Designation: E 1853M – 04

METRIC

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 that include 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 Human Reporter Gene System (HRGS) has shown concentration-response relationships using 2,3,7,8-TCDD, 5 coplanar PCBs, and several polycyclic aromatic hydrocarbons (PAHs) (**1-3**).[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 (**4, 5**) and established as EPA Method 4425 (**6**). 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:* [3]

D 3976 Practice for Preparation of Sediment Samples for Chemical Analysis

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

E 1391 Guide for Collection, Storage, Characterization, and Manipulation of Sediments for Toxicological Testing and for Selection of Samplers Used to Collect Benthic Invertebrates

[1] This guide is under the jurisdiction of ASTM Committee E47 on Biological Effects and Environmental Fate and is the direct responsibility of Subcommittee E47.02 on Terrestrial Assessment and Toxicology.

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

[3] For referenced ASTM standards, visit the ASTM website, www.astm.org, or contact ASTM Customer Service at service@astm.org. For *Annual Book of ASTM Standards* volume information, refer to the standard's Document Summary page on the ASTM website.

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

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

3.2.8 *Solvent Mix*—a mixture of DMSO (dimethylsulfoxide): isopropanol: toluene (2:1:1) used as the solvent for blanks, standards, and samples for application to the cells in this assay.

3.2.9 *Standard D/F mixture*—a commercially available mixture of 17 dioxins and furans used at 5, 10, 25, and 40 pg/mL in each assay to produce a calibration curve for adjusting the responses of the cells before producing TEQs for the samples.

3.2.10 *TCDD*—2,3,7,8-tetrachlorodibenzo-*p*-dioxin ranked as the most potent of the 17 hazardous dioxins and furans.

3.2.11 *TEQ*—Toxic equivalency quotient representing the potency of mixtures and coplanar PCBs, dioxins, and furans, based on the concentrations of the 12 PCB congeners and 17 dioxins and furans (D/F) and World Health Organization ranked potencies (**7**).

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 sensitivity of the 101L cell line to a series of planar organic compounds is shown in Annex A1, (Tables A1.3 and A1.4).

5. Summary of Guide

5.1 Test systems have been developed and tested previously based on transgenic cell lines (**1-3, 8-11**). 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. Dried extracts of environmental samples (see Practice D 3976) are dissolved in a solvent mix and added to individual wells of 6-well culture plates, containing approximately one million cells for an exposure time of 16 h. Volumes of solvent that produce a low background (blank) induction when applied to the 2 mL of culture medium range from 2 to 20 µL, but normally 10 µL are applied. After exposure, the cells are rinsed, and then lysed. The cell lysate is transferred to microcentrifuge tubes to sediment the cellular debris. Aliquots of the supernatant solution containing the soluble luciferase are transferred to 96 well luminometer plates to measure luminescence in relative light units (RLUs) upon the addition of the luciferase substrate (luciferin). With each set of test samples, luminescence is also measured in lysates from cells exposed to a solvent mix control and reference inducers (D/F standard, TCDD, benzo[a]pyrene, etc.). The mean RLUs of the control wells is set equal to unity. Mean RLUs of samples and standards are converted to Fold Induction by dividing by the mean RLUs of the solvent mix (control). This biochemical response represents the integrated induction from all planar organic compounds present in the extract, which bind to the Ah-receptor in the same manner as dioxin (**6**). Final results may be expressed as (B[a]PEq) or as the TEQ (toxic equivalent quotient). Extracts of environmental samples applied without any cleanup procedures are expressed as B[a]PEq (in µg/g), representing the sum of all inducing planar compounds. If extracts are first cleaned of PAHs by such procedures as a silica gel column, then the results are expressed as a TEQ (in ng/g), which represents the sum of the products of 17 dioxins and furans times their toxic equivalency factors (TEFs)(**7**). Used in the final calculations are the initial dry weight of the extracted sample, the final volume of the solvent mix containing the sample (normally 0.25 to 1.0 mL), and the amount applied (10 µL) to the cells.

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, furans, 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 (**12, 13**). 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 this Human Reporter Gene System (HRGS) 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 costly 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 HRGS 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 (**14**). Since samples are extracted with DCM, there is little chance for metals to be passed on to the organic solvent and thus interfere with the response. Tests with extracts of sediment, highly contaminated with a range of toxic metals (cadmium, copper, lead, zinc, and so forth), have shown strong induction and thus no indication of interference from sediment metals (**15**). Some studies have indicated that high levels of tributyl-tin (TBT) may inhibit the induction of CYP1A (**16**).

8. Apparatus

8.1 *Instruments*:
8.1.1 *Microcentrifuge*.
8.1.2 *Luminometer*, as Dynatech ML1000 or ML2251.
8.1.3 *Laminar Flow Hood*.
8.1.4 *Incubator*, with CO_2 regulation.
8.2 *Reagents*:
8.2.1 *D/F standard mixture*.
8.2.2 *DMSO*.
8.2.3 *Isopropanol*.
8.2.4 *Toluene*.
8.2.5 *B(a)P*.
8.2.6 *TCDD*.
8.2.7 *Luciferase Assay Kit* (with Luciferin).
8.2.8 *Luciferase Standard*.
8.3 *Supplies*:
8.3.1 *Sterile Centrifuge Tubes*.
8.3.2 *Sterile 6-well Culture Plates*, with covers.
8.3.3 *Sterile Tissue Culture Flasks*, 250 mL, 75 cm^2 canted neck, sterile, polystyrene.
8.3.4 *Sterile Human Hepatoma Cell Culture Media*, as Dulbecco's modified Eagle medium, with 4 mM glutamine, 1 mM pyruvate, 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 10-40 g of tissue, sediment (see Guide E 1391), or soil and 1 L of water are appropriate. After extraction the DCM can be evaporated to dryness in a small vial. The sample can later be taken up in a small volume of the solvent mix. The vials without solvent may readily be shipped from an extraction laboratory to the testing laboratory, if these are not the same. Sample extracts not receiving any cleanup, as with silica gel, should be expressed as B(a)P equivalents. Those sample extracts receiving cleanup procedures to remove PAHs should be expressed as TEQs. If the test sediments are highly contaminated, it may be possible to test the pore water directly for CYP1A induction. Sediments should be centrifuged to produce about 0.5 mL of pore water, which may be applied after sterilizing filtration 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, reducing 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 1.0 % 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 the buffered lysis 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 the solvent mix 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 final concentrations in the culture wells that are appropriate are 5 to 100 pg/mL 2,3,7,8-TCDD, 5 to 40 pg TEQ/mL of a dioxin/furan standard mixture, and 100 to 300 ng/mL benzo(a)pyrene. 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 5 % of the extracts of environmental samples to test for the possible reduction in HRGS response to a toxicant caused by the matrix of the sample. A matrix effect

TABLE 1 Example of a Spreadsheet Used in P450 HRGS Assay

Setup of plates				5/3/2004			2:45 PM	
Sample addition				5/6/2004			5:00 PM	
Assay harvest				5/7/2004			9:00 AM	
Client name and Project Date								
	16h	Vol. (uL)	RLU1	RLU2	Mean	St.Dev.	CV%	FOLD
Solv. Cntrl.	Solv. Mix	0	0.053	0.043	0.048	0.007	14.6	1.0
Ref. Toxicant	TCDD	0.05 ng/mL	1.721	1.510	1.616	0.149	9.2	33.4
D/F Mixture	TEQ	5 ng/mL	0.33	0.27	0.3	0.042	14.1	6.2
D/F Mixture	TEQ	10 ng/mL	0.68	0.61	0.645	0.049	7.7	13.4
D/F Mixture	TEQ	25 ng/mL	1.795	1.74	1.7675	0.039	2.2	36.6
D/F Mixture	TEQ	40 ng/mL	3.13	2.82	2.975	0.219	7.4	61.6
Applied 10 µL of extracts								
Client ID	Lab ID		RLU1	RLU2	Mean	St.Dev.	CV%	FOLD	Dry Wt. (g)
SO-1	555-1		0.260	0.215	0.238	0.03	13.4	4.9	18.0
SO-2	2		0.153	0.137	0.145	0.01	7.8	3.0	18.4
SO-3	3		0.219	0.202	0.211	0.01	5.7	4.4	18.1
SO-4	4		0.348	0.411	0.380	0.04	11.7	7.9	17.3
SO-5	5		0.321	0.286	0.304	0.02	8.2	6.3	17.1
SO-6	6		2.733	2.722	2.728	0.01	0.3	56.8	17.2
SO-7	7		3.343	3.421	3.382	0.06	1.6	70.5	18.4
SO-8	8		0.603	0.629	0.616	0.02	3.0	12.8	17.1
SO-9	9		0.086	0.089	0.088	0.00	2.4	1.8	18.7
SO-10	10		0.334	0.321	0.328	0.01	2.8	6.8	18.3
SD-1	11		0.186	0.197	0.192	0.01	4.1	4.0	15.9
SO-1 Dup	555-1 Dup		0.298	0.295	0.297	0.00	0.7	6.2	18.4
Std. Ref. Material	SRM1944		3.170	3.761	3.466	0.42	12.1	72.2	5.0
Method Blank	0221 MB		0.099	0.095	0.097	0.00	2.9	2.0	20.0
Lab Control	0221 LCS		1.079	1.133	1.106	0.04	3.5	23.0	20.0

is unlikely, since the primary matrix is the solvent mix and HRGS response to this solution is covered by the control samples. A high level of PAH in the sample matrix may somewhat reduce the HRGS 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 and coefficient of variation) for each reference toxicant and test sample. In the Annex, Tables A1.1 and A1.2 illustrate the procedures used for EPA Method 4425 to convert the data in Table 1 to either B[a]P equivalents or TEQs, respectively.

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. When fold induction is over 100, the extract should be diluted and re-tested. When testing cleaned extracts for TEQ values, the standard curve of the day for TEQ concentrations of a standard D/F mixture should produce an r^2 correlation value of 0.95 or greater. The coefficient of variation for replicates of a sample should be no greater than 20 %. Replicate plates may be 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.

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 will also be reported on a basis of equivalents of the reference toxicant (TEQ or benzo[a]pyrene) per gram dry weight of sample. The spreadsheets in Annex Tables A1.1 and A1.2 provide an examples of conversion of the raw data in Table 1 to reported values in either B[a]P equivalents or TEQs.

12. Keywords

12.1 Ah-receptor; benzo[a]pyrene; biomarker; carcinogenic; coplanar organic compound; CYP1A; dioxin; induction; P450 1A; reporter gene system; TCDD; toxic

ANNEX

(Mandatory Information)

A1. THE P450 REPORTER GENE SYSTEM, USING 101L CELLS (EPA 4425)

A1.1 Procedure

A1.1.1 *Overview*—This Annex describes the use of a transgenic cell line (101L)[4] derived from the human hepatoma cell line, HepG2 (**1**) to produce reports on the levels of B[a]P equivalents or Toxic Equivalency Quotients (TEQs) by EPA Method 4425. Fig. A1.1 provides an overview of the protocol used in testing the P450 HRGS response to either organic extracts or water (including pore water) 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 Since development of the HRGS assay several publications have appeared in the literature, which regard the use of this test system. Two methods papers were published (**4, 5**) earlier and the EPA Method was more recent (**6**). The results of many sediment testing programs (**17-22**) and a bivalve deployment investigation (**23**) have been published.

A1.1.2 *Culture Maintenance*—The maintenance of a vigorous cell line requires splitting the culture twice a week. The cells are removed from 75 cm^2 culture flasks by a brief exposure to trypsin. After centrifugation and replacement of medium, cells are re-seeded at 1.5×10^6 cells per flask in a total of 12 mL culture medium. Cultures 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 continue selection for cells that retain a stably integrated plasmid.

A1.1.3 *Calibration*—The Standard curve with dioxins/furans run with each assay will serve to demonstrate both the calibration of the cellular response and the performance of the luminometer, since an r^2 value of less than 0.95 is unacceptable. If this linearity and sensitivity (5 pgTEQ /mL) is not observed then there may be a need for luminometer calibration.

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 to approximately 1.0×10^6 cells/well and to ensure adhesion to the plastic well.

A1.1.4.3 Apply 10 µL of a range of concentrations of the D/F standard at 1-8 ng/mL to 2 mL in duplicate wells. This produces a final concentration range of 5 to 40 pg/mL in the culture wells.

A1.1.4.4 Apply 10 µL of 1.0 ng/µL solution of 2,3,7,8-TCDD to duplicate wells containing 2 mL of medium, producing a 50 pg/mL exposure in the wells.

A1.1.4.5 Apply 10 µL of the test samples in the solvent mix, or up 300 µL of water with microsyrinnge or positive displacement micropipettor to 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.

[4] The sole source of supply of this cell line known to the committee at this time is Battelle Pacific Northwest National Laboratory, Marine Sciences Laboratory, 1529 W. Sequim Bay Road, Sequim, WA 98382. 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.

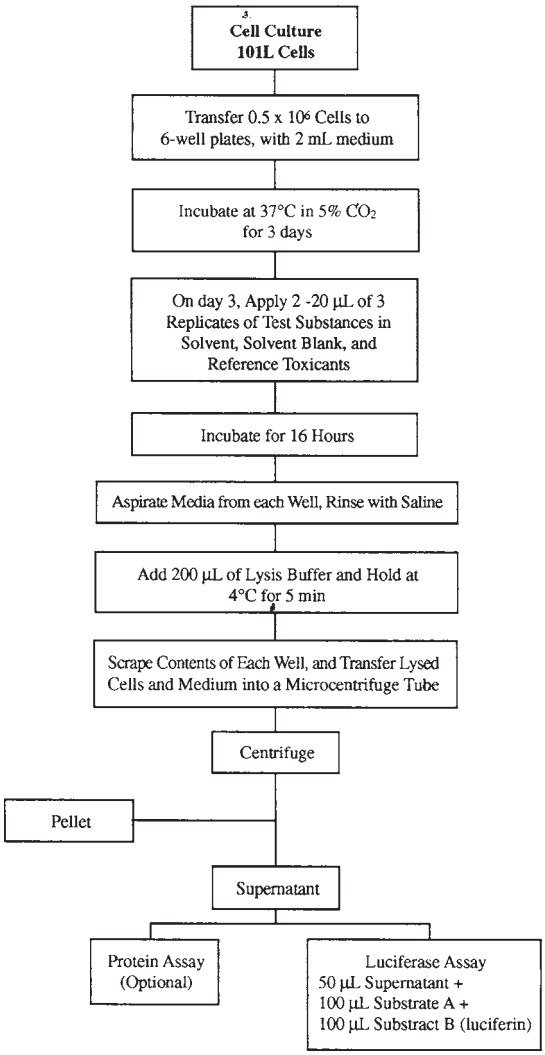

FIG. A1.1 Flow-chart for the P450 Reporter Gene System Assay

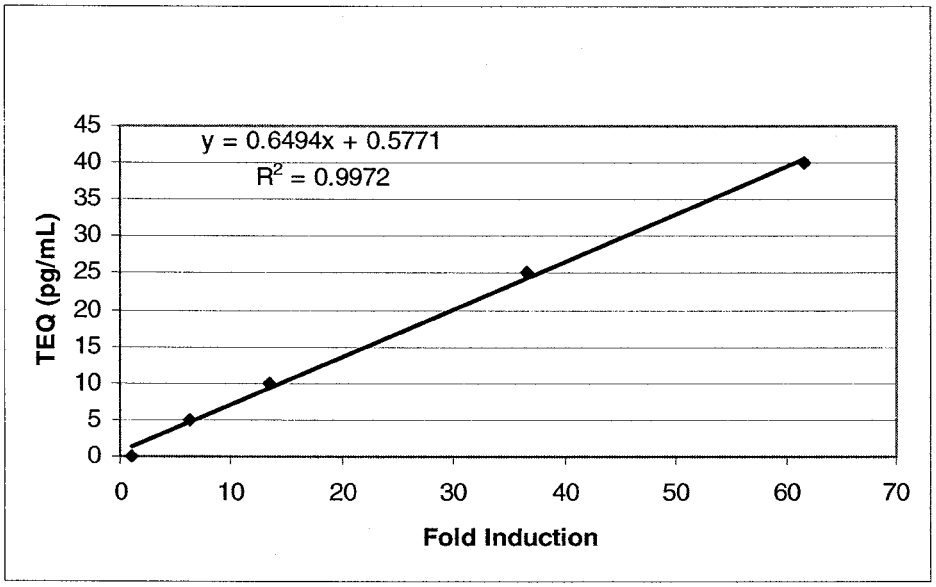

FIG. A1.2 Standard D/F Curve of the Day

TABLE A1.1 Calculation of Method 4425 B[a]P Equivalents in Solid Samples (Extracts Not Cleaned by Silica Gel)

Date		Fold Induction	Well Vol (mL)	Injection Vol (mL)	Dilution Factor	Extract Vol (mL)	Grams Extracted	Calculated µg/g B[a]PEq
Client ID	Sample ID							
SO-1	J2000555-1	4.9	2	0.01	1	1	18.0	0.92
SO-2	J2000555-2	3.0	2	0.01	1	1	18.4	0.55
SO-3	J2000555-3	4.4	2	0.01	1	1	18.1	0.81
SO-4	J2000555-4	7.9	2	0.01	1	1	17.3	1.52
SO-5	J2000555-5	6.3	2	0.01	1	1	17.1	1.23
SO-6	J2000555-6	56.8	2	0.01	5	1	17.2	55.05
SO-7	J2000555-7	70.5	2	0.01	5	1	18.4	63.90
SO-8	J2000555-8	12.8	2	0.01	1	1	17.1	2.50
SO-9	J2000555-9	1.8	2	0.01	5	1	18.7	1.62
SO-10	J2000555-10	6.8	2	0.01	1	1	18.3	1.24
SD-1	J2000555-11	4.0	2	0.01	1	1	15.9	0.84
SO-1-Dup	555-1 Dup	6.2	2	0.01	1	1	18.4	1.12
Std. Ref.	SRM 1944	72.2	2	0.01	1	1	5.0	48.13
Method Blank	0221 MB	2.0	2	0.01	1	1	20.0	0.34
Lab Control	0221 LCS	23.0	2	0.01	1	1	20.0	3.84

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 6000 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 a microwell of the 96-well plate, designed for the specific luminometer.

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. Auto injectors are often used to add either one or both of the substrates.

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, an aliquot of the supernatant of each sample may be analyzed for protein (**24**).

A1.2 Data Interpretation and Reporting

A1.2.1 Since this test detects both PAHs and chlorinated hydrocarbons (coplanar PCBs, dioxins, furans) it is appropriate to express the data based upon the sample preparation and cleanup techniques. When the sample extracts have been treated with silica gel to remove PAHs, it is typically reported as "Dioxin and Dioxin-Like Organic Compounds (TEQ)." When the sample extracts have not been cleaned-up with silica gel to separate organic compounds, it is typically reported as

TABLE A1.2 Calculation of Method 4425 TEQs in Solid Samples (Extracts Cleaned of PAHs by Silica Gel)

Date		Fold Induction	TEQ pg/mL	Well Vol (mL)	Injection Vol (mL)	Dilution Factor	Extract Vol (mL)	Grams Extracted	Calculated ng/g TEQ	Reporting Limit ng/g TEQ	Detection Limit ng/g TEQ	Report ng/g TEQ
Client ID	Sample ID											
SO-1	J2000555-1	4.9	3.8	2	0.01	1	1	18.0	0.042	0.042	0.017	0.042
SO-2	J2000555-2	3.0	2.5	2	0.01	1	1	18.4	0.028	0.042	0.017	0.028
SO-3	J2000555-3	4.4	3.4	2	0.01	1	1	18.1	0.038	0.042	0.017	0.038
SO-4	J2000555-4	7.9	5.7	2	0.01	1	1	17.3	0.066	0.044	0.018	0.066
SO-5	J2000555-5	6.3	4.7	2	0.01	1	1	17.1	0.055	0.045	0.018	0.055
SO-6	J2000555-6	56.8	37.5	2	0.01	5	1	17.2	2.178	0.222	0.089	2.178
SO-7	J2000555-7	70.5	46.3	2	0.01	5	1	18.4	2.521	0.208	0.083	2.521
SO-8	J2000555-8	12.8	8.9	2	0.01	1	1	17.1	0.104	0.045	0.018	0.104
SO-9	J2000555-9	1.8	1.8	2	0.01	5	1	18.7	0.094	0.204	0.082	0.094
SO-10	J2000555-10	6.8	5.0	2	0.01	1	1	18.3	0.055	0.042	0.017	0.055
SD-1	J2000555-11	4.0	3.2	2	0.01	1	1	15.9	0.040	0.048	0.019	0.040
1-Dup	555-1 Dup	6.2	4.6	2	0.01	1	1	18.4	0.050	0.042	0.017	0.050
Std. Ref.	SRM 1944	72.2	47.5	2	0.01	1	1	5.0	1.898	0.153	0.061	1.898
Method Blank	0221 MB	2.0	1.9	2	0.01	1	1	20.0	0.019	0.038	0.015	0.019
Lab Control	0221 LCS	23.0	15.5	2	0.01	1	1	20.0	0.155	0.038	0.015	0.155

Formula Used: ng/g TEQ = {(pg/mL TEQ)(Well Vol mL)(Dilution Factor)(Extract Vol mL)}/{(Inj Vol mL)(Grams)(1000 pg/ng)} (for example, Fold · 0.6494 + 0.5771)
Assume that 5 Fold Induction = Reporting Limit (that is, 3.82 pg/mL TEQ)
pg/mL TEQ = (0.6494)(Fold Induction) + 0.5771 r2 = 0.9972

TABLE A1.3 Relative Responses of the P450 HRGS Assay to Specific PAH Concentrations that 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	250	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 A1.4 Relative Response of the P450 HRGS to Chlorinated Hydrocarbon Concentrations that 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	62.5	2.5
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

"Planar Organic Compounds" (PAHs, PCBs, PCDDs/PCDFs) as B[a]P Equivalents. The formula below shows the simple calculation used in the spreadsheet of Table A1.1 to calculate B[a]PEq values from the raw data listed in Table 1. It should be recognized that the factor of 60 is used, since a concentration of 1.0 µg/mL B[a]P produces a fold induction of 60.

$$\text{HRGS B[a]P Eq (mg/kg)} = \text{Fold Induction} \times \frac{\text{(Dilution Factor)(Ext Vol in mL)}}{\text{60 (0.01 mL Inj. Vol) (Dry Grams Ext)}} \quad \text{(A1.1)}$$

A1.2.2 For extracts cleaned of PAHs by silica gel or other methods, the results from Table 1 can be calculated to produce TEQ values. The first step in this process is to evaluate the dioxin/furan calibration curve produced on the same day, as shown in Fig. A1.2. The correlation factor is acceptable, since it is above the required 0.95. The equation from the curve is used in the third column in the spreadsheet shown in Table A1.2 (pg/mL TEQ) to adjust the fold induction to the responses of the cells on that specific day. After that adjustment the remaining calculations are shown in the formula below.

$$\text{TEQ pg/mL} = (\text{Fold Induction x } 0.6494) + 0.5771 \quad \text{(A1.2)}$$

$$\text{TEQ mg/Kg} = \frac{\text{(pg/mL TEQ)(2 mL)(DF)(Ext Vol in mL)}}{\text{(0.01 mL Inj. Vol)(Grams Ext)(1000 pg/ng)}}$$

where:

pg/mL TEQ	=	pg/mL TEQ inducers calculated from the calibration curve,
2 mL	=	the induction well volume,
DF	=	dilution factor of the extract,
Ext Vol in mL	=	final volume of the same extract,
0.01 mL Inj. Vol.	=	10 µL of sample extract added to the induction wells,

Grams Ext.	=	grams of soil extracted (could be dry or as received weight), and
1000 pg/ng	=	conversion factor to convert pg to ng.

A1.3 Sensitivity

A1.3.1 Tables A1.3 and A1.4 list the concentrations of specific organic compounds that were found to produce a ten-fold induction of CYP1A, using the P450 HRGS assay. A ten-fold induction is a very strong response to these planar compounds, and a reporting level of induction is five-fold. The range of CYP1A1 induction has been from two to three fold for very low levels of inducing compounds, or from weak inducers to greater than 900-fold induction from extracts of highly contaminated marine sediments. However, induction responses greater than 100 may indicate saturation of the test system, so samples should be diluted and re-tested. Concentrations are listed in terms of nanograms per milliliter 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 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 by using a smaller final volume of the solvent mixture. It is recognized that there may be some competition for the Ah receptor by the mixtures of contaminants in environmental samples, however data generated over several years have indicated the responses are generally additive **(17, 20)**.

REFERENCES

(**1**) Anderson, J. W., Rossi, S. S., Tukey, R. H., Vu, T. P., and Quattrochi, L. C., "A Biomarker, P450 RGS, for Assessing the Induction Potential of Environmental Samples," *Environ. Toxicol. Chem.*, Vol 14(7), 1995, pp. 1159-1169.

(**2**) Postlind, H., Vu, T. P., Tukey, R. H., and Quattrochi, L. C., "Response of Human CYP1-Luciferase Plasmids to 2,3,7,8-tetrachlorodibenzo-p-dioxin and Polycyclic Aromatic Hydrocarbons," *Toxicol. Appl. Pharmacol.*, Vol 118, 1993, pp. 255–262.

(**3**) Jones, J. M., and Anderson, J. W., "Relative Potencies of PAHs and PCBs Based on the Response of Human Cells," *Environ. Toxicol. Pharmacol*., Vol 7, 1998, pp. 19-26.

(**4**) "P450 Reporter Gene Response to Dioxin-like Organics," *Method 8070, Standard Methods for the Examination of Water and Wastewater*, 20th Ed., Supplement, American Public Health Association, Washington, DC, 1998, pp. 8-36-37.

(**5**) Anderson, J. W., Bothner, K., Vu, T., and Tukey, R. H., "Using a Biomarker (P450 RGS) Test Method on Environmental Samples," *Techniques in Aquatic Toxicology*, G. K. Ostrander, Ed., Lewis Publishers, Boca Raton, FL, 1996, pp. 277-286.

(**6**) EPA Method 4425: Screening Extracts of Environmental Samples for Planar Organic Compounds (PAHs, PCBs, PCDDs/PCDFs) by a Reporter Gene on a Human Cell Line, EPA Office of Solid Waste, SW 846 Methods, Update IVB, 2000.

(**7**) Van den Berg, M., Birnbaum, L., Bosveld, A. T., Brunstrom, B., Cook, P., Feeley, M., Giesy, J. P., Hanberg, A., Hasegawa, R., Kennedy, S. W., Kubiak, T., Larsen, J. C., van Leeuwen, F. X., Liem, A. K., Nolt, C., Peterson, R. E., Poellinger, L., Safe, S., Schrenk, D., Tillitt, D., Tysklind, M., Younes, M., Waern, F., and Zacharewski, T., "Toxic Equivalency Factors (TEFs) for PCBs, PCDDs, PCDFs for Humans and Wildlife," *Environ Health Perspect*, 106, 1998, pp. 775-779.

(**8**) Houtman, C. J., Cenijn, P. H., Hamers, T., Lamoree, M. H., Legler, J., Murk, A. J., and Brouwer, A., "Toxicological Profiling of Sediments Using in vitro Bioassays with Emphasis on Endocrine Disruption," *Environ. Toxicol. Chem*. 23, 2004, pp. 32-40.

(**9**) Aarts, J. M. M. J. G., Denison, M. S., Cox, M. A., Schalk, J. A. C., Garrison, P. M., Tullis, K., De Haan, L. H. J., and Brouwer, A., "Species-specific Antagonism of Ah Receptor Action by 2,2′,5,5′-tetrachloro- and 2,2′,3,3′4,4′-hexachlorobiphenyl," *European J. Phamacol. Toxicol. Pharmacol. Section*, Vol 293, 1995, pp. 463-474.

(**10**) Denison, M. S., El-Fouly, M. H., Aarts, J. M. M. J. G., Brouwer, A., Richter, C., and Giesy, J. P., "Production of Novel Recombinant Cell Line Bioassay Systems for Detection of 2,3,7,8-Tetrachloridibenzo-p-dioxin-like Chemicals," Short Paper, Dioxin '93, 13th International Symposium on Chlorinated Dioxins and Related Compounds, Vienna, 1993.

(**11**) Garrison, P. M., Tullis, K., Aarts, J. M. M. J. G., Brouwer, A., Giesy, J. P., and Denison, M. S., "Species-specific Recombinant Cell Lines as Bioassay Systems for the Detection of 2,3,7,8-Tetrachlorodibenzo-p-dioxin-like Chemicals," *Fundam. Appl. Toxicol.*, Vol 30, 1996, pp. 194-203.

(**12**) Hahn, M. E., Poland, A., Glover, E., and Stegeman, J. J., "The Ah Receptor in Marine Animals: Phylogenetic Distribution and Relationship to Cytochrome P450 1A Inducibility," *Mar. Environ. Res.*, Vol 34, 1992, pp. 87-92.

(**13**) Stegeman, J., Brouwer, M., Di Giulio, R. T., Forlin, L., Fowler, B. A., Sanders, B. M., and Van Veld, P. A., "Molecular Responses to Environmental Contamination: Enzyme and Proteins Systems as Indicators of Chemical Exposure and Effect," *Biomarkers: Biochemical, Physiological, and Histological Markers of Anthropogenic Stress*, R. J. Huggett, R. A. Kimerle, P. M. Mehrle, Jr., and H. L. Bergman, Eds., Lewis Publishers, Boca Raton, FL, 1992, p. 235.

(**14**) Safe, S., "Polychlorinated Biphenyls (PCBs): Environmental Impact, Biochemical and Toxic Responses, and Implications for Risk Assessment," *Crit. Rev. Toxicol.*, Vol 24, 1994, pp. 87–149.

(**15**) Anderson, J. W., Zeng, E., and Jones, J. M., "Correlation Between the Response of a Human Cell Line (P450RGS) and the Distribution of Sediment PAHs and PCBs on the Palos Verdes Shelf, California," *Environ. Toxicol. Chem*., 18, 1999a, pp. 1506-1510.

(**16**) Fent, K., and Stegeman, J. J., "Effects of Tributyltin in vivo on Hepatic Cytochrome P450 Forms in Marine Fish," *Aquatic Toxicology*, Vol 24, 1993, p. 219.

(**17**) Anderson, J. W., Newton, F. C., Hardin, J., Tukey, R. H., and Richter, K. E., "Chemistry and Toxicity of Sediments from San Diego Bay, Including a Biomarker (P450 RGS) Response," *Environmental Toxicology and Risk Assessment: Biomarkers and Risk Assessment*, Vol 5, ASTM STP 1306, D. A. Bengtson, and D. S. Henshel, Eds., American Society for Testing and Materials, West Conshohocken, PA, 1996, pp. 53-78.

(**18**) Kim, G. B., Anderson, J. W., Bothner, K., Lee, J. H., Koh, C. H., and Tanabe, S., "Application of P450 RGS (Reporter Gene System) as a Bioindicator of Sediment PAH Contamination in the Vicinity of Incheon Harbor, Korea," *Biomarkers*, Vol 2, 1997, pp. 181-188.

(**19**) Anderson, J. W., Bothner, K., Edelman, D., Vincent S., Vu T., and Tukey, R. H., "A Biomarker, P450 RGS, for Assessing the Potential Risk of Environmental Samples," *Field Applications of Biomarkers for Agrochemicals and Toxic Substances*, J. Blancato, R. Brown, C. Dary, and M. Saleh, Eds., American Chemical Society, Washington, DC, 1996, pp. 150-168, Chp. 12.

(**20**) Anderson, J. W., Jones, J. M., Hameedi, J., Long, E., and Tukey, R. H., "Comparative Analysis of Sediment Extracts from NOAA's

Bioeffects Studies by the Biomarker, P450 RGS," *Mar. Environ. Res*., 48, 1999, pp. 389-405.

(**21**) McCoy, D. L., Jones, J. M., Anderson, J. W., Harmon, M., Hartwell, I., and Hameedi, M. J., "Distribution of Cytochrome P4501A1-inducing Chemicals in Sediments of the Delaware River-Bay System, USA,"*Environ. Toxicol. Chem*., 21, 2002, pp. 1618-1627.

(**22**) Anderson, J. W., Jones, J. M., McCoy, D. L., Fujita, A., Yamamoto, T., and Iijima, S., "Comparison Between a Rapid Biological Screening Method (EPA 4425) for TCDDs/TCDFs and Chemical Analytical Methods," *Organohalogen Compounds*, 60, 2003, pp. 271-274.

(**23**) Anderson, J. W., Jones, J. M., Steinert, S., Sanders, B., Means, J., McMillin, D., Vu, T., and Tukey, R., "Correlation of CYP1A1 Induction, as Measured by the P450 RGS Biomarker Assay, with High Molecular Weight PAHs in Mussels Deployed at Various Sites in San Diego Bay in 1993 and 1995," *Mar. Environ. Res*., 48, 1999, pp. 407-425.

(**24**) Bradford, M. M., "A Rapid and Sensitive Method for the Quantitation of Microgram Quantities of Protein Utilizing the Principle of Protein-dye Binding," *Anal. Biochem.*, Vol 72, 1976, pp. 248-254.