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Standard Guide for Measuring the Presence of Planar Organic Compounds Which Induce CYP1A, Using Reporter Gene Test Systems (Metric)¹

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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*

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

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:

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2. Referenced Documents

2.1 ASTM Standards:³

D3976 Practice for Preparation of Sediment Samples for Chemical Analysis

E1023 Guide for Assessing the Hazard of a Material to Aquatic Organisms and Their Uses

E1391 Guide for Collection, Storage, Characterization, and Manipulation of Sediments for Toxicological Testing and for Selection of Samplers Used to Collect Benthic Invertebrates

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—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 D3976](#)) 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 μ L, but normally 10 μ L 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 μ g/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 μ L) 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 [2018-01-01](#)

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 [E1023](#)). 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 CO₂ 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 cm² 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 [E1391](#)), 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 the