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Designation: ~~E1439 – 98 (Reapproved 2004)~~ E1439 – 12

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

This standard is issued under the fixed designation E1439; 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 procedures for obtaining laboratory data concerning the developmental toxicity of a test material. The test utilizes embryos of the ~~South~~ 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 ~~extracts of water-insoluble materials,~~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 E47 on Biological Effects and Environmental Fateand is the direct responsibility of Subcommittee E47.01 on Aquatic Assessment and Toxicology. 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.

Current edition approved ~~April 1, 2004~~Dec. 1, 2012 Published ~~April 2004~~January 2013. Originally approved in 1991. Last previous edition approved in ~~1998~~2004 as ~~E1439 – 98.~~E1439 – 98 (2004). DOI: ~~10.1520/E1439-98R04.~~10.1520/E1439-12.

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

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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 ~~divided by~~ 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.

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4. Summary of Guide

4.1 In FETAX, range-finding and ~~three replicate~~ 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 ~~two replicate dishes~~ 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 ~~probit~~ 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 ~~toxicants.~~ 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.

[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.

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.

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.

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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 ~~23~~21 ± 3°C.

7.1.1 Two types of breeding aquaria have been used successfully. ~~A5~~A 5 or 10-gal ~~glass~~ 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 ~~24~~23 ± ~~2°C.~~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 ~~simple darkroom enlarger~~ 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. ~~The microcomputer is also used in data analysis.~~

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) hydro-chloric acid to remove scale, metals, and bases.

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

7.4.1.5 Rinse once with full strength reagent-grade[4] acetone to remove organic compounds.

7.4.1.6 Rinse well with hot ASTM Type II water.

7.4.1.7 Rinse well with ASTM Type I water or FETAX solution.

7.4.1.8 Heat the glassware in an oven at 150° C for 3 h to drive off any residual acetone. Toxicity problems have occurred in experiments when this glassware washing procedure was omitted.

7.5 *Acceptability*—Before FETAX is conducted in new test facilities it is desirable to conduct a "non-toxicant" test, in which all test chambers contain FETAX solution with no added test material. The embryos should grow, develop, and survive in numbers consistent with an acceptable test (see 14.1). The magnitude of the chamber-to-chamber variation should be evaluated.

8. Water for Culturing *Xenopus* Adults

8.1 *Requirements*—Besides being available in adequate supply, the water should allow satisfactory survival and reproduction of the adults, be of uniform quality, and not necessarily affect results of the test.

8.2 *Source:*

8.2.1 Natural water is preferred for adult culture. It should be obtained from an uncontaminated source that provides uniform quality. The quality of water from a well or spring is usually more uniform than that of a surface water. If a surface water is used as a source of fresh water, the intake should be positioned to minimize fluctuations in quality and the possibility of contamination and to maximize the concentration of dissolved oxygen to help ensure low concentrations of sulfide and iron. FETAX solution is acceptable for adult culture. The cost and formulation time make it suitable only for small colonies. Water temperature should be adjusted to ~~23~~21 ± 3°C before being used to culture adults.

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8.2.2 Dechlorinated water can be used to culture adults as long as residual chlorine and its oxidants are reduced to levels that do not affect survival and reproduction. ~~Dechlorinated water should only be used as a last resort because dechlorination is often incomplete.~~ Sodium bisulfite is probably better for dechlorinating water than sodium sulfite and both are more reliable than carbon filters, especially for removing chloramines (**9**). Fluorides can be removed by passage over activated alumina columns (**10**). In addition to residual chlorine, chloramines, and fluoride, municipal drinking water often contains unacceptably high concentrations of copper, lead, and zinc, and quality is often rather variable. Excessive concentrations of most metals can usually be removed with a chelating resin (**11**).

8.3 *Treatment:*

8.3.1 A continuous flow system for culturing adults is recommended although a static system has proven successful. Water for culturing adults should be aerated by the use of air stones or surface aerators. Air used for aeration should be free of fumes, oil, and water. Compressed air supplies might be contaminated with oil or water containing rust or sludge. Some compressed air supplies might also have a high level of carbon monoxide. A low-pressure blower will provide high-quality air without the problems associated with a high-pressure air supply as long as its air supply is uncontaminated. Adequate aeration will stabilize pH, bring concentrations of dissolved oxygen and other gases into equilibrium with air, and minimize oxygen demand and concentrations of volatiles. However, it is not absolutely necessary to aerate the water for *Xenopus* adults (**12**).

8.3.2 Filtration through bag, sand, sock, or depth-type cartridge filters may be used to keep the concentration of particulate matter acceptably low and as a pretreatment before filtration through a finer filter. Organics may be removed by filtration through activated ~~charcoal~~carbon filtration. ~~Charcoal~~Carbon filters should be changed on a monthly ~~basis.~~basis, or when residual chlorine is detected.

8.3.3 Water that might be contaminated with facultative pathogens may be passed through a properly maintained ultraviolet sterilizer (**13**) equipped with an intensity meter and flow controls, or passed through a filter with a pore size of 0.45 µm or less.

8.4 *Characterization:*

8.4.1 The following items should be measured at least ~~quarterly:~~annually: pH, total dissolved solids (TDS), total organic carbon (TOC), organophosphorus pesticides, organic chlorine (or organochlorine pesticides plus PCBs), chlorinated phenoxy herbicides,

[4] "Reagent Chemicals, American Chemical Society Specifications," Am. Chemical Soc., Washington, DC. For suggestions on the testing of reagents not listed by the American Chemical Society, see "Analar Standards for Laboratory U.K. Chemicals," BDH Ltd., Poole, Dorset, and the "United States Pharmacopeia."

ammonia, bromide, beryllium, cadmium, chromium, copper, iron, lead, manganese, mercury, nickel, selenium, silver, and zinc. For each method used the detection limit should be below the concentration in the water or the lowest concentration that has been shown to adversely affect the test species.

8.4.2 Physical and chemical limits on water: pH should be between 6.5 and 9 (**14**). The TOC should be less than 10 mg/L, while alkalinity and hardness both should be between 16 and 400 mg/L as $CaCO_3$ (**15**).Table 1 shows the recommended maximum concentrations for some contaminants that have often been found to be in excess concentration in laboratory water supplies. The values reported are one tenth of the minimum concentration that inhibits growth. While these data are not indicative of the effect of long-term exposure of adults on reproductive success, they, nonetheless, serve as a guide for limiting adult exposure to these metals. The maximum quantity of the other contaminants listed in 8.4.1 should meet EPA freshwater chronic water quality criteria (**14**).

9. FETAX Solution Water

9.1 *Requirements*—FETAX solution should be used for ~~breeding and~~ static ~~or~~ renewal assays. FETAX solution should also be used for flow-through experiments whenever possible. However, ~~should the~~ larger volumes need for breeding or flow-through exposure may require the use of natural water sources, such as well water for dechlorinated tap water. Should the need for a large volume preclude the use of FETAX solution, then water conforming to the specifications listed in Section 8 may be used. The water must allow embryonic growth at the same rate as FETAX solution and there should be no differences between control mortality and malformation rates.

9.2 *Formulation*—FETAX solution is composed of 625 mg NaCl, 96 mg $NaHCO_3$, 30 mg KCl, 15 mg $CaCl_2$, 60 mg $CaSO_4 \cdot 2H_2O$, and 75 mg $MgSO_4$ per litre of deionized or distilled water. The pH of the final solution should be 7.6 to 7.9. All chemicals should be reagent-grade[4] or better. Deionizedor distilled water must conform to Type I ASTM water (Specification D1193).

10. Test Material

10.1 *General*—The test material should be reagent-grade[4] or better unless a specific test involves an unknown complex mixture, formulation, commercial product, or technical-grade or use-grade material. Before a test is begun, the following should be known about the test material:

10.1.1 Identities and concentrations of major ingredients and major impurities, for example, impurities constituting more than about 1 % of the material.

10.1.2 Solubility and stability in water.

10.1.3 Estimate of toxicity to humans.

10.1.4 Recommended handling procedures (see Section 6).

10.1.5 For unknown samples much of the information specified in 10.1.1-10.1.4 will be lacking, but the pH, hardness, alkalinity, and conductivity of the sample should be measured.

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10.2 *Stock Solution:*

10.2.1 If the test material can not be directly added to the test vessel, a stock solution should be prepared. Various dilutions of the stock solution should be prepared in a separate vessel prior to introduction to the Petri dish so as to expose the embryos to a homogeneous mixture without concentration gradients. If a stock solution is used, the concentration and stability of the test material in it should be determined before the beginning of the test. Stock solutions should be prepared daily unless analytical data indicate the solution is stable with time. If the test material is subject to photolysis, the stock solution should be shielded from light.

10.2.2 Except possibly for tests on hydrolyzable, oxidizable, and reducible materials, the preferred solvent is FETAX solution. Alternatively, dechlorinated tap water or well water may be used if adequately characterized. The minimum necessary amount of a strong acid or base may be used in the preparation of an aqueous stock solution, but such acid or base might affect the pH of test solutions appreciably. Use of a more soluble form of the test material, such as chloride or sulfate salts of organic amines, sodium or potassium salts of phenols or organic acids, and chloride or nitrate salts of metals, might affect the pH more than the use of minimum necessary amount of a strong acid or base. Any adjustments of pH can send the test material through a transition

TABLE 1 Recommended Maximum Concentrations of Some Metals

Metal[A]	Recommended Maximum Concentration (µg/L)
Cadmium (2)	10.0
Lead (2)	5.0
Mercury (2)	0.144
Nickel (2)	25.0
Selenium (unpublished)	140.0
Zinc (2)	70.0

[A] Tested in FETAX at 100 mg/L hardness as $CaCO_3$. Values reported are one tenth of the minimum concentration to inhibit growth.

to affect changes in such properties as solubility or degree and type of dissociation, or both. Prior to testing, as much chemical and physical data as are available on the test material should be obtained and considered prior to making decisions on pH adjustments.

10.2.2.1 If a solvent other than FETAX solution is used, its concentration in test solutions should be kept to a minimum and should be low enough that it does not affect *Xenopus* embryo growth and survival. Because of its low toxicity, low volatility, and high ability to dissolve many organic chemicals, triethylene glycol is often a good organic solvent for preparing stock solutions. Other water-miscible organic solvents such as dimethyl sulfoxide and acetone also may be used as solvents. Concentrations of triethylene glycol, dimethyl sulfoxide, and acetone in test solutions should be <1.6 %, <1.1 %, and, <1.1 % *v/v*, respectively. These concentrations have been found not to cause any adverse effects in FETAX (**16**). At times, concentrations approaching 1 % solvent are necessary to keep test materials in solution for FETAX. This is often the case when the assay is used in testing pure compounds for the purpose of comparing test results with mammalian data. If possible, it is desirable to perform the test using two different solvents and compare the results. This will help in identifying possible interactions between a solvent and test material.

10.2.2.2 Ethanol is not recommended because its teratogenic index (TI) in FETAX is approximately 1.4. Methanol has high toxicity in FETAX. Acetone might stimulate the growth of microorganisms and is quite volatile. If an organic solvent is used it should be reagent-grade[4] or better. A surfactant should not be used in the preparation of a stock solution because it might affect the form and toxicity of the test material in the test solutions.

10.2.3 If a solvent other than dilution-water or FETAX solution is used, at least one solvent control, using solvent from the same batch used to make the stock solution, must be included in the test and a dilution-water or FETAX solution control should be included in the test. If no solvent other than dilution-water or FETAX solution is used, then a dilution-water or FETAX solution control must be included in the test.

10.2.3.1 The concentration of solvent must be the same in all test solutions that contain test material and the solvent control must contain the same concentration of solvent.

10.2.3.2 If the test contains both a dilution-water or a FETAX-solution control and a solvent control, the mortality, malformation, and growth inhibition should be compared using a two-tailed t-test. If a statistically significant difference in either mortality, malformation, or growth inhibition is detected between the two controls, only the solvent control may be used as the basis for comparison in the calculation of results.

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10.2.3.3 If a solvent other than dilution-water or FETAX solution is used to prepare a stock solution, it might be desirable to conduct simultaneous tests using chemically unrelated solvents or two different concentrations of the same solvent to obtain information concerning possible effects of solvents on results of the test.

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11. Test Organisms

11.1 *Species*—FETAX is designed to use embryos of the ~~South~~ African clawed frog *Xenopus laevis* (Daudin). Information regarding the basic biology and development of this species has been reported by Deuchar (**17, 18**). However, use of the South African clawed frog, *Silurana tropicalis* is also acceptable. (Appendix X1). Appendix X1 also lists other North American species that can be used in situations where *Xenopus laevis* or *Silurana tropicalis* cannot, although there will be differences in the rate of development and the method of inducing breeding. Many anurans only breed in a specific season during the year. The length of exposure might have to be altered to allow proper organogenesis.

11.2 *Source*—For breeding, adult frogs may be obtained from various supply houses or independent suppliers. Proven breeders should be requested from the supplier. Each animal should be thoroughly examined upon arrival for skin lesions or red patches on the ventral surfaces. Skin lesions are indicative of nematode infection while the red patches indicate *Aeromonas* infection. Care should be taken to ensure that only healthy, sexually mature frogs are placed in the colony.

11.3 *Adults:*

11.3.1 *Selection—Xenopus* males should be 7.5 to 10 cm in crown-rump length and at least two years of age. Males have dark arm pads on the underside of each forearm and lack cloacal lips. Females should be 10 to 12.5 cm in length and at least three years old. Females are always larger than males and easily identified by the presence of fleshy cloacal lips.

11.3.2 *Diet*—The minimum recommended diet for adults should be three feedings per week of ground adult beef liver. ~~Alternatively, a 2:1 mixture of ground beef liver to beef lung may be used.~~ Finely diced liver is an acceptable diet, especially for small colonies. Liquid multiple vitamins should be added to the ground beef liver. The concentration of vitamins is shown in Table 2. Concentrations of vitamins from 0.05 to 0.075 cm^3/5 g liver are appropriate. Alternatively, #3 size trout or salmon pellets obtained commercially may be used. All food should be screened for the test material if the test material is present in the environment. All liver beef must meet USDA standards for human consumption.

11.3.3 *Temperature*—Adults should be kept at ~~23~~21 ± 3°C.

11.3.4 *Circadian Rhythm*—Adults should be kept on a 12-h day/12-h night cycle. The role that circadian rhythm plays in *Xenopus* reproduction has not been investigated. Thus, a consistent photoperiod should be maintained.

11.4 *Breeding*—Males and females are bred as a single pair. The frogs should be bred in the same water in which the test is to be conducted. Water temperature should be held at ~~21~~20 ± 2°C. To induce breeding, the male and the female should receive ~~250~~150 to ~~500~~350 and ~~500~~350 to ~~1000~~700 IU, respectively, of human chorionic gonadotropin by way of injection into the dorsal lymph sac. The hormone concentration should be 1000 IU/mL in sterile 0.9 % NaCl. A 1-mL tuberculin syringe fitted with a ½-in.

TABLE 2 Recommended Concentration of Vitamins[A]

Vitamin A, IU	1500.
Vitamin D, IU	400.
Vitamin E, IU	5.
Vitamin C, mg	35.
Thiamine, mg	0.5
Riboflavin, mg	0.6
Niacin, mg	8.
Vitamin B_6, mg	0.4
Vitamin B_{12}, mcg	2.

[A] Per millilitre of solution.

long, 26-gage needle should be used to make the injection. Larger bore needles might allow leakage of hormone from the injection site. The amount of human chorionic gonadotropin injected depends on the time of year and condition of the adults. Lower doses are usually used in spring and higher doses in fall. Amplexus normally ensues within 2 to 6 h and egg deposition about 9 to 12 h after injection. The eggs should be immediately inspected for fertility and quality. The fertility rate should be > 75 %. Eggs laid in "strings" or not perfectly round should not be used because they develop abnormally.

11.5 *Embryos:*

11.5.1 *Removal of Jelly Coat*—De-jellying of embryos should begin immediately following the end of egg laying. ~~Dejellying~~De-jellying of embryos should be carried out by gentle swirling for 1 to 3 min in a 2 % w/v L-cysteine (CAS #52-90-4) solution prepared in FETAX solution. The cysteine solution should be adjusted to pH 8.1 with 1 N NaOH. The solution should be made up immediately prior to use. De-jellying should be monitored continuously and the process stopped just after all jelly is removed. Care should be taken not to treat the embryos too long because survival will be reduced.

11.5.2 *Staging of Embryos*—Nieuwkoop and Faber must be used in all staging of embryos (**19**).

11.5.3 *Embryo Selection*—Normally cleaving embryos must be selected for use in testing. The "Atlas of Abnormalities"[5] should be consulted in order to determine which embryos are normal (**19**). It is best to use two levels of selection. In double selection, normally cleaving embryos are first sorted into dishes containing fresh FETAX solution. After a short period during which cleavage continues, embryos are again sorted ensuring that only normal embryos are selected. Abnormal pigmentation should be viewed as an indicator of bad embryos. Either Nieuwkoop and Faber (**19**) or the "Atlas of Abnormalities" can be used as a reference to determine whether the cleavage pattern is normal. Mid blastula (stage 8) to early gastrula (stage 11) must be used to start the test. Embryos chosen prior to stage 8 might develop abnormal cleavage patterns later whereas embryos selected after stage 11 have commenced organogenesis. A large bore blood bank Pasteur pipet can be used to transfer embryos at this stage without harm. The sorting should be done in 100-mm Petri dishes.

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12. Procedure

12.1 *Experimental Design*—FETAX is a 96-h renewal whole embryo assay that can be used to evaluate the developmental toxicity of a test material. Exposure is continuous throughout the test. For each concentration two ~~dishes~~vessels each containing a maximum of 25 embryos and 10 mL of test solution are used. For each control, four ~~dishes of~~ vessels of a maximum of 25 embryos each are used. Embryos must be randomly assigned to test ~~dishes~~vessels except when a forced air incubator is used, in which there are no hot or cold locations. ~~Dishes~~ Test vessels must be randomly assigned to their positions in the incubator. In order to properly evaluate developmental toxicity, mortality, malformation, and growth-inhibition, data must be collected. In most tests it will be possible to generate concentration-response curves for mortality, malformation, and growth inhibition. The mortality and malformation concentration-response curves should then be used to estimate the concentration that would affect 50 % of the exposed embryos. At least 90 % of the FETAX-solution controls must have attained stage 46 at 96 h (**19**).

12.2 *Temperature and pH Requirements:*

12.2.1 *Temperature*—~~24~~22 ± 2°C must be maintained throughout the 96-h test. Temperatures higher than 26°C cause malformation whereas low temperatures prevent the controls from reaching stage 46 in 96 h.

12.2.2 *pH*—The pH of the stock and test solutions should be 7.7 and must be between 6.5 and 9.0 (**14**). The pH of a control ~~dish~~vessel and the pH of the highest test concentration should be measured at the beginning of the test and every 24 h thereafter to determine if they have changed.

12.3 *Beginning the Test:*

12.3.1 *Recommended Protocol for Testing New Materials*—The following sequence should be followed when testing a new test material to determine the 96-h LC50 and the 96-h EC50 (malformation)(termed the 96-h EC50). This procedure will guide initial range-finding experiments and help reduce replicate test variability. The procedure is iterative and designed to produce test concentrations for definitive experiments that will yield 96-h LC50 and 96-h EC50 values with narrow confidence intervals. This

[5] Available from John A. Bantle, Dept. of Zoology, 430 LSW, Oklahoma State University, Stillwater, OK 74078.

should be accomplished by defining several concentrations between the 16 and 84 % effect concentrations (at least 3, preferably 5). The procedure is designed to account for different slopes of concentration-response curves.

12.3.1.1 *Goal of Range-Finding and Definitive Tests*—Range-finding tests are to be used whenever possible to find the best approximation of the 96-h LC50 and EC50 for definitive testing. Once the data are collected from the range-finding tests, the expected 96-h LC50 and EC50 are estimated using probit analysis, trimmed Spearman-Karber analysis, or the two-point graphical method. The graphical method is used only when regular statistical analyses fail to generate useful data. If the data allow probit analysis or trimmed Spearman-Karber methods to be used, then probit analysis may be used when the data meet normal distribution and homogeneity of variance assumptions. Trimmed Spearman-Karber is used when the data fail to meet these assumptions. Range-finding tests may bypass the homogeneity of variance requirements here but not in definitive tests discussed in 12.3.2. Definitive test data are analyzed similarly but may not bypass homogeneity of variance requirements. Growth inhibition data are not collected from range-finding tests. Once the definitive test concentrations are selected, three definitive tests are performed that will yield 96-h LC50 and EC50 information with acceptable repeatability (see 12.3.2). Prior testing suggests that intraboratory variability should yield a coefficient of variation less than 100 %.

12.3.1.2 *Selection of Concentrations*—Concentration selection is a multistep process depending on the nature of the test material and the results of the first test in this series. The first test simply consists of a series of at least seven concentrations that differ by a factor of ten. If a metabolic activation system (MAS) is to be used to assess possible effects on mammals or for human health hazard assessment, all tests should be performed with and without the metabolic activation system. This is usually adequate to delineate the concentration range to establish the approximate MAS and No MAS 96-h LC50 and EC50 values. The second range-finding test series is performed using the sliding scale of concentrations presented in Appendix X2. The table presents concentration values from 0.001 to 100; in steps of 0.0005 between 0.001 and 0.1, steps of 0.05 between 0.1 and 1, in steps of 0.5 between 1 and 10, and in steps of 5 between 10 and 100. Using the sliding scale, the value closest to the MAS and No MAS 96-h LC50 should be identified and then three values immediately below and three values immediately above the LC50 point chosen. The same method should be used to estimate concentrations surrounding the 96-h EC50. A test is performed and the data collected. The 96-h LC50 and EC50 values with confidence limits should then be calculated. From the data obtained above, the 96-h LC5, LC16, LC50, LC84 and LC95 and the EC5, EC16, EC50, EC84, and EC95 may be calculated. By determining these values, the concentrations to be tested in the definitive tests below are established and the slopes of the concentration-response curves are taken into consideration. Additional concentrations between the EC16 and EC84 are highly recommended to ensure obtaining a 96-h LC50 and EC50 values. However, the same concentrations must be used for each replicate (definitive) test. Interlaboratory studies indicated a reduction in intralaboratory test variability when the above procedures were used. For some test materials it may be necessary to use the results of the first definitive experiment as another range-finder and readjust the test concentrations again.

12.3.2 *Replicate-Definitive Tests:*

12.3.2.1 *Number of Tests and Data Collection*—~~Three~~ At least two replicate-definitive tests are then performed with and without an exogenous MAS each with a separate clutch of embryos (see 12.3.3.2). The minimum five concentrations for each endpoint determined above are used with and without MAS. The experiments should yield acceptable MAS and NO MAS 96-h LC50 and EC50 values. If they do not, the tests should be repeated. In some cases where test variability is extremely high, it may be necessary to determine whether the test material is rapidly degrading, salting out or volatilizing out of solution. Remember that MAS is only used when assessing mammalian developmental toxicity.

12.3.2.2 *Ensuring Adequate Embryo Supply and Maintenance of Separate Clutches*—To ensure an adequate supply of normal embryos for each test, three mating pairs should be induced and clutches harvested separately. Embryos should be sorted to ensure viability prior to testing. Each test uses early embryos derived from a single mating pair; if the controls from a particular mating pair indicate a problem with fertility or viability of early embryos, the test will be unacceptable for that particular clutch. Each individual test will yield data that will be used to generate concentration-response curves for mortality, malformation, and growth inhibition. It is necessary to keep clutches separate because embryos from a particular mating pair might develop poorly although they initially appear acceptable. This would cause all the embryos to be discarded if embryos are mixed from different mating pairs.

Each test should be performed with embryos derived from a single mating pair regardless of the number of replicate dishes. The selection of experimental design and statistical methods required to evaluate mortality, malformation, and growth-inhibition data, should consider the type of compound or chemical mixture being evaluated and the limitations that sample or time availability might imply as far as appropriate statistical techniques (**20**).

12.3.3 *Reference Toxicant*—For a positive control or reference toxicant, 6-aminonicotinamide presents a mortality and malformation database convenient for reference purposes. Commercial sources for the 6-aminonicotinamide (CAS #329-89-5; formula weight, 137.14) should specify the physicochemical data and the purity for the compound which ensure its being comparable to that readily available to other laboratories (for example, UV spectroscopic characterization: at A_{257} and pH 1.8, a 9.74 µg/mL solution of 6-aminonicotinamide has an absorbance of 1 and an absorbance ratio (A_{257}/A_{302}) of 2.28). The purity should be >99 %. From this published database, the 96 h LC50 is ~~2.23 mg/mL~~ approximately 2,230 mg/L and the 96 h EC50 (malformation) is ~~0.005 mg/mL~~ approximately 5.0 mg/L yielding a TI of 446 (**21**). The MCIG was ~~1.15 mg/mL.~~1,150 mg/L. At least quarterly concentration-response experiments must be performed and the results of these tests compared with historical tests in order to judge the laboratory quality of FETAX data. The reference toxicant test must produce data within ±2 SD of the

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historical mean values (14.1.17). This procedure follows standard EPA toxicity testing procedures for aquatic tests (**21**). Only those biological responses related to mortality and malformation are considered in this analysis; growth inhibition is not considered in regard to responses to 6-aminonicotinamide.

12.4 *Renewal*—The renewal procedure should be used for the standard FETAX test. The renewal procedure entails fresh replacement of test material every 24 h during the test. Just prior to this change it is advisable to measure the pH of the control and the highest test dishes in order to determine if significant changes occurred. Renewal should be accomplished by removing the test solution with a Pasteur pipet. The orifice of the Pasteur pipet should be enlarged and fire-polished to accommodate embryos without damage in case the embryos are accidentally picked up. This procedure should proceed quickly in order to minimize embryo desiccation. This is the standard procedure for FETAX but two other variants are allowed as described in the Appendix X2. Variations to the renewal procedure must be reported.

12.5 *Duration of the Test*—The standard exposure time for FETAX is 96 h and the attainment of stage 46 in controls. If 90 % of the controls have not reached stage 46 by 96h, then the test may be extended by 3 h in order for the controls to reach stage 46. Deviations from this standard exposure time must be reported as deviating from standard FETAX conditions.

12.6 *Exogenous Metabolic Activation System (MAS)*—An exogenous MAS must be used when FETAX is used to evaluate developmental toxicity for human health hazard assessment. The MAS is composed of rat liver microsomes and a nicotinamide adenine dinucleotide (reduced form) [NADPH] generator system which simulates mammalian metabolism. Since early *Xenopus* embryos have limited xenobiotic metabolic capabilities, particularly cytochrome (CYP) P-450, the incorporation of the exogenous system into the standard assay protocol is warranted. Aroclor 1254 may be used as a broad-spectrum inducing agent and used in the majority of situations. Isoniazid induction or uninduced microsomes may be used in those cases where Aroclor 1254 induction may repress specific P-450 isozymes. The nature of the test material may suggest which inducing system to use. In cases where limited information is available concerning test material biotransformation, a set of Aroclor 1254 and isoniazid-induced rat liver microsomes mixed in equivalent activity ratios may be used.

12.6.1 Sterile plastic Petri dishes should be used to minimize bacterial contamination, although the volume is only 8 mL instead of the 10 mL in the glass dishes. Antibiotics are required to inhibit bacterial growth and these may interact with the test substance. Microsomal protein can slow growth and development at concentrations greater than 60 µg/mL. NADPH, which is required for microsomal activity, can also cause abnormal development and its concentration must also be kept low. Despite these drawbacks, the MAS improves the predictive accuracy of FETAX and provides repeatable and reliable data.

12.6.1.1 The P-450 activities of each lot of microsomes prepared will vary. The P-450 activity of each lot must be measured and a standard amount added to each dish. It is important to include a MAS-only (microsomes and generator system without test material) negative control. The bioactivation positive control is 4 mg/mL cyclophosphamide with and without MAS. The MAS-only control should result in less than 10 % mortality and malformation. The 4 mg/mL cyclophosphamide-only control should result in less than 10 % mortality. With MAS, bioactivated 4 mg/mL cyclophosphamide should kill 100 % of the embryos in 96 h while there should be less than 10 % mortality without MAS. A final control is needed to demonstrate that the cytochrome P-450 system is responsible for the observed bioactivation. For this control, a small amount of dithionite may be added directly to the microsomes followed by bubbling carbon monoxide through the microsomal protein at a steady rate for 3 min to inactivate P-450. This procedure must be performed in a safety hood.

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12.6.2 *Microsome Preparation:*

12.6.2.1 *Animal Treatment*—Male Sprague-Dawley rats (200 to 250 g) should be used. For Aroclor 1254-induced microsomes, an intraperitoneal injection of 500 mg/kg body weight should be given five days prior to isolation. The Aroclor 1254 stock solution should be prepared in corn oil (500 mg/mL). For isoniazid induction, 0.1 % w/v isoniazid in 5 % sucrose may be administered in the drinking water for ten consecutive days.

12.6.2.2 *Preparation*—Rats are killed by cervical dislocation. All buffers and tissue samples should be maintained at 4°C. Livers are perfused using a peristaltic pump via the hepatic portal vein with Buffer 2 (Appendix X4). Perfusion takes place until the liver is well blanched (approximately 50 mL). The liver is excised and homogenized in seven volumes of Buffer 3 (Appendix X4.1) using a tissue homogenizer. Several styles of homogenizers may be used, but a motorized homogenizer with ~~Teflon~~TFE-fluorocarbon pestle is adequate for preparing microsomes from young rat livers. Centrifuge first at 900 ~~x~~× g avg. for 10 min, then increase speed to 9000 ~~x~~× g avg. for an additional 15 min. Remove S-9 supernatant to another tube and centrifuge the S-9 supernatant at 105 000 ~~x~~× g avg. for 1 h. Discard supernatant and resuspend pellet in Buffer 2. Centrifuge again at 105 000 ~~x~~× g avg. for an additional h. Resuspend microsomal pellet in 20 to 30 mL of Buffer 1. ~~A 1~~A 1 mL sample should be removed for Nash and protein content assays, snap frozen in liquid nitrogen and frozen at –80°C until analyzed. Homogenize again with two to three strokes using a tissue homogenizer. Aliquot samples into microcentrifuge tubes or cryovials, and snap freeze in liquid nitrogen. Protein concentration and P-450 activities should be measured prior to use in testing.

12.6.2.3 *Additional MAS Components of FETAX*—The various concentrations of the test solutions should be prepared in separate Erhlenmeyer flasks to avoid exposing the embryos to individual components of the system. The following order of the addition of MAS components should be observed to maximize the productivity of the MAS. To prepare 20 mL of test solution, place appropriate volume of FETAX solution (for example, 19 mL) into a 50 mL flask, add the MAS components and the appropriate volume of test material stock to give the desired concentration and, finally, adjust the final volume to 20 mL with