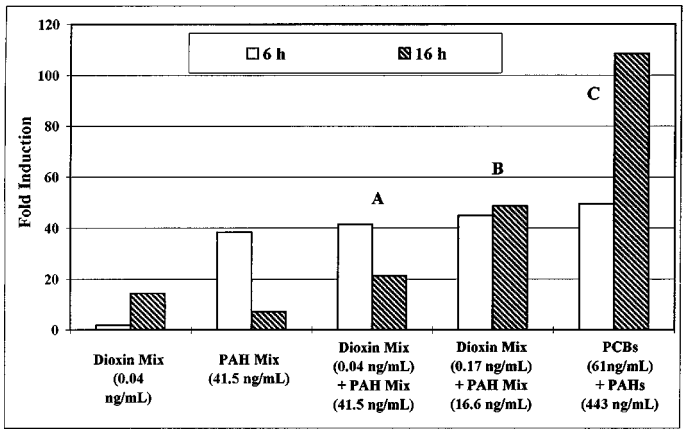
TABLE A1.2 Relative Response of the P450 RGS to Chlorinated Hydrocarbons Concentrations which Produce a 10-Fold Induction (10 times background)

Chemical	Concentration		
	Medium (ng/mL)	Soil (ng/g)	Water (ng/L)
2,3,7,8-TCDD	0.004	0.02	0.0008
2,3,7,8-TCDF	0.025	0.125	0.005
Octa-CDDs	12.50	6250	250
Octa-CDFs	50	250	10
Dioxin/Furan Mixture	0.07	0.35	0.014
PCB Congener # **(18)**			
81	0.5	2.5	0.1
126	4	20	0.8
77	250	1250	50
114	250	1250	50
118	1250	6250	250
123	1250	6250	250
169	7500	37 500	1500
105	>7500	>37 500	>1500
156	>7500	>37 500	>1500
157	>7500	>37 500	>1500
167	>7500	>37 500	>1500
180	>7500	>37 500	>1500
189	>7500	>37 500	>1500

extraction of a 40-g sample and the reduction of the solvent to 1 mL, with the application of 20 µL to 2 mL of medium. Lower detection limits are possible by extracting larger samples and evaporating the solvent to a smaller volume.

A1.3 Time-Dependent Responses

A1.3.1 Investogators have shown that standard solutions and environmental samples, applied to the cells for both 6 and 16 h of exposure, produce responses (fold induction) which differ in relation to the relative concentrations and potencies of the PAHs and chlorinated hydrocarbons (coplanar PCBs, dioxins, furans) present in the solutions. PAHs applied to the cells are apparently being degraded by P450 enzymes over the 16-h time period, such that the maximal response is observed at 6 h of exposure. After 16 h, the magnitude of response to these PAHs has decreased to approximately 20 % of the 6-h response. On the other hand, chlorinated hydrocarbons only reach approximately 20 % of their potential for induction at 6 h, so that compounds as dioxin (TCDD) and PCB #126 (3, 3', 4, 4', 5-pentachlorobipiphenyl) produce about 5 times higher induction at 16 h, compared with the 6-h response. When the objectives of a project include differentiation between the contribution of PAHs and chlorinated hydrocarbons to the total observed induction, it is recommended that the two replicates of the sample be tested at 6 h and two additional replicates at 16 h of exposure. The illustration in Fig. 1 provides a means of interpreting the results of these exposures. The only change in the testing protocol is to apply samples to four wells, and terminate two wells after 6 h and two remaining wells after 16 h of exposure.



NOTE 1—General trends in RGS time course data: When only PAHs are present, fold induction will always decrease (5 times or more) from 6 h to 16 h. When only chlorinated compounds are present, fold induction will always increase from 6 h to 16 h. When both PAHs and chlorinated compounds are present, depending on the concentrations and potencies of the chlorinated compounds, fold induction from 6 h to 16 h will: (A) decrease less than 5 times (low concentrations); (B) not change (moderate concentrations), or (C) increase significantly (high concentrations; actual field sample).

FIG. A1.2 Time-Dependent Responses to PAHs and Chlorinated Compounds.

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