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Standard Guide for Conducting the Frog Embryo Teratogenesis Assay-Xenopus (FETAX)[1]

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1. Scope

1.1 This guide covers procedures for obtaining laboratory data concerning the developmental toxicity of a test material. The test utilizes embryos of the African clawed frog, *Xenopus laevis* and is called FETAX (Frog Embryo Teratogenesis Assay-*Xenopus*) (**1**).[2] Some of these procedures will be useful for conducting developmental toxicity tests with other species of frogs although numerous modifications might be necessary. A list of alternative anurans is presented in Appendix X1.

1.2 A renewal exposure regimen and the collection of the required mortality, malformation, and growth-inhibition data are described. Special needs or circumstances might require different types of exposure and data concerning other effects. Some of these modifications are listed in Appendix X2 although other modifications might also be necessary. Whenever these procedures are altered or other species used, the results of tests might not be comparable between modified and unmodified procedures. Any test that is conducted using modified procedures should be reported as having deviated from the guide.

1.3 These procedures are applicable to all chemicals either individually or in formulations, commercial products or mixtures that can be measured accurately at the necessary concentrations in water. With appropriate modification these procedures can be used to conduct tests on the effects of temperature, dissolved oxygen, pH, physical agents, and on materials such as aqueous effluents (see Guide E1192), surface and ground waters, leachates, aqueous and solid phase extracts, and solid phase samples, such as soils and sediments, particulate matter, sediment, and whole bulk soils and sediment.

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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[1] This guide is under the jurisdiction of ASTM Committee E50 on Environmental Assessment, Risk Management and Corrective Action and is the direct responsibility of Subcommittee E50.47 on Biological Effects and Environmental Fate. A standard guide is a document, developed using the consensus mechanisms of ASTM, that provides guidance for the selection of procedures to accomplish a specific test but which does not stipulate specific procedures.

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

2. Referenced Documents

2.1 *ASTM Standards:*[3]

D1193 Specification for Reagent Water

E729 Guide for Conducting Acute Toxicity Tests on Test Materials with Fishes, Macroinvertebrates, and Amphibians

E943 Terminology Relating to Biological Effects and Environmental Fate

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

E1192 Guide for Conducting Acute Toxicity Tests on Aqueous Ambient Samples and Effluents with Fishes, Macroinvertebrates, and Amphibians

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

E1525 Guide for Designing Biological Tests with Sediments

E1706 Test Method for Measuring the Toxicity of Sediment-Associated Contaminants with Freshwater Invertebrates

IEEE/ASTM SI 10 American National Standard for Use of the International System of Units (SI): The Modern Metric System

3. Terminology

3.1 *Definitions of Terms Specific to This Standard:*

3.1.1 The words "must," "should," "may,"" can," and "might," have very specific meanings in this guide. "Must" is used to express an absolute requirement, that is, to state that the test ought to be designed to satisfy the specified condition, unless the purpose of the test requires a different design. "Must" is only used in connection with factors that directly relate to the acceptability of the test (see Section 14). "Should" is used to state that the specified condition is recommended and ought to be met if possible. Although violation of one "should" is rarely a serious matter, 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."

3.1.2 A developmental toxicant is a test material that affects any developmental process. Therefore, a developmental toxicant affects embryo mortality and malformation, and causes growth inhibition. A teratogen is a test material that causes abnormal morphogenesis (malformation). The Teratogenic Index or TI is a measure of potential developmental hazard (**1**). TI values higher than 1.5 signify larger separation of the mortality and malformation concentration ranges and, therefore, a greater potential for all embryos to be malformed in the absence of significant embryo mortality. The TI is defined as the ratio of the 96-h LC50 and the 96-h EC50 (malformation).

3.1.3 For definitions of other terms used in this guide, refer to Guides E729 and E1023, also Terminology E943. For an explanation of units and symbols, refer to IEEE/ASTM SI 10.

4. Summary of Guide

4.1 In FETAX, range-finding and definitive tests are performed on each test material. A control in which no test material has been added is used to provide 1) a measure of the acceptability of the test by indicating the quality of embryos and the suitability of the FETAX solution, test conditions and handling procedures, and 2) a basis for interpreting data from other treatments. Each test consists of several different concentrations of test material with at least two replicates of each concentration. Each of the three tests is conducted using embryos from a different male/female pair of *Xenopus laevis.* A reference toxicant (6-aminonicotinamide) should be used as a quality control measure. The 96-h LC50 and 96-h EC50 (malformation) are determined by an appropriate statistical analysis and the TI (Teratogenic Index) is calculated by dividing the 96-h LC50 by the 96-h EC50. Growth inhibition is determined by measuring the head-tail length of each embryo and determining whether growth at a particular concentration is significantly different from that of the control. Other useful data can be collected (for example, pigmentation, locomotion, and hatchability) to expand the utility of the test.

5. Significance and Use

5.1 FETAX is a rapid test for identifying potential developmental toxicity. Data may be extrapolated to other species including mammals. FETAX might be used to prioritize samples for further tests which use mammals. Validation studies using compounds with known mammalian or human developmental toxicity, or both, suggest that the predictive accuracy will exceed 85 % (**2**). When evaluating a test material for mammalian developmental toxicity, FETAX must be used with and without a metabolic activation system (MAS). Use of this exogenous MAS should increase the predictive accuracy of the assay to approximately 95 %. The accuracy rate compares favorably with other currently available " *in vitro* teratogenesis screening assays" (**3**). Any assay employing cells, parts of embryos, or whole embryos other than *in vivo* mammalian embryos is considered to be an *in vitro* assay.

5.2 It is important to measure developmental toxicity because embryo mortality, malformation, and growth inhibition can often occur at concentrations far less than those required to affect adult organisms.

5.3 Because of the sensitivity of embryonic and early life stages, FETAX provides information that might be useful in estimating the chronic toxicity of a test material to aquatic organisms.

5.4 Results from FETAX might be useful when deriving water quality criteria for aquatic organisms (**4**).

5.5 FETAX results might be useful for studying structure-activity relationships between test materials and for studying bioavailability.

[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.

6. Safety Precautions

6.1 Many materials can affect humans adversely if precautions are inadequate. Therefore, skin contact with all test materials and solutions of them should be minimized by such means as wearing appropriate protective gloves (especially when washing equipment or putting hands in test solutions), laboratory coats, aprons, and safety glasses, and using pipets to remove organisms from test solutions. Special precautions, such as covering test chambers and ventilating the area surrounding the chambers and the use of fume hoods, should be taken when conducting tests on volatile materials. Information provided in Material Safety Data Sheets on toxicity to humans (**5**), recommended handling procedures (**6**), and chemical and physical properties of the test material should be studied before a test is begun. Special procedures might be necessary with radiolabeled test materials (**7**) and with test materials that are, or are suspected of being, carcinogenic (**8**).

6.2 Although disposal of stock solutions, test solutions, and test organisms poses no special problems in most cases, health and safety precautions and applicable regulations should be considered before beginning a test. Removal or degradation of test material might be desirable before disposal of stock and test solutions.

6.3 Cleaning of equipment with a volatile solvent such as acetone should be performed only in a fume hood.

6.4 To prepare dilute acid solutions, concentrated acid should be added to water, not vice versa. Opening a bottle of concentrated acid and adding concentrated acid to water should be performed only in a fume hood.

6.5 Because FETAX solution and test solutions are usually good conductors of electricity, use of ground fault systems and leak detectors should be considered to help avoid electrical shocks.

7. Apparatus

7.1 *Facilities for Maintaining and Breeding Xenopus*—Adults should be kept in an animal room that is isolated from extraneous light which might interfere with a consistent photoperiod of 12-h day/12-h night. The role that circadian rhythm plays in *Xenopus* reproduction has not been investigated. A consistent photoperiod is therefore recommended so that *Xenopus* can be bred year-round. Adults can be kept in large aquaria or in fiberglass or stainless steel raceways at densities of 4 to 6 per 1800 cm^2 of water surface area. The sides of tanks should be opaque and at least 30 cm high. The water depth should be between 7 and 14 cm. Water temperature for adults should be 21 ± 3°C.

7.1.1 Two types of breeding aquaria have been used successfully. A 5 or 10-gal aquarium may be used if fitted with a 1-cm mesh suspended about 3-cm from the bottom of the aquarium so that deposited eggs will lie undisturbed on the bottom of the aquarium. Hardware cloth or other metal mesh must not be used. Nylon or plastic mesh is recommended. The sides of the breeding aquarium should be opaque and an optional bubbler may be fitted to oxygenate the water. The top of the aquarium should be covered with an opaque porous material such as a fiberglass furnace filter. Alternatively, an adequate breeding tank can be constructed from two plastic dish pans (at least 38 by 38 cm) stacked one in the other. The floor of the topmost pan is perforated. A cork borer can be used to create 1.5-cm holes for the eggs to fall through.

7.2 *Facilities for Conducting FETAX*—A constant temperature room or a suitable incubator for embryos is required although a photoperiod is unnecessary. The incubator must be capable of holding 23 ± 1°C. Abnormal development will occur at temperatures greater than 26°C. Covered 60-mm glass Petri dishes should be used as test chambers except that disposable 55-mm polystyrene Petri dishes should be used if a substantial amount of the test material binds to glass but not to polystyrene. A binocular dissection microscope capable of magnifications up to 30× is required to count and evaluate abnormal embryos. A digital camera with adequate zoom is used to enlarge embryo images two to three times for head-tail length measurements. It is also possible to measure embryo length through the use of a map measurer or an ocular micrometer. However, the process is greatly facilitated by using a digitizer interfaced to a microcomputer.

7.3 *Construction Materials*—Equipment and facilities that contact stock solutions, test solutions, or water in which embryos will be placed should not contain substances that can be leached or dissolved by aqueous solutions in amounts that would adversely affect embryo growth or development. Additionally, items that contact stock solutions or test solutions should be chosen to minimize sorption of most test materials from water. Glass, Type 316 stainless steel, nylon, and fluorocarbon plastic should be used whenever possible to minimize dissolution, leaching, and sorption. Rigid plastics may be used for holding, acclimation, and in the water supply system, but they should be soaked for a week before use in water used for adult maintenance.

7.3.1 FETAX solution, stock solutions, or test solutions should not contact brass, copper, lead, galvanized metal, or natural rubber before or during the test. Items made of neoprene rubber or other materials not mentioned above should not be used unless it has been shown that their use will not adversely affect either survival or growth of the embryos and larvae of the test species.

7.4 *Cleaning*—At the end of each test, all glass dishes and other glassware that are to be used again should be immediately emptied, rinsed with water, and cleaned by the following procedure.

7.4.1 *Glassware Washing Procedure:*

7.4.1.1 Soak 15 min, and scrub with tissue culture compatible detergent in tap water.

7.4.1.2 Rinse twice with tap water.

7.4.1.3 Rinse once with fresh, dilute (10 %, v/v) hydrochloric acid to remove scale, metals, and bases.

7.4.1.4 Rinse twice with water conforming to Type II ASTM water (Specification D1193).