Draft Method for the Amphibian Metamorphosis Assay

Overview of the Method

The Amphibian Metamorphosis Assay (AMA) is a screening assay intended to empirically identify substances which may interfere with the normal function of the hypothalamic-pituitary-thyroid (HPT) axis. The AMA represents a generalized vertebrate model to the extent that it is based on the conserved structures and functions of the HPT axis. It is an important assay because amphibian metamorphosis provides a well-studied, thyroid-dependent process which responds to substances active within the HPT axis, and it is the only assay that detects thyroid activity in a developing animal.

The general experimental design entails exposing *Xenopus laevis* tadpoles at stage 51 to four different concentrations of a test chemical and a dilution water control for 21 days. There are four replicates of each test treatment. Larval density at test initiation is 20 tadpoles per test tank for all treatment groups. The primary endpoints are hind limb length, body length (whole body length [WBL] and snout to vent [SVL]), developmental stage, wet weight, thyroid histology, and daily observations of mortality.

Study Design

Test Species

Xenopus laevis is routinely cultured in laboratories worldwide and is easily obtainable through commercial suppliers. Reproduction can be easily induced in this species throughout the year using human chorionic gonadotropin (hCG) injections and the resultant larvae can be routinely reared to selected developmental stages in large numbers to permit the use of stage-specific test protocols. Larvae used for the assay should be from in-house adults as opposed to shipping larval stages of frogs for direct use in the study.

Equipment and Supplies

The following equipment and supplies are needed for the conduct of this assay:
• Exposure system (see description below);
• Glass aquaria (see description below);
• Breeding tanks
• L-cysteine (optional)
• NaOH
• Temperature controlling apparatus (e.g., heaters or coolers (adjustable to 22º ± 1°C));
• Thermometer;
• Binocular dissection microscope;
• Digital camera with at least 4 megapixel resolution and micro function;
• Image digitizing software;
• Petri dish (100 x 15 mm) or transparent plastic chamber of comparable size;
• Analytical balance capable of measuring to 3 decimal places (mg);
• Dissolved oxygen meter;

• pH meter;
• Light intensity meter capable of measuring in lux units;
• Miscellaneous laboratory glassware and tools;
• Adjustable pipetters (10 to 5,000 µL) or assorted pipettes of equivalent sizes.
- Test chemical in sufficient quantities to conduct the study, preferably of one lot #
- Analytical instrumentation appropriate for the chemical on test or contracted analytical services

Chemical Testability

This test is based upon an aqueous exposure protocol whereby test chemical is introduced into the test chambers via a flow through system. Flow-through methods however, introduce constraints on the types of chemicals that can be tested, as determined by the physicochemical properties of the compound. Therefore, prior to using this protocol, baseline information about the chemical shall be obtained that is relevant to determining the testability, and the OECD Guidance Document on Aquatic Toxicity Testing of Difficult Substances and Mixtures should be consulted[2]. Characteristics which indicate that the chemical may not be testable include: high octanol water partitioning coefficients (log K_{ow}), high volatility, susceptibility to hydrolysis, and susceptibility to photolysis under ambient laboratory lighting conditions. Other factors may also be relevant to determining testability and should be determined on a case by case basis. If a successful test is not possible for the chemical using a flow-through test system, a static renewal system may be employed. If neither system is capable of accommodating the test chemical, then the negative default is to not test it using this protocol.

If the flow-through system can accommodate the chemical and maintain constant concentrations of the chemical, conduct the test according to the method.

Exposure System

A flow-through diluter system is preferred, when possible, over a static renewal system. If physical and/or chemical properties of any of the test substances are not amenable to a flow-through diluter system, then an alternative exposure system (e.g., static-renewal) can be employed. The system components should have water-contact components of glass, stainless steel, and/or Teflon®. However, suitable plastics can be utilized if they do not compromise the study. Exposure tanks should be glass aquaria (with approximate measurements of 22.5 x 14.0 x 16.5 cm deep) equipped with standpipes that result in an approximate tank volume of 4.0 L and minimum water depth of 10 to 15 cm. The system should be capable of supporting all exposure concentrations and a control, with up to four replicates per treatment. The flow rate to each tank should be 25 mL/min. The treatment tanks shall be randomly assigned to a position in the exposure system in order to reduce potential positional effects, including slight variations in temperature, light intensity, etc. Fluorescent lighting should be used to provide a photoperiod of 12 hr light: 12 hr dark at an intensity that ranges from 600 to 2,000 lux (lumens/m2) at the water surface. Water temperature should be maintained at 22º ± 1°C, pH maintained between 6.5 to 8.5, and the dissolved oxygen (DO) concentration > 3.5 mg/L (> 40% of the air saturation) in each test tank and should be measured daily. Table 1 outlines the experimental conditions under which the protocol should be executed.

Iodine Concentration in Test Water

In order for the thyroid gland to synthesize TH, sufficient iodide needs to be available to the larvae through a combination of aqueous and dietary sources. Currently, there are no empirically derived guidelines for minimal iodide concentrations. However, iodide availability may affect the responsiveness of the thyroid system to thyroid active agents and is known to modulate the basal activity of the thyroid gland, an aspect that deserves attention when interpreting the results from thyroid histopathology. Therefore, determination of aqueous iodide concentrations must be considered as an important measurement in any bioassay used for the detection of thyroid active substances. Ideally, the minimum iodine concentration in the test water should 0.5 μ/L. If the test water is reconstituted from deionized water, iodine must be added at a minimum concentration of 0.5 μ/L. Any additional supplementation of the test water with iodine or other salts must be noted in the report.

Table 1. Experimental Conditions for the 21-day Amphibian Metamorphosis Assay.

Test Animal		*Xenopus laevis* larvae	
Initial Larval Stage		NF stage 51	
Exposure Period		21 days	
Larvae Selection Criteria		Developmental stage and total length (optional)	
Test Concentrations		Minimum of 3 concentrations spanning approximately one order of magnitude	
Exposure Regime		flow-through (preferred) and/or static-renewal	
Test System Flow-Rate		25 mL/min (complete volume replacement ca. every 2.7 h)	
Primary Endpoints / Determination Days		Mortality	Daily
		Developmental Stage	D 7 and 21
		Hind Limb Length	D 7 and 21
		Whole Body Length	D 7 and 21
		Snout-Vent Length	D 7 and 21
		Wet Body Weight	D 7 and 21
		Thyroid Histology	D 21
Dilution Water / Laboratory Control		Dechlorinated tap water (charcoal-filtered) or the equivalent laboratory source	
Larval Density		20 larvae / test vessel (5 / L)	
Test Solution / Test Vessel		4 L (10-15 cm minimum water) / Glass Aquaria (22.5 cm x 14 cm x 16.5 cm)	
Replication		4 replicate tanks / test concentration and control	
Acceptable Mortality Rate in Controls		< 10% overall	
Thyroid Fixation	Number Fixed	5 / Replicate tank, 20 total/treatment	
	Region	Head or whole body	
	Fixation Fluid	Davidson's fixative	
Feeding	Food	Sera Micron®	
	Amount / Frequency	See table below for feeding regime	
Lighting	Photoperiod	12 h Light : 12 h dark	
	Intensity	600 to 2000 lux (Measured at Water Surface)	
Water Temperature		22º ± 1ºC	
pH		6.5 – 8.5	
Dissolved Oxygen (DO) Concentration		>3.5 mg/L (>40% Air Saturation)	
Analytical Chemistry Sample Schedule		Once / Week (4 Sample Events / Test)	

Adult Care and Breeding

Adult care and breeding is conducted in accordance with standard guidelines [1] and the reader is directed to the standard guide for performing FETAX for more detailed information . The *X. laevis* larvae used for the study should be obtained exclusively from in-house cultures. To induce breeding, pairs (3-5) of adult females and males are injected with human chorionic gonadotropin (hCG). Female and male specimens are injected with approximately 800 IU and 600 IU, respectively, of hCG dissolved in 0.9% saline solution. Breeding pairs are held in large tanks, undisturbed and under static conditions in order to promote amplexus. The bottom of each breeding tank should have a false bottom of stainless steel or plastic mesh which permits the egg masses to fall to the bottom of the tank. Frogs injected in the late afternoon will usually deposit most of their eggs by mid morning of the next day. After a sufficient quantity of eggs are released and fertilized, adults shall be removed from the breeding tanks.

Larval Care and Selection

After the adults are removed from the breeding tanks, the eggs are collected and evaluated for viability using a representative sub-set of the embryos from all breeding tanks. The best spawn(s) (1 required; 2-3 recommended) should be retained based upon embryo viability and the presence of an adequate number (minimum of 1500) of embryos. The embryos are removed from the breeding tank and treated with a 2% L-cysteine solution for approximately two minutes to remove the adhesive jelly coat. Treatment with 2% L-cysteine is optional, however if used, the residual L-cysteine should be removed by rinsing the embryos with clean culture water multiple times. The embryos are transferred into a large flat pan or dish and all obvious dead or abnormal eggs are removed using a pipette or eyedropper. The sound embryos from each spawn are transferred into three hatching tanks. Four days after being placed in the hatching tanks, the best spawn, based on viability and hatching success, is selected and the larvae are transferred into an appropriate number of rearing tanks at approximately 22 °C. In addition, some additional larvae are moved into extra tanks for use as replacements in the event that mortalities occur in the rearing tanks during the first week. This procedure maintains consistent organism density and thereby reduces developmental divergence within the cohort of a single spawn. All rearing tanks shall be siphoned clean daily. Mortalities shall be removed daily and replacement larvae shall be added back to maintain the organism density during the first week. Larvae are fed twice/day Monday through Friday and once/day on the weekends and holidays, as appropriate.

During the pre-exposure phase, tadpoles are acclimated to the conditions of the actual exposure phase including; the type of food, temperature, light-dark cycle, and the culture medium. If a static culture system is used for maintaining tadpoles during the pre-exposure phase, the culture medium should be replaced completely at least twice per week. Crowding, caused by high larval densities during the pre-exposure period, should be avoided because such effects could markedly affect tadpole development during the subsequent testing phase. Therefore, the rearing density should not exceed approximately four tadpoles/L culture medium (static exposure system) or 10 tadpoles/L culture medium (50 mL/min flow-through exposure system). Under these conditions, tadpoles should develop from stages 45/46 to stage 51 within approximately two weeks.

Representative tadpoles of this stock population should be inspected daily for developmental stage in order to estimate the appropriate time point for initiation of exposure. All tadpoles that are used as test organisms should be derived from the same spawn.

Larval Culture and Feeding

Tadpoles are fed Sera Micron® (Sera GmbH, Heinsberg, Germany) throughout the pre-exposure period (after NF stage 46) and during the entire test period of 21 days. Sera Micron®, a commercially available tadpole food that has been shown to support proper growth and development of *X. laevis* tadpoles, is a fine particulate that stays suspended in the water column for a long period of time and is subject to washing out with the flow. Therefore, the total daily amount of food should be divided into smaller portions and fed twice daily. A recommended feeding regime is outlined in Table 2 for the course of the in-life portion of the study. Feeding rates are recorded. Sera Micron® can be fed dry or as a stock solution prepared in dilution water. Such a stock solution should be freshly prepared every other day.

Table 2. Feeding regime for *X. laevis* tadpoles during the in-life portion of the AMA.

Study Day	Food ration (mg Sera Micron/animal/day)
0-4	30
5-7	40
7-10	50
11-14	70
15-21	80

Analytical Chemistry

Test solutions from each replicate tank at each concentration shall be sampled for analytical chemistry at test initiation (day 0), and weekly during the test for a minimum of four samples. It is also recommended that each test concentration be analyzed during system preparation, prior to test initiation, to verify system performance. In addition, it is recommended that stock solutions be analyzed when they are changed, especially if the volume of the stock solution does not provide adequate amounts of chemical to span the duration of routine sampling periods. In the case of chemicals which cannot be detected at some or all of the concentrations used in a test, stock solutions shall be measured and system flow rates recorded in order to calculate nominal concentrations. Also, prior to conducting a study, the stability of the test compound shall be evaluated using existing information on its solubility, degradability, and volatility.

Chemical Delivery

The method used to introduce the test chemical to the system can vary depending on the physicochemical properties of the test chemical. Water soluble compounds can be dissolved in aliquots of test water at a concentration which allows delivery at the target test concentration in a flow through system. Chemicals which are liquid at room temperature and sparingly soluble in water can be introduced using liquid:liquid saturator methods. Chemicals which are solid at

room temperature and are sparingly soluble in water can be introduced using glass wool column saturators. The preference is to use a carrier-free test system, however different test chemicals will possess varied physicochemical properties that will likely require different approaches for preparation of chemical exposure water. It is preferred that effort be made to avoid solvents or carriers because: (1) certain solvents themselves may result in toxicity and/or undesirable or unexpected endocrinological responses, (2) testing chemicals above their water solubility (as can frequently occur through the use of solvents) can result in inaccurate assessments of risk from the perspective of contaminant bioavailability, and (3) the use of solvents in longer-term tests can result in a significant degree of "biofilming" associated with microbial activity. For difficult to test substances, a solvent may be employed as a last resort, and the OECD guidance document on aquatic toxicity testing of difficult substances and mixtures should be consulted [2] to determine the best method. If solvent carriers are used, appropriate solvent controls must be evaluated in addition to non-solvent controls. If it is not possible to administer a chemical via the water, either because of physicochemical characteristics (low solubility) or limited chemical availability, introducing it via the diet may be considered. Preliminary work has been conducted on dietary exposures, however, this route of exposure is not commonly used. The choice of method shall be documented and analytically verified.

Establishing the High Test Concentration

For the purposes of this test, the high test concentration shall be set by the maximum tolerated concentration (MTC) for acutely toxic chemicals or 100 mg/L, whichever is lowest.

The MTC is defined as the highest test concentration of the chemical which results in less than 10% acute mortality. Using this approach assumes that there are existing empirical acute mortality data from which the MTC can be estimated. Estimating the MTC can be inexact and typically requires some professional judgment. Although the use of regression models may be the most technically sound approach to estimating the MTC, a useful approximation of the MTC can be derived from existing acute data by using 1/3 of the acute LC50 value. However, acute toxicity data may be lacking for the species on test. If species specific acute toxicity data are not available, then a 96 hour LC50 test can be completed with organisms that are representative of those on test in the AMA. Optionally, if data from other aquatic species are available (e.g. LC50 studies in fish or other amphibian species), then professional judgment may be used to estimate a likely MTC based on inter-species extrapolation.

Alternatively, if the chemical is not acutely toxic and is soluble above 100 mg/L, then 100 mg/L shall be considered the HTC as this concentration is typically considered "practically non-toxic."

Static renewal methods may be used where flow-through methods are inadequate to achieve the MTC. If static renewal methods are used, then the stability of the test chemical concentration must be documented and remain within the performance criteria limits. Twenty-four hour renewal periods are recommended. Renewal periods exceeding 72 hours are not acceptable. Additionally, water quality parameters (e.g. DO, temperature, pH, etc.) must be measured at the end of each renewal period, immediately prior to renewal.

Test Concentration Range

There is a required *minimum* of three test concentrations and a clean water control (and vehicle control if necessary). The minimum test concentration differential between the highest and lowest shall be at least one order of magnitude. The maximum dose separation is 0.33 and the minimum is 0.5. For example, if using three test concentrations, the concentrations may be 1, 0.33, and 0.11 the high test concentration.

Determination of Biological Endpoints

During the 21 day exposure phase, measurement of primary endpoints is performed on days 7 and 21, however daily observation of test animals is necessary. Table 3 provides an overview of the measurement endpoints and the corresponding observation time points. More detailed information for technical procedures for measurement of apical endpoints and histological assessments is available in the attached guidance documents.

Table 3. Observation time points for primary endpoints for the AMA.

Apical Endpoints	Daily	Day 7	Day 21
-Mortality	●		
-Developmental Stage		●	●
-Hind Limb Length		●	●
-Whole Body Length		●	●
-Snout-Vent Length		●	●
-Wet Body Weight		●	●
-Thyroid Gland Histology			●

Apical Endpoints

Developmental stage, hind limb length, WBL, SVL and wet weight are the apical endpoints of the AMA, and each is briefly discussed below. Further technical information for collecting these data is available in the guidance documents attached including procedures for computer-assisted analysis which are recommended for use.

Developmental Stage

The developmental stage of *X. laevis* tadpoles is determined using the staging criteria of Nieuwkoop and Faber [3].

Hind Limb Length

Differentiation and growth of the hind limbs are under control of thyroid hormones and are major developmental landmarks already used in the determination of developmental stage. Hind limb development is used qualitatively in the determination of developmental stage, but is considered here as a quantitative endpoint. Therefore, hind limb length is measured as an endpoint to detect effects on the thyroid axis (Figure 1).

Body Length and Wet Weight

Determinations of body length (whole and SVL) and wet weight are included in the test protocol to assess possible effects of test substances on the growth rate of tadpoles in comparison to the control group. Because the removal of adherent water for weight determinations can cause stressful conditions for tadpoles and may cause skin damage, these measurements are only performed on day 7 histology samples and all remaining tadpoles at test termination (day 21).

Two different approaches to assess tadpole growth via body length measurements can be used: 1) whole body length; and 2) SVL length (Figure 1).

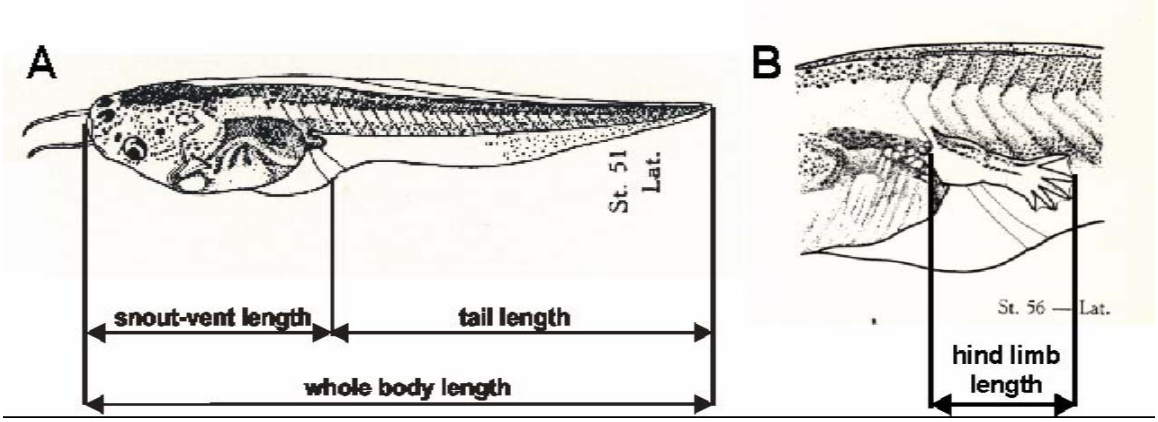

Figure 1. (A) Types of body length measurements and (B) Hind limb length measurements for *X. laevis* tadpoles (1).

Thyroid Gland Histology

While developmental stage and hind limb length are important endpoints to evaluate exposure-related changes in metamorphic development, developmental delay cannot, by itself, be considered a diagnostic indicator of anti-thyroidal activity. Some changes may only be observable by routine histopathological analysis. Please refer to "Amphibian Metamorphosis Assay: Part 1 - Technical guidance for morphologic sampling and histological preparation" [4] (Appendix 1) and "Amphibian Metamorphosis Assay: Part 2 – Approach to reading studies, diagnostic criteria, severity grading and atlas" [5] (Appendix 2) for information on obtaining samples for histological analysis and for performing histologic analyses on tissue samples. Overt and significant changes in apical endpoints indicating developmental acceleration may preclude

the necessity to perform histopathological analysis of the thyroid glands. However, absence of overt morphological changes or evidence of developmental delays warrants histological analyses.

Additional Observations

All test tanks should be checked daily for dead tadpoles and the numbers recorded for each tank. Dead animals should be removed from the test tank as soon as observed. Mortality rates exceeding 10% may indicate inappropriate test conditions or toxic effects of the test chemical. Further, cases of abnormal behavior (e.g., uncoordinated swimming, hyperventilation, atypical quiescence, non-feeding, etc.) and grossly visible malformations (e.g., limb deformities, etc.) should be recorded.

Test Initiation and Conduct

Day 0

The exposure shall be initiated when a sufficient number of tadpoles in the pre-exposure stock population have reached developmental stage 51, according to Nieuwkoop and Faber [3]. For selection of test animals, healthy and normal looking tadpoles of the stock population should be pooled in a single vessel containing an appropriate volume of dilution water. For developmental stage determination, tadpoles should be individually removed from the pooling tank using a small net or strainer and transferred to a transparent measurement chamber (e.g., 100 mm Petri dish) containing dilution water. For stage determination, one may anesthetize the tadpoles with 100 mg/L tricaine methanesulfonate (MS-222), appropriately buffered with sodium bicarbonate, before handling. Animals should be carefully handled during this transfer in order to minimize handling stress and to avoid any injury.

The developmental stage of the animals is determined using a binocular dissection microscope. In order to reduce the ultimate variability in developmental stage, it is important that this staging be conducted as accurately as possible. According to Nieuwkoop and Faber [3], the primary developmental landmark for selecting stage 51 organisms is hind limb morphology. The morphological characteristics of the hind limbs should be examined under the microscope. Because the morphological appearance of the hind limbs at stage 51 differs markedly from the limb morphology at stages 50 and 52, only little experience is needed to correctly distinguish the different stages of the tadpoles.

In addition to the developmental stage selection, an optional size selection of the experimental animals may be used. For this purpose, the whole body length should be measured at day 0 for a sub-sample of approximately 20 NF stage 51 tadpoles. After calculation of the mean whole body length for this group of animals, minimum and maximum limits for the whole body length of experimental animals can be set by allowing a range of the mean value ± 3 mm (mean values of WBL range between 24.0 and 28.1 mm for stage 51 tadpoles). However, developmental staging is the primary parameter in determining the readiness of each test animal. Tadpoles exhibiting grossly visible malformations or injuries should be excluded from the assay.

Tadpoles that meet the stage criteria described above are held in a tank of clean culture water until the staging process is completed. Once the staging is completed, the larvae are randomly distributed to exposure treatment tanks (containing 4.0 L treatment solution and labeled accordingly) until each tank contains 20 larvae. Each treatment tank is then inspected for animals with abnormal appearance (e.g., injuries, abnormal swimming behavior, etc.). Overtly unhealthy looking tadpoles should be removed from the treatment tanks and replaced with larvae newly selected from the pooling tank.

Day 7 Measurements

On day 7, five randomly chosen tadpoles per replicate are removed from each test tank and anesthetized in 150 to 200 mg/L MS-222, appropriately buffered with sodium bicarbonate. Tadpoles are rinsed in water and blotted dry, followed by body weight determination to the nearest milligram. Hind limb length, body length (WBL and SVL), and developmental stage (using a binocular dissection microscope) are determined for each tadpole. Each tadpole removed from the study should be humanely euthanized.

Day 21 Measurements (Test Termination)

At test termination (day 21), the remaining tadpoles are removed from the test tanks and euthanized in 150 to 200 mg/L MS-222, appropriately buffered with sodium bicarbonate. Tadpoles are rinsed in water and blotted dry, followed by body weight determination to the nearest milligram. Developmental stage, WBL, SVL, and hind limb lengths are measured for each tadpole.

All larvae are placed in Davidson's fixative for 48 to 72 hours either as whole body samples or as trimmed head tissue samples containing the lower jaw for histological assessments. Five tadpoles are selected from each of the treatment tanks (20/dose group total) for subsequent histopathological analysis of the thyroid gland. The remaining samples should be preserved in 10% formalin (neutral buffered) for archival storage and retained until the study is deemed acceptable. If warranted, these archived samples may also be processed and analyzed.

Since follicular cell height is stage dependent [6], the most appropriate sampling approach is to use stage-matched individuals, whenever possible. In order to select stage-matched individuals, all larvae must first be staged prior to selection and subsequent processing for data collection and preservation. This is necessary because normal divergence in development will result in differential stage distributions within each replicate tank. After the larvae are segregated by stage, the distribution of stages across the test must be evaluated. Larvae should then be sampled from the most advanced stage with sufficient number of larvae (n=5) in all replicates. If there are more than five larvae from each replicate at the appropriate stage, then five are randomly selected. If there are fewer than five larvae, then randomly selected individuals from the next lower developmental stage bin with adequate numbers of larvae should be used to reach a total sample size of five/replicate.

Data Collection, Analysis and Reporting

Data Collection

All data shall be collected using electronic or manual systems which conform to good laboratory practices (GLP) and meet the specific quality assurance plan for the study. Data from these studies generally fall into the following categories:

Operational records: These consist of observations pertaining to the functioning of the test system and the supporting environment and infrastructure. Typical records include: ambient temperature, test temperature, photoperiod, status of critical components of the exposure system (e.g. pumps, cycle counters, pressures), flow rates, water levels, stock bottle changes, and feeding records.

Biological observations and data: These include daily observations of mortality, food consumption, abnormal swimming behavior, lethargy, loss of equilibrium, malformations, lesions, etc. Observations and data collected at predetermined intervals include: developmental stage, hindlimb length, whole body length, snout vent length, and wet weight.

Histological data: These include narrative descriptions, as well as graded severity and prevalence scores of specific observations, as detailed in the histopathology guidance document.

Chemical observations and data: These data fall into two distinct areas, one pertaining to general water quality and the other to the test chemical. General water quality parameters include: pH, DO, conductivity, total iodine, alkalinity, and hardness. Test chemical information includes actual and nominal concentrations of the test chemical, and in some cases, non-parent chemical, as appropriate. Test chemical measurements may be required for stock solutions as well as for test solutions.

Deviations from the test method: This information should include any information or narrative descriptions of deviations from the test method.

Ad hoc observations: These observations should include narrative descriptions of the study that do not fit into the previously described categories.

Statistical analyses

Statistical analyses of the data shall follow guidelines described in the OECD Current Approaches in the Statistical Analysis of Ecotoxicity Data: A Guidance to Application [7]. For all quantitative endpoints (mortality, HHL, SVL, WBL, wet weight) that are normally distributed, perform an ANOVA followed by Dunnetts or Williams' test (if there is a linear dose response). For endpoints where the data are not normally distributed, the Mann-Whitney U test can be used or the Jonkheere-Terpstra test if there is a linear dose response. For developmental stage data

that are reported as medians instead of means, the Mann-Whitney U test can be used or the Jonkheere-Terpstra test if there is a linear dose response.

Data reporting

Appendix 3 contains daily data collection spreadsheets that can be used as guidance for raw data entry and for calculations of summary statistics. Additionally, reporting tables are provided that are convenient for communicating summaries of endpoint data. Reporting tables for histological assessments can be accessed at http://webdomino1.oecd.org/comnet/env/tf-edta.nsf under "Useful Documents" or for hard copy, are in Appendix 3.

Performance Criteria

Generally, gross deviations from the test method will result in unacceptable data for interpretation or reporting. Therefore, the following criteria have been developed as guidance for determining the quality of the test performed, the general performance of the control organisms, and for the acceptability of data acquired from a test.

Table 4. Performance criteria for the AMA.

Criterion	Acceptable limits
Test concentrations	Maintained at ≤ 20% CV(variability of measured test concentration) over the 21 day test
Mortality in controls	≤10%
Minimum median developmental stage of controls at end of test	57
Dissolved Oxygen	≥ 40% air saturation
pH	pH should be maintained between 6.5-8.5. The inter-replicate/inter-treatment differentials should not exceed 0.5.
Water temperature	22º ± 1ºC The inter-replicate/inter-treatment differentials should not exceed 0.2 ºC
Test concentrations without overt toxicity	≥ 1
Replicate performance	< 2 replicates across the test can be compromised
Special conditions for use of a solvent	If a carrier solvent is used, both a solvent control and water control must be used and results reported
	No statistically significant differences between solvent control and water control groups can be detected with any endpoint
Special conditions for static renewal system	Representative chemical analyses before and after renewal must be reported
	Ammonia levels must be measured immediately prior to renewal
	All water quality parameters listed in Table 1 must be measured immediately prior to renewal
	Renewal period must not exceed 72 hours

REFERENCES

1. ASTM. Standard Guide for Conducting the Frog Embryo Teratogenesis Assay - *Xenopus* (FETAX). E 1439-98. 2004.
2. OECD. Guidance Document on Aquatic Toxicity Testing of Difficult Substances and Mixtures. 23. 2000.
3. Nieuwkoop,P.D. & Faber,J. Normal Table of *Xenopus Laevis*. Garland Publishing, New York (1994).
4. OECD. Amphibian Metamorphosis Assay: Part 1 - Technical guidance for morphologic sampling and histological preparation. 2007.
5. OECD. Amphibian Metamorphosis Assay Histopathology Part 2 - Approach to reading studies, diagnostic criteria, severity grading and atlas. 2007.

6. Dodd,M.H.I. & Dodd,J.M. Physiology of Amphibia. Lofts,B. (ed.), pp. 467-599 (Academic Press, New York,1976).
7. OECD. Current Approaches in the Statistical Analysis of Ecotoxicity Data: A Guidance to Application. No. 54, ENV/JM/MONO(2006)18. 2006.

Appendix 1

Amphibian Metamorphosis Assay: Part 1 - Technical guidance for morphologic sampling and histological preparation

Find a current version of this document at http://webdomino1.oecd.org/comnet/env/tf-edta.nsf under "Useful Documents".

Appendix 2

Amphibian Metamorphosis Assay Histopathology: Part 2 – Approach to reading studies, diagnostic criteria, severity grading, and atlas

Find a current version of this document at http://webdomino1.oecd.org/comnet/env/tf-edta.nsf under "Useful Documents".

Appendix 3

Reporting tables for raw data and summary data.

Appendix 3 Table 1. General test chemical information.

Chemical Information:

Enter Test Substance, Concentration Units, and Treatments

Test substance:	
Concentration units:	
Treatment 1:	
Treatment 2:	
Treatment 3:	
Treatment 4:	

Date (day 0):	00/00/00	Enter date (mm/dd/yy)
Date (day 7):	00/00/00	Enter date (mm/dd/yy)
Date (day 21):	00/00/00	Enter date (mm/dd/yy)

Appendix 3 Table 2. Raw data collection sheets for days 7 and 21.

DAY X
DATE 00/00/00

ROW	Concentration TRT	Treatment Number TRT #	Replicate Number REP	Individual Number IND	Individual Identifier ID #	Developmental Stage STAGE	Body Length (mm) BL	Hindlimb Length (mm) HLL	Whole Organism Wet Weight (mg) WEIGHT
1	0.00	1							
2	0.00	1							
3	0.00	1							
4	0.00	1							
5	0.00	1							
6	0.00	1							
7	0.00	1							
8	0.00	1							
9	0.00	1							
10	0.00	1							
11	0.00	1							
12	0.00	1							
13	0.00	1							
14	0.00	1							
15	0.00	1							
16	0.00	1							
17	0.00	1							
18	0.00	1							
19	0.00	1							
20	0.00	1							
21	0.00	2							
22	0.00	2							
23	0.00	2							
24	0.00	2							
25	0.00	2							
26	0.00	2							
27	0.00	2							
28	0.00	2							
29	0.00	2							
30	0.00	2							
31	0.00	2							
32	0.00	2							
33	0.00	2							
34	0.00	2							
35	0.00	2							
36	0.00	2							
37	0.00	2							
38	0.00	2							
39	0.00	2							
40	0.00	2							
41	0.00	3							
42	0.00	3							
43	0.00	3							
44	0.00	3							
45	0.00	3							
46	0.00	3							
47	0.00	3							
48	0.00	3							
49	0.00	3							
50	0.00	3							
51	0.00	3							
52	0.00	3							
53	0.00	3							
54	0.00	3							
55	0.00	3							
56	0.00	3							
57	0.00	3							
58	0.00	3							
59	0.00	3							
60	0.00	3							
61	0.00	4							
62	0.00	4							
63	0.00	4							
64	0.00	4							
65	0.00	4							
66	0.00	4							
67	0.00	4							
68	0.00	4							
69	0.00	4							
70	0.00	4							
71	0.00	4							
72	0.00	4							
73	0.00	4							
74	0.00	4							
75	0.00	4							
76	0.00	4							
77	0.00	4							
78	0.00	4							
79	0.00	4							
80	0.00	4							

Appendix 3 Table 3. Calculated summaries for endpoint data from days 7 and 21.

		Developmental Stage			Body Length (mm)		Hindlimb Length (mm)		Weight (mg)	
TRT	REP	MIN	MEDIAN	MAX	MEAN	STD DEV	MEAN	STD DEV	MEAN	STD DEV
1	1	0	#NUM!	0	#DIV/0!	#DIV/0!	#DIV/0!	#DIV/0!	#DIV/0!	#DIV/0!
1	2	0	#NUM!	0	#DIV/0!	#DIV/0!	#DIV/0!	#DIV/0!	#DIV/0!	#DIV/0!
1	3	0	#NUM!	0	#DIV/0!	#DIV/0!	#DIV/0!	#DIV/0!	#DIV/0!	#DIV/0!
1	4	0	#NUM!	0	#DIV/0!	#DIV/0!	#DIV/0!	#DIV/0!	#DIV/0!	#DIV/0!
2	1	0	#NUM!	0	#DIV/0!	#DIV/0!	#DIV/0!	#DIV/0!	#DIV/0!	#DIV/0!
2	2	0	#NUM!	0	#DIV/0!	#DIV/0!	#DIV/0!	#DIV/0!	#DIV/0!	#DIV/0!
2	3	0	#NUM!	0	#DIV/0!	#DIV/0!	#DIV/0!	#DIV/0!	#DIV/0!	#DIV/0!
2	4	0	#NUM!	0	#DIV/0!	#DIV/0!	#DIV/0!	#DIV/0!	#DIV/0!	#DIV/0!
3	1	0	#NUM!	0	#DIV/0!	#DIV/0!	#DIV/0!	#DIV/0!	#DIV/0!	#DIV/0!
3	2	0	#NUM!	0	#DIV/0!	#DIV/0!	#DIV/0!	#DIV/0!	#DIV/0!	#DIV/0!
3	3	0	#NUM!	0	#DIV/0!	#DIV/0!	#DIV/0!	#DIV/0!	#DIV/0!	#DIV/0!
3	4	0	#NUM!	0	#DIV/0!	#DIV/0!	#DIV/0!	#DIV/0!	#DIV/0!	#DIV/0!
4	1	0	#NUM!	0	#DIV/0!	#DIV/0!	#DIV/0!	#DIV/0!	#DIV/0!	#DIV/0!
4	2	0	#NUM!	0	#DIV/0!	#DIV/0!	#DIV/0!	#DIV/0!	#DIV/0!	#DIV/0!
4	3	0	#NUM!	0	#DIV/0!	#DIV/0!	#DIV/0!	#DIV/0!	#DIV/0!	#DIV/0!
4	4	0	#NUM!	0	#DIV/0!	#DIV/0!	#DIV/0!	#DIV/0!	#DIV/0!	#DIV/0!

Note: Cell calculations are associated with data entries into table 2.

Appendix 3 Table 4. Daily mortality data.

Test Day	Date	1	2	3	4	1	2	3	4	1	2	3	4	1	2	3	4
0	00/00/00																
1	#VALUE!																
2	#VALUE!																
3	#VALUE!																
4	#VALUE!																
5	#VALUE!																
6	#VALUE!																
7	#VALUE!																
8	#VALUE!																
9	#VALUE!																
10	#VALUE!																
11	#VALUE!																
12	#VALUE!																
13	#VALUE!																
14	#VALUE!																
15	#VALUE!																
16	#VALUE!																
17	#VALUE!																
18	#VALUE!																
19	#VALUE!																
20	#VALUE!																
21	#VALUE!																
Replicate count		0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
Treatment count		0				0				0				0			

Note: Cell calculations are associated with data entries into table 1.

Appendix 3 Table 5. Summary chemistry data.

Chemical name:																				
CAS #:																				
Test Day	Date	1	2	3	4	1	2	3	4	1	2	3	4	1	2	3	4	1	2	3	4
0	00/00/00																				
1	#VALUE!																				
2	#VALUE!																				
3	#VALUE!																				
4	#VALUE!																				
5	#VALUE!																				
6	#VALUE!																				
7	#VALUE!																				
8	#VALUE!																				
9	#VALUE!																				
10	#VALUE!																				
11	#VALUE!																				
12	#VALUE!																				
13	#VALUE!																				
14	#VALUE!																				
15	#VALUE!																				
16	#VALUE!																				
17	#VALUE!																				
18	#VALUE!																				
19	#VALUE!																				
20	#VALUE!																				
21	#VALUE!																				

Note: Cell calculations are associated with data entries into table 1.

Appendix 3 Table 6. Histopathology reporting tables for core criteria.

Date: Chemical: Pathologist:

		Thyroid gland hypertrophy	Thyroid gland atrophy	Follicular cell hypertrophy	Follicular cell hyperplasia
Control Animal ID - replicate 1					
Control Animal ID - replicate 2					
Total:					

		Thyroid gland hypertrophy	Thyroid gland atrophy	Follicular cell hypertrophy	Follicular cell hyperplasia
____ Dose Animal ID - replicate 1					
____ Dose Animal ID - replicate 2					
Total:					

		Thyroid gland hypertrophy	Thyroid gland atrophy	Follicular cell hypertrophy	Follicular cell hyperplasia
____ Dose Animal ID - replicate 1					
____ Dose Animal ID - replicate 2					
Total:					

		Thyroid gland hypertrophy	Thyroid gland atrophy	Follicular cell hypertrophy	Follicular cell hyperplasia
____ Dose Animal ID - replicate 1					
____ Dose Animal ID - replicate 2					
Total:					

Appendix 3 Table 7. Additional histopathology criteria.

Date: Chemical: Pathologist:

		Follicular lumen area increase	Follicular lumen area decrease
Control Animal ID - replicate 1			
Control Animal ID - replicate 2			
Total:			

		Follicular lumen area increase	Follicular lumen area decrease
____ Dose Animal ID - replicate 1			
____ Dose Animal ID - replicate 2			
Total:			

		Follicular lumen area increase	Follicular lumen area decrease
____ Dose Animal ID - replicate 1			
____ Dose Animal ID - replicate 2			
Total:			

		Follicular lumen area increase	Follicular lumen area decrease
____ Dose Animal ID - replicate 1			
____ Dose Animal ID - replicate 2			
Total:			

Appendix 3 Table 8. Narrative descriptions for histopathological findings.

Date:
Chemical:
Pathologist:

		Narrative description
Control Animal ID - replicate 1		
Control Animal ID - replicate 2		
____ Dose ____ Animal ID - replicate 1		
____ Dose ____ Animal ID - replicate 2		
____ Dose ____ Animal ID - replicate 1		
____ Dose ____ Animal ID - replicate 2		

____ Dose ____ Animal ID - replicate 1		
____ Dose ____ Animal ID - replicate 2		

Appendix 3 Table 9. Summary reporting table template for day x (7 or 21) of the AMA.

		Control				Dose 1					Dose 2					Dose 3				
Endpoint	**Replicate**	**Mean**	**SD**	**CV**	**N**	**Mean**	**SD**	**CV**	**N**	***p*-value**	**Mean**	**SD**	**CV**	**N**	***p*-value**	**Mean**	**SD**	**CV**	**N**	***p*-value**
Hind Limb Length (mm)	1																			
	2																			
	3																			
	4																			
	Mean:																			
Whole Body Length (mm)	1																			
	2																			
	3																			
	4																			
	Mean:																			
Snout Vent Length (mm)	1																			
	2																			
	3																			
	4																			
	Mean:																			
Wet Weight (mg)	1																			
	2																			
	3																			
	4																			
	Mean:																			

Appendix 3 Table 10. Summary reporting table template for day x (7 or 21) developmental stage data for the AMA.

		Control				**Dose 1**					**Dose 2**					**Dose 3**				
	Replicate	**Median**	**Min**	**Max**	**N**	**Median**	**Min**	**Max**	**N**	***p*-value**	**Median**	**Min**	**Max**	**N**	***p*-value**	**Median**	**Min**	**Max**	**N**	***p*-value**
Developmental Stage	1																			
	2																			
	3																			
	4																			
	Mean:																			