



Standard Guide for Measuring the Presence of Planar Organic Compounds Which Induce CYP1A, Using Reporter Gene Test Systems¹

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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, 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:

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2. Referenced Documents

2.1 ASTM Standards:

- D 3976 Practice for Preparation of Sediment Samples for Chemical Analysis³
- E 1023 Guide for Assessing the Hazard of a Material to Aquatic Organisms and Their Uses⁴
- E 1391 Guide for Collection, Storage, Characterization, and Manipulation of Sediments for Toxicological Testing⁴

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

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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.

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-Methylcholanthrene*—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 culture plates, containing approximately one million cells, and the

exposure time is 16 h. Volumes of solvent successfully tested are 2 to 20 μ L, 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 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 μ L) 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