Designation: E 1439 – 98

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

This standard is issued under the fixed designation E 1439; 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 E 1192), surface and ground waters, leachates, aqueous extracts of water-insoluble materials, 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:



[1] This guide is under the jurisdiction of ASTM Committee E-47 on Biological Effects and Environmental Fate and 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.

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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:*

D 1193 Specification for Reagent Water[3]

E 380 Practice for Use of the International System of Units (SI) (the Modernized Metric System)[4]

E 729 Guide for Conducting Acute Toxicity Tests with Fishes, Macroinvertebrates and Amphibians[5]

E 943 Terminology Relating to Biological Effects and Environmental Fate[5]

E 1023 Guide for Assessing the Hazard of a Material to Aquatic Organisms and Their Uses[5]

E 1192 Guide for Conducting Acute Toxicity Tests on Aqueous Effluents with Fishes, Macroinvertebrates and Amphibians[5]

E 1391 Guide for Collection, Storage, Characterization, and Manipulation of Sediments for Toxicological Testing[5]

E 1525 Guide for Designing Biological Tests with Sediments[5]

E 1706 Test Methods for Measuring the Toxicity of Sediment-Associated Contaminants with Freshwater Invertebrates[5]

## 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 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 96-h LC50 divided by the 96-h EC50 (malformation).

3.1.3 For definitions of other terms used in this guide, refer to Guides E 729 and E 1023, also Terminology E 943. For an explanation of units and symbols, refer to Practice E 380.

## 4. Summary of Guide

4.1 In FETAX, range-finding and three replicate 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 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 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 developmental toxicants. 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] *Annual Book of ASTM Standards,* Vol 11.01.
[4] *Annual Book of ASTM Standards,* Vol 14.02.
[5] *Annual Book of ASTM Standards,* Vol 11.05.

## 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 23 ± 3°C.

7.1.1 Two types of breeding aquaria have been used successfully. 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 ± 2°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 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) hydrochloric acid to remove scale, metals, and bases.

7.4.1.4 Rinse twice with water conforming to Type II ASTM water (Specification D 1193).

7.4.1.5 Rinse once with full strength reagent-grade[6] acetone to remove organic compounds.

[6] "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."

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 ± 3°C before being used to culture adults.

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 filtration. Charcoal filters should be changed on a monthly basis.

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: pH, total dissolved solids (TDS), total organic carbon (TOC), organophosphorus pesticides, organic chlorine (or organochlorine pesticides plus PCBs), chlorinated phenoxy herbicides, 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 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

**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.

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[6] or better. Deionizedor distilled water must conform to Type I ASTM water (Specification D 1193).

## 10. Test Material

10.1 *General*—The test material should be reagent-grade[6] 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.

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. 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 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[6] 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.

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.

## 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)**. Appendix X1 lists other North American species that can be used in situations where *Xenopus* 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. 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 ± 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 ± 2°C. To induce breeding, the male and the female should receive 250 to 500 and 500 to 1000 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. 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.

**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.

11.5 *Embryos*:

11.5.1 *Removal of Jelly Coat*—Dejellying of embryos should begin immediately following the end of egg laying. Dejellying 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. Dejellying 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"[7] 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.

## 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 each containing 25 embryos and 10 mL of test solution are used. For each control, four dishes of 25 embryos each are used. Embryos must be randomly assigned to test dishes except when a forced air incubator is used, in which there are no hot or cold locations. Dishes 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 ± 2°C must be maintained throughout the 96-h test. Temperatures higher than 26°C cause

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

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 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 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 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 and the 96 h EC50 (malformation) is 0.005 mg/mL yielding a TI of 446 **(21)**. The MCIG was 1.15 mg/mL. 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 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 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.

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 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 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 FETAX solution. This test mixture should then be divided between replicate Petri dishes to which the embryos will be added (10 mL each for glass dishes, 8 mL each for plastic dishes.

12.6.2.4 A penicillin-streptomycin antibiotic solution (100 U/mL each, final concentration) NADPH generator stock (3.6 mM glucose-6-phosphate, 0.1 mM NADP, and 7.0 µM NADPH final concentration), microsomes (0.4 U/mL N-demethylase activity, not to exceed 60 µg/mL protein, final concentration), and glucose-6-phosphate dehydrogenase (0.31 U/mL final demethylase activity. Each test should consist of at least duplicate concentrations of test material with and without the exogenous MAS.

12.7 *Biological Data*:

12.7.1 *Mortality*—Dead embryos must be removed at the end of each 24-h period during the 96-h test at the time solutions are changed. If dead embryos are not removed, microbial growth can occur that might kill live embryos. Death at 24 h (stage 27) is ascertained by the embryo's skin pigmentation, structural integrity, and irritability. At 48 h (stage 35), 72 h (stage 42), and 96 h (stage 46) the lack of heartbeat serves as an unambiguous sign of death. At 96 h of exposure or stage 46 of controls, the number dead is recorded. Dead embryos are removed and the remaining live embryos fixed in 3 % formalin.

12.7.2 *Malformation*—Malformations must be recorded at the end of 96 h. The "Atlas of Abnormalities" should be used in scoring malformations, particularly slight malformations. Embryos exposed to the test material should also be compared to appropriate controls. The number of malformations in each category should be reported in standard format for ease of interlaboratory comparison (Fig. 1).

12.7.3 *Growth Inhibition*—The ability of a material to inhibit embryonic growth is often the most sensitive indicator of developmental toxicity. Head-tail length data (growth) must be collected at the end of each test. If the embryo is curved or kinked, then the measurement should follow the contour of the embryo (see 7.2). Measurement should be made after embryos are fixed in 3 % formalin. No significant length reductions due to formalin fixation have been observed. The minimum concentration to inhibit growth (MCIG) is the minimum concentration of test material that significantly inhibits growth as determined by measurement of head-tail length. A significant difference in growth should be determined by the t-Test for grouped observations at the $p = 0.05$ level **(22)**.

12.7.4 *Additional Data*—Different types of data have been collected in FETAX and may, at the user's option, be used in addition to the mortality, malformation, and growth inhibition data listed above (see Appendix X2).

12.7.5 *Criteria for Estimating Developmental Toxicity and Teratogenic Hazard*:

12.7.5.1 FETAX provides concentration-response data for mortality, malformation and growth inhibition. These data can be compared with similar data on a molar basis using other pure test materials to yield a relative ranking of toxicity.

12.7.5.2 A test material is considered a developmental toxicant when it causes any deficit in an embryo, especially at concentrations lower than those required to cause adult toxicity. In comparison, a teratogen causes some observable abnormality in embryonic development. Three separate criteria have been developed to identify teratogens using FETAX **(22)**.

12.7.5.3 The three criteria are based on empirical evidence based on over 100 test materials tested in FETAX. Any one criterion is sufficient to identify a potential teratogenic hazard. The TI, severity of malformations caused and growth inhibition are the criteria by which potential teratogens are identified.

12.7.5.4 The TI presents a relative ranking of hazard from nearly 1 to several thousand. The hazard becomes a concern when the mean TI of three definitive tests is $> 1.5$. The mortality and malformation concentration-response curves should have similar slopes with acceptable confidence limits when compared to data from 6-aminonicotinamide positive control experiments. The TIs of different test materials may be compared to generate relative rankings.

12.7.5.5 A 96 h-LC50 can be established for most test materials tested in FETAX. Nonteratogens only cause slight to moderate malformations at concentrations near the 96-h LC50. Teratogens generally cause moderate to severe malformations at these concentrations. Comparison can be made to the positive control 6-aminonicotinamide which causes severe malformations in order to identify what constitutes a severe malformation. The "Atlas of Abnormalities" is also available for judging the severity of malformation **(19)**.

12.7.5.6 While growth inhibition probably does not play a significant role in mammalian teratogenesis, it is correlated to teratogenesis in FETAX. Endpoints in *in vitro* developmental toxicity tests do not have to emulate mammalian endpoints, only predict hazard. Teratogenic hazard becomes apparent when growth is significantly affected at concentrations below 30 % of the 96 h-LC50. When using this criterion, it is important to ensure that the test concentrations selected are adequate to define the MCIG. A test material poses some teratogenic hazard when any one of the three criteria are met.

## 13. Analytical Methodology

13.1 The methods used to analyze test solutions might determine the usefulness of the test results if the results are based on measured concentrations. For example, if the analytical method measures any impurities or reaction or degradation products along with the parent test material, results can be

Directions: Place a check in each box for each type of malformation. The resultant scoresheet reads like a histogram.

INVESTIGATOR _____ COMPOUND _____

DATE _____/_____/_____ CONCENTRATION _____

TOTAL SURVIVING _____ TEST # _____

% MALFORMED _____ DISH # _____

| *Malformations:* | 1 | 2 | 3 | 4 | 5 | 6 | 7 | 8 | 9 | 0 | 1 | 2 | 3 | 4 | 5 | 6 | 7 | 8 | 9 | 0 | 1 | 2 | 3 | 4 | 5 |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| Severe | | | | | | | | | | | | | | | | | | | | | | | | | |
| Stunted | | | | | | | | | | | | | | | | | | | | | | | | | |
| Gut | | | | | | | | | | | | | | | | | | | | | | | | | |
| Edema (multiple) | | | | | | | | | | | | | | | | | | | | | | | | | |
| a. cardiac | | | | | | | | | | | | | | | | | | | | | | | | | |
| b. abdominal | | | | | | | | | | | | | | | | | | | | | | | | | |
| c. facial | | | | | | | | | | | | | | | | | | | | | | | | | |
| d. cephalic | | | | | | | | | | | | | | | | | | | | | | | | | |
| e. optic | | | | | | | | | | | | | | | | | | | | | | | | | |
| Axial malformations | | | | | | | | | | | | | | | | | | | | | | | | | |
| A. tail | | | | | | | | | | | | | | | | | | | | | | | | | |
| B. notocord | | | | | | | | | | | | | | | | | | | | | | | | | |
| C. fin | | | | | | | | | | | | | | | | | | | | | | | | | |
| Face | | | | | | | | | | | | | | | | | | | | | | | | | |
| Eye | | | | | | | | | | | | | | | | | | | | | | | | | |
| Brain | | | | | | | | | | | | | | | | | | | | | | | | | |
| Hemorrhage | | | | | | | | | | | | | | | | | | | | | | | | | |
| Cardiac | | | | | | | | | | | | | | | | | | | | | | | | | |
| Blisters | | | | | | | | | | | | | | | | | | | | | | | | | |
| Other (specify) | | | | | | | | | | | | | | | | | | | | | | | | | |

TOTAL SURVIVING _____ TEST # _____

% MALFORMED _____ DISH # _____

| *Malformations:* | 1 | 2 | 3 | 4 | 5 | 6 | 7 | 8 | 9 | 0 | 1 | 2 | 3 | 4 | 5 | 6 | 7 | 8 | 9 | 0 | 1 | 2 | 3 | 4 | 5 |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| Severe | | | | | | | | | | | | | | | | | | | | | | | | | |
| Stunted | | | | | | | | | | | | | | | | | | | | | | | | | |
| Gut | | | | | | | | | | | | | | | | | | | | | | | | | |
| Edema (multiple) | | | | | | | | | | | | | | | | | | | | | | | | | |
| a. cardiac | | | | | | | | | | | | | | | | | | | | | | | | | |
| b. abdominal | | | | | | | | | | | | | | | | | | | | | | | | | |
| c. facial | | | | | | | | | | | | | | | | | | | | | | | | | |
| d. cephalic | | | | | | | | | | | | | | | | | | | | | | | | | |
| e. optic | | | | | | | | | | | | | | | | | | | | | | | | | |
| Axial malformations | | | | | | | | | | | | | | | | | | | | | | | | | |
| A. tail | | | | | | | | | | | | | | | | | | | | | | | | | |
| B. notocord | | | | | | | | | | | | | | | | | | | | | | | | | |
| C. fin | | | | | | | | | | | | | | | | | | | | | | | | | |
| Face | | | | | | | | | | | | | | | | | | | | | | | | | |
| Eye | | | | | | | | | | | | | | | | | | | | | | | | | |
| Brain | | | | | | | | | | | | | | | | | | | | | | | | | |
| Hemorrhage | | | | | | | | | | | | | | | | | | | | | | | | | |
| Cardiac | | | | | | | | | | | | | | | | | | | | | | | | | |
| Blisters | | | | | | | | | | | | | | | | | | | | | | | | | |
| Other (specify) | | | | | | | | | | | | | | | | | | | | | | | | | |

NOTE 1—Directions: Place a check in each box for each type of malformation. The resultant scoresheet reads like a histogram.

**FIG. 1 Scoresheet of Malformations at 96 h**

calculated only for the whole group of materials, and not for the parent material by itself, unless it is demonstrated that such impurities and products are not present.

13.2 If samples of stock solutions or test solutions cannot be analyzed immediately, they should be handled and stored appropriately **(23)** to minimize loss of test material by such things as microbial degradation, hydrolysis, oxidation, photolysis, reduction, sorption, and volatilization.

13.3 Chemical and physical data should be obtained using appropriate ASTM standards whenever possible. For those measurements for which ASTM standards do not exist, methods should be obtained from other reliable sources **(24)**.

## 14. Acceptability of Test

14.1 A test using embryos from a single mating pair should be considered unacceptable if one or more of the following occurred.

14.1.1 Embryos were used from more than one mating pair.

14.1.2 Hardware cloth or metal mesh was used as a support in the breeding aquarium.

14.1.3 In the controls, either the mean survival is < 90 % or the mean malformation in embryos is > 10 %, or both.

14.1.4 If 90 % of the FETAX-solution-only controls do not reach stage 46 by the end of 96 to 99h. The primary cause of control embryos not reaching stage 46 is low temperature (see 12.2.1).

14.1.5 If dilution water was used in the test, and it did not allow embryonic growth at the same rate as FETAX solution.

14.1.6 The demonize or distilled water does not conform to Type I ASTM standard.

14.1.7 A required dilution-water, FETAX solution, or MAS control or solvent control was not included in the test.

14.1.8 The concentration of solvent was not the same in all treatments, except for a dilution-water or FETAX-solution control.

14.1.9 Staging of embryos was performed using a reference other than Nieuwkoop and Faber **(19)**.

14.1.10 The test either was started with less than stage 8 blastulae or greater than stage 11 gastrulae.

14.1.11 All Petri dishes were not physically identical throughout the test.

14.1.12 Petri dishes were not randomly assigned to their positions in a non-forced air incubator.

14.1.13 The embryos were not randomly assigned to the Petri dishes.

14.1.14 Required data concerning mortality, malformation, and growth were not collected.

14.1.15 The pH of the test solution was < 6.5 or > 9.0 in the control or highest test concentration.

14.1.16 Dead embryos were not removed after each 24-h (± 2 h) interval.

14.1.17 Consistently deviating from the temperature limits as stated in 12.2.1. A short-term deviation of more than ± 2°C might be inconsequential.

14.1.18 If the reference toxicant study produced significant variability (± 2 SD units from the historical mean values) compared to historical data plotted on a control chart.

## 15. Documentation

15.1 The record of the results of an acceptable FETAX should include the following information either directly or by reference to existing publications.

15.1.1 Name of test material, investigator(s) name, location of laboratory, and dates of initiation and termination of test.

15.1.2 Source of test material, its lot number, composition (identities and concentrations of major ingredients and major impurities), known chemical and physical properties, and the identity and concentration(s) of any solvent used. For some complex environmental mixtures a great deal of this information might be lacking.

15.1.3 If a dilution water other than FETAX solution is used, its chemical characteristics and a description of any pretreatment.

15.1.4 Recent analyses of FETAX solution and adult culture water.

15.1.5 pH measurements of control and of the highest test concentrations at the end of each 24-h time period. Available data on sample hardness, alkalinity, conductivity, total organic carbon (TOC), concentration of dissolved oxygen, and metal content.

15.1.6 The mortality, malformation rates, and the mean embryo length at 96 h in the dilution-water, FETAX solution, or solvent control.

15.1.7 The mortality and malformation results obtained for the 6-aminonicotinamide positive control. If a full concentration-response curve was performed, then the 96-h LC50, the 96-h EC50 (malformation), and their confidence limits should be reported.

15.1.8 The 96-h LC50, the 96-h EC50 (malformation), the TI (96-h LC50/96-h EC50 (malformation)), and the minimum concentration to inhibit growth (MCIG) for each test. The geometric means of these values and their 95 % confidence limits. Concentration-response data for mortality, malformation, and growth inhibition may be provided.

15.1.9 A table for each test that lists the percent mortality, percent malformation, and the head-tail length at each concentration tested.

15.1.10 The names of the statistical tests employed, the alpha-levels of the tests, and some measure of the variability of the hypothesis tested.

15.1.11 The types, frequency, and severity of malformations. The types of malformations and their severity might differ over the different concentrations tested. It might be best to define ranges of concentrations tested and create a summary table that lists the malformations that occurred in each concentration range.

15.1.12 Any deviations from standard FETAX (see Appendix X1 and Appendix X2).

## 16. Keywords

16.1 amphibia; developmental toxicity; FETAX; screening test; short-term chronic test; teratogenicity; *Xenopus*

# APPENDIXES

**(Nonmandatory Information)**

## X1. LIST OF ALTERNATIVE SPECIES

X1.1 *Use of Alternative Species*—Although FETAX was designed expressly for the use of *Xenopus laevis*, it might be necessary to use endemic species when required by regulations or other considerations. Users are cautioned that many endemic species of frogs are threatened by pollution and habitat loss and the user should carefully consider the environmental consequences of large-scale collection of local anuran species. Deviations from standard procedures must be reported (see Section 15) and it will be difficult to compare data between standard FETAX and data derived using an alternative species.

X1.2 *Recommended Anurans*—Members of the family Ranidae (for example, *Rana pipiens*) and Bufonidae (for example, *Bufo fowleri*) might be best suited for FETAX, because the number of eggs or the seasonal availability, or both, are more limited for other species. Seasonal availability can be extended by two to three months using human chorionic gonadotropin injection. *Rana catesbiena* and *Bufo americanus* are likely as well suited as *Rana pipiens* and *Bufo fowleri* **(25)**. High egg production, geographical range, short hatching periods, and other factors would indicate that these four species could serve as alternatives. Comparative sensitivities to inorganic mercury have been reported for some of these species **(25)**. These studies have reported a range in sensitivity to inorganic mercury which should be taken into account when comparing data with other amphibian species.

## X2. ADDITIONAL DATA AND ALTERNATIVE EXPOSURES

X2.1 *Additional Data*—Other types of data can be collected in FETAX that increases its versatility. The types of data listed below represent some that have been collected in past experiments. In the case of pigmentation and locomotion, scoring is subjective.

X2.1.1 *Pigmentation*—Collecting data on pigmentation might be useful for measuring neural damage because it is thought that the size of the pigment patches is under nervous control. Agents that affect these nerves cause smaller pigment patches and the overall color of the 96-h larvae will pale. Comparison to the standard“ Atlas of Abnormalities” and suitable controls must be made in order to determine abnormal pigmentation. Other causes of depigmentation are possible including loss of melanin production. A concentration-response curve can be generated and an EC50 (pigmentation) determined.

X2.1.2 *Locomotion*—Collecting locomotion data is potentially useful in measuring specific neural or muscle damage since larvae with substantial cellular damage swim poorly, erratically or not at all. The ability to swim properly should be determined by comparison to appropriate controls. A concentration-response curve can be generated and an EC50 (locomotion) determined **(26)**.

X2.1.3 *Hatchability*—The embryos hatch from the fertilization membrane between 18 and 30 h. The number failing to hatch at 48 h should be recorded. Delay or failure indicates a slowing of developmental processes. This is analogous to staging the embryos at the end of the 96-h time period except that it is much easier to score hatching. A concentration-response curve can be generated and an EC50 (hatching) determined.

X2.2 *Additional Exposures*:

X2.2.1 *Additional Exposure Length*—In special circumstances, exposure periods exceeding 96 h or pulse exposures, or both, may be performed. Data so collected should be reported as deviating from standard FETAX.

X2.2.2 *Static*—In the static technique, the test material is added at the beginning of the test and not changed. It should be recognized that many test materials will degrade in a short period of time. The static technique should only be used for materials that are extremely stable and do not volatilize or sorb to the test dishes. The cost or the size of the sample might also dictate that the static technique be used. This variation in procedure must be reported as deviating from standard FETAX.

X2.2.3 *Flow-Through*—A toxicant-delivery system is used to continuously deliver toxicant and dilution water to the embryos. Small glass containers with bottom screening are used to contain the embryos in a larger diluter apparatus. The flow-through technique is recommended for chemicals that degrade quickly or are volatile or for large volume environmental samples. Every attempt should be made to use FETAX solution as the diluent. This variation in procedure must be reported as deviating from standard FETAX.

X2.2.4 *Solid Phase Sample Testing*:

X2.2.4.1 *Sample Handling*—Approximately 1 kg of soil or sediment should be collected and expediently sent to the laboratory to minimize holding time. Prior to testing, soil or sediment subsamples should be thoroughly homogenized. Composites usually consist of three to six grab samples. Subsamples are collected with a non-reactive sampling device and placed in a non-reactive storage container. Subsamples are mixed and stirred until the texture and color are uniform (see 9.7 of Guide E 1391). The samples are then stored at 4°C until FETAX testing is initiated. It is recommended that samples be

tested within two weeks of receipt unless specific circumstances delay testing (see 10.5 of Guide E 1391).

X2.2.4.2 *Assay Methods*— FETAX studies should be performed in accordance with the methods cited in the previous sections with the following modifications for whole soil or sediment testing. Testing may be performed in 250 mL specimen bottles or similar capped vessels equipped with a 55 mm glass tube with Teflon® mesh insert as the exposure chamber. Stainless steel mesh (100 µ pores) can be substituted for Teflon® mesh. For screening tests, 35 g of sediment (dry weight) should be placed in the bottom of the vessel, the exposure insert added, and filled with 140 mL of FETAX Solution (dilution water)(see X2.2.5.1). It is essential that the dilution soil be non toxic and as chemically and physically similar to the test soil as possible. Care must be taken in interpreting results of soil/sediment dilution experiments in that toxicity results may be altered because of the nature of the soil/sediment used for dilution **(27)**. The sample must be equilibrated. The top edge of the glass tube must be higher than the water level to prevent larvae swimming out after day two. This represents four parts of dilution water to one part of soil or sediment. Blastulae stage embryos are placed directly on the mesh insert that rests directly over the top of the soil or sediment in the sediment/water interface region. The test consists of 25 embryos placed in each of four replicates (total of 100 embryos exposed to FETAX Solution), a minimum 25 embryos exposed to blasting sand (artificial sediment) in each of three replicates (minimum 50 total), and 25 embryos exposed to the soil or sediment sample in each of three replicates (minimum 50 embryos total). Blasting or beach sand should be extensively tested beforehand to ensure that there is less than 10 % mortality or malformation after 96 h. There should also be a reference soil/sediment tested that is nontoxic but represents the soil/sediment characteristics of the site (see Test Method E 1706). Dilutions of the soil or sediment should be prepared by mixing the sample with uncontaminated site soil or laboratory reference soil. Four to six dilutions ranging from 0 to 100 % soil sample and a FETAX Solution control are typically tested. Screening tests (control and 100 % sample) may be performed prior to multi-concentration definitive testing. Each sample should be tested in triplicate. Solutions and soils or sediments should be changed every 24 h of the 4-d test by moving the insert containing the embryos to a fresh jar of diluent water and soil/sediment sample. Dead embryos are removed at this time. Dissolved oxygen and pH should be measured prior to renewal and in the waste solutions from each successive day. Dissolved oxygen, pH, conductivity, hardness, alkalinity, ammonia-nitrogen, and residual chlorine should be measured on separate aliquots of the batches of FETAX Solution used during the study. The measurements must be conducted after the conclusion of the exposure period and oxygen content must be greater than 5.5 mg/L.

X2.2.4.3 *Data Analysis*— At the conclusion of the test, embryos should be preserved in 3 % (w/v) formalin (pH 7.0) and morphological characteristics evaluated using a dissecting microscope. If only screening tests are performed, determination of LC50 and EC50 (malformation) is not possible and responses may be reported as a percent effect. Growth achieved by the embryos may be determined using a digitizing software package. Statistical evaluation of differences in response between the control and treated groups may be evaluated using parametric or non-parametric hypothesis tests for the mortality and malformation responses, and a grouped t-test for the growth data ($P < 0.05$ for all tests).

X2.2.5 *Aqueous Extracts of Solid Samples*:

X2.2.5.1 *Moisture Fraction Determination*—The initial moisture content of the bulk soil samples should be determined to calculate the dry weight of each soil sample. A 25 g aliquot of the bulk soil or sediment sample should be placed in a clean, crystallizing dish and weighed to obtain the initial wet weight for moisture content calculations. The combined weight of the sample and dish equals the initial wet weight. The sample should be dried at 103 - 105°C for 24 h. After drying the sample should be placed into a desiccator to cool. After cooling, the dried sample should be weighed again. The combined weight of the dish and the dried sample equals the final dry weight. The moisture fraction of the sample may be calculated using the following formula:

$$MF = (I - F)/[A - (I - F)]*100 \quad \text{(X2.1)}$$

where:

*MF* = moisture fraction of bulk soil (in %),
*I* = initial wet weight of sample + crucible (in g),
*F* = final dry weight of sample + crucible (in g), and
*A* = initial aliquot weight (in g).

X2.2.5.2 *Extract Preparation*—The following procedure may be used to prepare the soil elutriates. ASTM Type I water or FETAX Solution should be used to prepare all elutriates from soil samples. If ASTM Type I water is used to extract the samples, a control (reference site) sample must also be extracted; pH, hardness, alkalinity, and conductivity measured; and controls tested in the FETAX assay to ensure that the extract contains sufficient minerals and nutrients to support normal embryo development. Any other water used for extraction should first be tested in FETAX to show that it supports normal survival, development and growth as well as FETAX solution does alone. A weight of dilution water equal to four times the dry weight of the soil sample should be added to an appropriate mixing vessel. This mixture should then be shaken for 48-h (30 ± 2 rpm) at a constant temperature (22 ± 2°C) in darkness. A rotary extractor (end-over-end mixing) or similar apparatus may be used. Zero head space conditions should be used, if possible, in the mixing containers to prevent loss of volatile substances. The shaken sample should be allow to settle overnight in refrigerated (4 ± 2°C) storage, decanted, and pH recorded. Place the mixture in a refrigerated centrifuge (4°C) for about 20 min at 8000 rpm (5500 to 6000 x g-force) or until supernatant is clear. The elutriate should not be filtered because filtering may remove particulate material that may have toxicants absorbed onto it, resulting in an underestimation of toxicity. Sufficient quantities of elutriate should be stored for the first 24-h testing period in a vented refrigerated (4 ± 2°C) until needed. This solution should be used within 24-h. The temperature of the elutriate must be equilibrated to 24 ± 2°C before adding embryos. Divide the remaining elutriate samples into aliquots of 10 mL with no head space. The aliquots should be refrigerated (4 ± 2°C) with no headspace until needed for

the daily test renewal or physical/chemical testing. The elutriate can be used in the presence of the metabolic activation system to bioactivate toxicants. See Guide E 1391 for additional soil/sediment preparation techniques.

## X3. CONCENTRATION STEPS FOR RANGE FINDING TESTS

**TABLE X3.1 Concentration Steps for Range Finding Tests**

| 0.001 to 0.01 Range | 0.01 to 0.1 to Range | 0.1 to 1 Range | 1 to 10 Range | 10 to 100 Range |
|---|---|---|---|---|
| 0.001 | 0.01 | 0.1 | 1.0 | 10 |
| 0.0015 | 0.015 | 0.15 | 1.5 | 15 |
| 0.002 | 0.02 | 0.2 | 2.0 | 20 |
| 0.0025 | 0.025 | 0.25 | 2.5 | 25 |
| 0.003 | 0.03 | 0.3 | 3.0 | 30 |
| 0.0035 | 0.035 | 0.35 | 3.5 | 35 |
| 0.004 | 0.04 | 0.4 | 4.0 | 40 |
| 0.0045 | 0.045 | 0.45 | 4.5 | 45 |
| 0.005 | 0.05 | 0.5 | 5.0 | 50 |
| 0.0055 | 0.055 | 0.55 | 5.5 | 55 |
| 0.006 | 0.06 | 0.6 | 6.0 | 60 |
| 0.0065 | 0.065 | 0.65 | 6.5 | 65 |
| 0.007 | 0.07 | 0.7 | 7.0 | 70 |
| 0.0075 | 0.075 | 0.75 | 7.5 | 75 |
| 0.008 | 0.08 | 0.8 | 8.0 | 80 |
| 0.0085 | 0.085 | 0.85 | 8.5 | 85 |
| 0.009 | 0.09 | 0.9 | 9.0 | 90 |
| 0.0095 | 0.095 | 0.95 | 9.5 | 95 |
| . . . | . . . | . . . | . . . | 100 |

## X4. MICROSOME ISOLATION REAGENTS AND NADPH GENERATING SYSTEM COMPONENTS

X4.1 *Buffer*:

X4.1.1 1. 0.05 M Tris-HCl - Adjust to pH 7.5. Store at 4°C.

X4.1.2 1.12 % w/v KCl in 0.05 M Tris-HCl: Adjust pH to 7.5. Store at 4°C.

X4.1.3 1.15 % w/v KCl in 0.02 M Tris-HCl with 0.5 % w/v bovine serum. Adjust pH to 7.5. Store at 4°C.

X4.2 *Metabolic Activation Generator System*—The following components may be added individually to the Petri dish or as a combined generator stock solution (see below). For routine work, the combined generator stock should be used.

| Component | Concentration in Petri Dish |
|---|---|
| Glucose-6-Phosphate | 3.6 mM |
| Glucose-6-Phosphate Dehydrogenase | 0.31 U/mL |
| Nicotinamide Adenine Dinucleotide (NADP) | 0.1 mM |
| Reduced Nicotinamide Adenine Dinucleotide (NADPH) | 7.0 µM |

X4.2.1 To prepare the combined generator solution, 1.85 g of glucose-6-phosphate, 132 mg NADP, and 8.4 mg NADPH to should be added 16.8 mL of FETAX solution. This will supply 50 dishes. Glucose-phosphate dehydrogenase is added separately. The generator stock should be stored at –20°C and 77 µL of the generator stock preparation is pipetted to each dish which contains a total of 8 mL of solution.

X4.3 *Antibiotics*—A stock solution of 10 000 U/mL penicillin G and 10,000 U/mL streptomycin sulfate should be prepared in FETAX solution. A final concentration of 100 U/mL of each antibiotic per Petri dish is recommended. The stock should be stored at 4°C.

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