INTRODUCTION

1. This Guideline is based on a decision from 1998 to develop new or revise existing OECD Guidelines for the screening and testing of potential endocrine disrupters. The Fish Sexual Development Test (FSDT) was identified as a promising test method covering a sensitive fish life stage responsive to both oestrogen and androgen-like chemicals. The test method went through an inter-laboratory validation exercise from 2006 to 2010 where Japanese medaka (Oryzias latipes) and zebrafish (Danio rerio) were fully validated. The protocol is in principle an enhancement of OECD Guideline No. 210 ‘Fish, Early Life Stage Toxicity Test’[OECD TG 210 1992], where the exposure is continued until the fish are sexually differentiated, i.e. about 60 days post-hatch for Japanese medaka and zebrafish (The exposure period can be shorter or longer for other species that are validated in the future). The FSDT assesses early life-stage effects and potential adverse consequences of putative endocrine disrupting chemicals (e.g., oestrogens, androgens and steroidogenesis inhibitors) on sexual development. The combination of the two core endocrine endpoints, vitellogenin (VTG) concentration and the population-relevant sex ratio enable the test to be used for hazard and risk assessment when the chemical mode of action is known.

2. The protocol is based on chemicals exposure via water to fish during the sex labile period in which the fish is expected to be most sensitive toward the effects of endocrine disrupting chemicals. Two core endpoints are measured as indicators of endocrine-associated developmental aberrations, the VTG concentrations; and sex ratios (proportions of sex) determined via gonad histology. Gonadal histopathology (evaluation and staging of oocytes and spermatogenetic cells) is optional. Additionally the genetic sex is determined whenever possible (e.g. in Japanese medaka). Other apical endpoints measured include hatching rate, survival, length and body weight. The test method might also be adaptable to other species than those mentioned above, provided a validation equal to the one accomplished for Japanese medaka and zebrafish and provided that fish are sexually differentiated at the end of the test, VTG levels are sufficiently high to detect significant chemical-related variations, and sensitivity of the test system is established using proficiency chemicals.

INITIAL CONSIDERATIONS AND LIMITATIONS

3. VTG is normally produced by the liver of female oviparous vertebrates in response to circulating endogenous oestrogen [Jobling et al. 1996]. It is a precursor of egg yolk proteins and, once produced in the liver, travels in the bloodstream to the ovary, where it is taken up and modified by developing eggs. The VTG synthesis is very limited, though detectable, in immature fish and adult male fish because they lack sufficient circulating oestrogen; however, the liver is capable of synthesizing and secreting VTG in response to exogenous oestrogen stimulation [Sumpter and Jobling 1995; Tyler et al. 1999; Holbech et al. 2001a].

4. The measurement of VTG serves for the detection of chemicals with oestrogenic, anti-oestrogenic, androgenic and anti-androgenic modes of action and chemicals that interfere with steroidogenesis as for
example aromatase inhibitors. The detection of oestrogenic chemicals is possible via the measurement of VTG induction in male fish, and it has been abundantly documented in the scientific peer-reviewed literature. VTG induction has also been demonstrated following exposure to aromatizable androgens [Andersen et al. 2003; Orn et al. 2003]. A reduction in the circulating level of oestrogen in females, for instance through the inhibition of the aromatase converting the endogenous androgen to the natural oestrogen 17β-estradiol, causes a decrease in the VTG concentration, which is used to detect chemicals having aromatase inhibiting properties or steroidogenesis inhibitors more broadly [Kinnberg et al. 2007]. The biological relevance of the VTG response following oestrogenic/aromatase inhibition is established and has been broadly documented [Panter et al. 2002; Sun et al. 2007]. However, it is possible that production of VTG in females can also be affected by general toxicity and non-endocrine toxic modes of action, e.g. hepatotoxicity.

5. Several measurement methods have been successfully developed and standardised for routine use to quantify VTG. This is the case for all four FSDT validation species of species-specific Enzyme-Linked Immunosorbent Assay (ELISA) methods using immunochemistry for the quantification of VTG from blood, liver, whole body- or head/tail homogenate samples collected from individual [Parks et al. 1999; Holbech et al. 2001a; Brion et al. 2002; Nishi et al. 2002; Hahlbeck et al. 2004; Tatarazako et al. 2004; Eidem et al. 2006; Jensen and Ankley 2006]. In Japanese medaka and zebrafish, there is a good correlation between VTG measured from blood plasma, liver and homogenate samples although homogenate tend to show slightly lower values than plasma [Holbech et al. 2001b; Nilsen et al. 2004; Orn et al. 2006]. Annex 4 provides the recommended procedures for sample collection for VTG analysis. Species-specific and validated ELISA-kits for the measurement of VTG are widely available. The changes in VTG concentrations are already in use in TG 229 and TG 230.

6. Change in the sex ratio (proportions of sex) is an endpoint reflecting phenotypic sex reversal. In principle, oestrogens, anti-oestrogens, androgens, anti-androgens and steroidogenesis inhibiting chemicals can affect the sex ratio of developing fish [Scholz and Kluver 2009]. It has been shown that this sex reversal is partly reversible in zebrafish [Fenske et al. 2005] following oestrogenic-related chemical exposure, whereas sex reversal following androgenic-related chemical exposure is permanent [Morthorst et al. 2010]. The sex is defined as either female, male, intersex (both oocytes and spermatogenetic cells in one gonad) or undifferentiated determined in individual fish via histological examination of the gonads. Guidance is given in ANNEX 7 and “OECD Guidance Document for the Diagnosis of Endocrine-Related Histopathology of Fish Gonads” [OECD et al. 2010].

7. Genetic sex is examined via genetic markers when they exist in a given fish species. In Japanese medaka the female XX or male XY genes can be detected by PCR or the Y-linked DM domain gene (DMY) can be analysed (DMY negative or positive) as described by [Kobayashi et al. 2004; Shinomiya et al. 2004]. Where the genetic sex can be individually linked to the phenotypic sex, the power of the test is improved and therefore genetic sex must be determined in species with documented genetic sex markers.

8. The two core endocrine endpoints; VTG and sex ratio can in combination demonstrate the endocrine mode of action of the chemical (}
Table 1). The sex ratio is a population relevant biomarker [Kidd et al. 2007; Palace et al. 2009] and for some well defined modes of action, the FSDT results may be used for hazard and risk assessment purposes. These modes of action are at present oestrogens, androgens and steroidogenesis inhibitors.

9. The FSDT does not cover the reproductive life stage of the fish and therefore chemicals that are suspected to affect reproduction at lower concentrations than sexual development should be examined in a test that covers reproduction.
Table 1: Reaction of the endocrine endpoints to different modes of action of chemicals: ↑=increasing, ↓=decreasing, -=unchanged, ?=unknown.

<table>
<thead>
<tr>
<th>MOA</th>
<th>Vtg ♂</th>
<th>Vtg ♀</th>
<th>Sex ratio</th>
<th>references</th>
</tr>
</thead>
<tbody>
<tr>
<td>Weak oestrogen agonist</td>
<td>↑</td>
<td>↑</td>
<td>↑♀</td>
<td>[Panter et al. 2006]</td>
</tr>
<tr>
<td>Strong oestrogen agonist</td>
<td>↑</td>
<td>↑</td>
<td>↑♀, No ♂</td>
<td>[Holbech et al. 2006]</td>
</tr>
<tr>
<td>Oestrogen antagonist</td>
<td>?</td>
<td>↓</td>
<td>↓♀, ↑Undiff.</td>
<td>[Andersen et al. 2004]</td>
</tr>
<tr>
<td>Androgen agonist</td>
<td>↓ or -</td>
<td>↓ or -</td>
<td>↑♂, No ♀</td>
<td>[Holbech et al. 2006; Morthorst et al. 2010]</td>
</tr>
<tr>
<td>Androgen antagonist</td>
<td>-</td>
<td>↑</td>
<td>↑♀</td>
<td>[Kiparissis et al. 2003; Panter et al. 2004]</td>
</tr>
<tr>
<td>Aromatase inhibitor</td>
<td>↓</td>
<td>↓</td>
<td>↓♀</td>
<td>[Kinnberg et al. 2007]</td>
</tr>
</tbody>
</table>

10. Definitions in this Test Guideline are given in Annex 1.

11. The in vivo FSDT is intended to detect chemicals with androgenic or oestrogenic properties as well as anti-androgenic, anti-oestrogenic and aromatase inhibiting properties.

**PRINCIPLE OF THE TEST**

12. In the test, fish are exposed from newly fertilized egg until sexual differentiation has finished to at least three concentrations of the test chemical dissolved in water. The test conditions should be flow-through unless impossible due to the availability or nature of the exposure chemical. The test starts with the placing of a minimum of 160 newly fertilized eggs per treatment divided between at least 4 replicates. For the validated fish species Japanese medaka and zebrafish, the test is terminated at 60 days post hatch (dph). At test termination, all fish are euthanized humanly. A biological sample (blood plasma, liver or head/tail homogenate) is collected for VTG analysis from each fish and the remaining part is fixed for histological evaluation of the gonads to determine the phenotypic sex; optionally histopathology (e.g. staging of gonads, severity of intersex) can be performed. A biological sample (the anal- or the dorsal fin) for the determination of the genetic sex is also taken in species possessing appropriate markers (ANNEX 9). The combination of VTG measurements and sex ratio (analysed as proportion of sex) adds weight to the assessment of effects and likely mode of action (as seen in
Table 1) of the chemical in many cases.

13. An overview of relevant test conditions specific for two validated species: Japanese medaka and zebrafish is provided in ANNEX 2.

INFORMATION ON THE TEST CHEMICAL

14. Results from an acute toxicity test or other short-term toxicity assay (e.g., OECD TG 204, TG 212, TG 215) preferably performed with the species chosen for this test, should be available. This implies that the water solubility and the vapor pressure of the test chemical are known and a reliable analytical method for the quantification of the chemical in the test chambers with known and reported accuracy and limit of detection is available.

15. Useful information includes the structural formula, purity of the chemical, stability in water and light, pKₐ, Pₗₒ and results of a test for ready biodegradability (TG 301) [OECD 1992].

TEST ACCEPTANCE CRITERIA

16. For the test results to be acceptable the following conditions apply:

- The test should be performed in a laboratory capable of following the principles of Good Laboratory Practice (GLP)
- the dissolved oxygen concentration should be at least 60 per cent of the air saturation value throughout the test;
- the water temperature should not differ by more than ± 1.5 °C between test chambers at any one time during the exposure period and be maintained within a range of 2°C within the temperature ranges specified for the test species (Annex 2)
- A validated method for analysis of the exposure chemical with a detection limit well below the lowest nominal concentration should be available and evidence should be gathered to demonstrate that the concentrations of the test chemical in solution have been satisfactorily maintained within ±20% of the mean measured values;
- overall survival of fertilized eggs in the controls and, where relevant, in the solvent controls should be greater than or equal to the limits defined in ANNEX 2
- acceptance criteria related to growth and proportions of sex (control group/solvent control group mean values, based on data pooled from replicates at termination of the test):

<table>
<thead>
<tr>
<th></th>
<th>Japanese medaka</th>
<th>Zebrafish</th>
</tr>
</thead>
<tbody>
<tr>
<td>Growth (weight (wet weight*))</td>
<td>&gt;150 mg</td>
<td>&gt;75 mg</td>
</tr>
<tr>
<td>Length (standard length)</td>
<td>&gt;20mm</td>
<td>&gt;14 mm</td>
</tr>
<tr>
<td>Sex ratio (% males or females)</td>
<td>30-70 %</td>
<td>30-70 %</td>
</tr>
</tbody>
</table>

(*wet weight = Fish wet weight (blotted dry))

- When a solvent is used it should have no significant effect on survival and should not produce any endocrine disrupting effects or other adverse effects on the early-life stages as revealed by a solvent control.
DESCRIPTION OF THE METHOD

Test chambers

17. Any glass, stainless steel or other chemically inert chambers can be used. The dimensions of the chambers should be large enough to allow compliance with loading rate criteria given below. It is desirable that test chambers be randomly positioned in the test area. A randomized block design with each treatment being present in each block is preferable to a completely randomized design. The test chambers should be shielded from unwanted disturbance.

Selection of species

18. Recommended fish species are given in ANNEX 2. The test has been fully validated using the Japanese medaka (Oryzias latipes) and the zebrafish (Danio rerio). The test might be adaptable to other species provided i) they undergo a validation equal to the one accomplished for Japanese medaka and zebrafish, ii) fish are sexually differentiated at the end of the test, iii) VTG concentrations are sufficiently high to detect significant chemical-related variations, and iii) sensitivity of the test system is established using proficiency chemicals. The rationale for the selection of the species and the experimental method should be reported in this case.

Holding of the brood fish

19. Details on holding the brood stock under satisfactory conditions may be found in OECD TG 210. Brood fish should be fed twice a day (ANNEX 2 for details). Parental fish should be fed once or twice a day with appropriate food. To avoid genetic bias, eggs are collected from a minimum of three breeding pairs or groups, mixed and randomly selected to initiate the test.

Handling of embryos and larvae

20. Initially, embryos and larvae may be exposed within a main chamber in smaller glass or stainless steel chambers, fitted with mesh sides or ends to permit a flow of test chemical through the chamber. Non-turbulent flow through these small chambers may be induced by suspending them from an arm arranged to move the chamber up and down but always keeping the organisms submerged.

21. Where egg containers, grids or meshes have been used to hold eggs within the main test chamber, these restraints should be removed after the larvae hatch, except that meshes should be retained to prevent the escape of the fish. If there is a need to transfer the larvae, they should not be exposed to the air and nets should not be used to release fish from egg containers. The timing of this transfer varies with the species and transfer may not always be necessary.

Water

22. Any water in which the test species shows control survival at least as good as that described in ANNEX 3 is suitable as test water. It should be of constant quality during the period of the test. In order to ensure that the dilution water will not unduly influence the test result (for example by complexation of the test chemical) or adversely affect the performance of the brood stock, samples should be taken at intervals for analysis. Total organic carbon, conductivity, pH and suspended solids should be measured, for example every three months where dilution water is known to be relatively constant in quality. Measurements of heavy metals (e.g. Cu, Pb, Zn, Hg, Cd, Ni), major anions and cations (e.g. Ca, Mg, Na, K, Cl, SO₄) and pesticides should be done, if water quality is questionable. Details about chemical analysis and water collection can be found in Paragraph 34.
Test solutions

23. Flow-through system is recommended. For flow-through tests a system, which continually dispenses and dilutes a stock solution of the test chemical (e.g. metering pump, proportional diluter, and saturator system) is required to deliver a series of concentrations to the test chambers. The flow rates of stock solutions and dilution water should be checked at intervals during the test and should not vary by more than 10% throughout the test. A flow rate equivalent to at least five test chamber volumes per 24 hours has been found suitable [OECD TG 210 1992]. Care should be taken to avoid the use of plastic tubing or other materials, some of which may contain biologically active chemicals or may adsorb the test chemical.

24. The stock solution should preferably be prepared without the use of solvents by simply mixing or agitating the test chemical in the dilution water by using mechanical means (e.g. stirring or ultrasonication). If the test chemical is difficult to dissolve in water, procedures described in the OECD Guidance for handling difficult substances should be followed ([OECD GD 2000]). The use of solvents should be avoided but may be required in some cases in order to produce a suitably concentrated stock solution. Examples of suitable solvents are given in [OECD GD 2000].

25. Semi-static test conditions should be avoided unless there are compelling reasons associated with the test chemical (e.g., stability, limited availability, high cost or hazard). For the semi-static technique, two different renewal procedures may be followed. Either new test solutions are prepared in clean chambers and surviving eggs and larvae gently transferred into the new chambers, or the test organisms are retained in the test chambers whilst a proportion (at least two thirds) of the test water is changed daily.

PROCEDURE

Conditions of Exposure

Duration

26. The test should start as soon as possible after the eggs have been fertilized, the embryos preferably being immersed in the test solutions before cleavage of the blastodisc commences, or as close as possible after this stage. The test should continue until sexual differentiation in the control group is completed (60 days post hatch for Japanese medaka and zebrafish).

Loading

27. The number of fertilized eggs at the start of the test should be at least 160 per treatment divided between a minimum of 4 replicates. The eggs should be randomly distributed (by using statistical tables for randomization) among treatments. The loading rate (biomass per volume of test solution) should be low enough in order that a dissolved oxygen concentration of at least 60% of the air saturation value (ASV) can be maintained without directly aeration of the chambers. For flow-through tests, a loading rate not exceeding 0.5 g/l per 24 hours and not exceeding 5 g/l of solution at any time has been recommended.

Light and temperature

28. The photoperiod and water temperature should be appropriate for the test species (see ANNEX 2).

Feeding

29. Food and feeding are critical, and it is essential that the correct food for each stage should be supplied from an appropriate time and at a level sufficient to support normal growth. Feeding should be ad libitum whilst minimizing the surplus. To obtain a sufficient growth rate it is recommended that fish are fed at least
twice daily (accepting once daily in weekends), separated by at least three hours between each feeding. Surplus food and faeces should be removed as necessary to avoid accumulation of waste. As experience is gained, food and feeding regimes are continually being refined to improve survival and optimize growth. Effort should therefore be made to confirm the proposed regime with acknowledged experts. Recommendations from an OECD Guidance Document on fish testing, which is under development, should be taken into account. Feeding should be withheld 24 hours before ending the test. Examples of appropriate food items are listed in ANNEX 2.

**Test concentrations**

30. Test chemicals should be spaced as described in ANNEX 4. A minimum of three test concentrations in at least four replicates should be used. The curve relating LC$_{50}$ to period of exposure in the acute studies available should be considered when selecting the range of test concentrations. Five test concentrations are recommended if the data are to be used for risk assessment.

31. Concentrations of the chemical higher than 10% of the acute adult LC$_{50}$ or 10 mg/l, whichever is the lower, need not be tested. The maximum test concentration should be 10% of the LC$_{50}$ on the larval/juvenile life-stage (the discussion on upper limit test concentrations for ED TG’s is ongoing within the OECD and any change in this relation should be followed).

**Controls**

32. One set of dilution-water control replicates (≥ 4) and also, if relevant, one set of control replicates (≥ 4) containing the solvent should be run in addition to the test series. In relation to control groups and the statistical analysis of these, state-of-time OECD recommendations should be followed. Only solvents that have been investigated not to have any significant influence on the test endpoints should be used in the test.

33. Where a solvent is used, its final concentration should not be greater than 0.1 ml/L [OECD 2000] and it should be the same concentration in all test chambers. However, every effort should be made to avoid the use of such materials or keep concentrations to a minimum.

**Frequency of Analytical Determinations and Measurements**

34. Chemical analysis of the test chemical water concentration should be performed before initiation of the test to check compliance with the acceptance criteria. All replicates should be analyzed individually at the beginning and termination of the test. One replicate per test concentration should be analyzed at least once per week during the test, changing systematically between replicates (1,2,3,4,1,2…. ) (if other sampling frequencies are recommended by the OECD these should be followed). If samples are stored to be analyzed at a later point, the storage method of the samples should be previously validated. Samples should be filtered (e.g. using a 0.45 m pore size) or centrifuged to ensure that the determinations are made on the chemical in true solution.

35. During the test, dissolved oxygen, pH, total hardness, conductivity and salinity (if relevant) and temperature should be measured in all test chambers. As a minimum dissolved oxygen, salinity (if relevant) and temperature should be measured weekly, and pH, conductivity and hardness at the beginning and at the end of the test. Temperature should preferably be monitored continuously in at least one test chamber.

36. Results should always be based on mean measured concentrations and included in reporting.
Observations

37. **Stage of embryonic development**: The exposure should begin as fast as possible post fertilisation and no later than 24 h post fertilisation to ensure exposure during early embryonic development.

38. **Hatching and survival**: Observations on hatching and survival should be made at least once daily and numbers recorded. Dead embryos, larvae and juvenile fish should be removed as soon as observed since they can decompose rapidly and may be broken up by the actions of the other fish. Extreme care should be taken when removing dead individuals not to knock or physically damage adjacent eggs/larvae, these being extremely delicate and sensitive. Criteria for death vary according to life stage:

   - for eggs: particularly in the early stages, a marked loss of translucency and change in coloration, caused by coagulation and/or precipitation of protein, leading to a white opaque appearance;
   - for larvae and juvenile fish: immobility and/or absence of respiratory movement and/or absence of heart-beat and/or white opaque coloration of central nervous system and/or lack of reaction to mechanical stimulus.

39. **Abnormal appearance**: The number of larvae or fish showing abnormality of body form should be recorded at appearance and the nature of the abnormality described. It should be noted that abnormal embryos and larvae occur naturally and can be of the order of several per cent in the control(s) in some species. Abnormal animals should only be removed from the test chambers on death. However, this may be in conflict with some local regulations, therefore if considerable suffering (very severe and death can be reliably predicted) is observed, animals should be anaesthetized and euthanized according to the description in paragraph 33 and treated as mortality for data analysis.

40. **Abnormal behaviour**: Abnormalities, e.g. hyperventilation, uncoordinated swimming, atypical quiescence and atypical feeding behaviour should be recorded at appearance.

41. **Weight**: At the end of the test all surviving fish should be euthanized (anaesthetized if blood samples should be taken), blotted dry and individually weighed.

42. **Length**: At the end of the test, measurement of individual lengths (standard length) is recommended.

43. These observations will result in some or all of the following data being available for reporting:

   - cumulative mortality;
   - numbers of healthy fish at end of test;
   - time to start of hatching and end of hatching;
   - length and weight of surviving animals;
   - numbers of deformed larvae;
   - numbers of fish exhibiting abnormal behaviour.
Sampling of fish

44. **Fish** sampling is performed at termination of the test. Sampled fish should be euthanized with e.g. MS-222 (100-500 mg per L buffered with 200 mg NaHCO₃ per L) or FA-100 (4-allyl-2-methoxyphenol: eugenol) and individually measured and weighed as wet weight (blotted dry) or anaesthetized if a blood sample should be taken (see paragraph 49).

Sampling for VTG analysis and sex determination via histological evaluation

45. All fish should be sampled and prepared for analysis of sex and VTG. All fish should be analyzed histologically to determine sex. For the VTG measurements, a sub-sampling from at least 10 fish from each replicate is accepted. More fish should be analyzed for VTG if the results of the sub-sampling turn out to be unclear.

46. The sampling procedure for VTG and sex determination is dependant on the VTG analysis method:

*Head/tail homogenate method for VTG analysis*

47. The fish is euthanized. Head and tail of each fish are separated from the body of the fish by cuts made: right behind the pectoral fins, and right behind the dorsal fin, using a scalpel. See Figure 1. The head and tail part from each fish are pooled, weighed and individually numbered, frozen in liquid nitrogen and stored at -70° or less for VTG analysis. The body part of the fish is numbered and fixed in an appropriate fixative for histological evaluation [OECD et al. 2010]. By use of this method VTG and histopathology are evaluated on each individual and a possible change in the VTG level can thus be related to the phenotypic sex of the fish or genetic sex (Japanese medaka) of the fish (for further information sees the SOP for homogenization (ANNEX 5) and the SOP for VTG quantification (ANNEX 6).

*Liver homogenate method for VTG analysis*

48. The fish is euthanized. The liver is dissected out and stored at -70° C or less. Standard operating procedures for liver excision and pre-treatment are available from the protocol on the OECD Guidelines 229 or 230, and will be added as an annex to this protocol if there is sufficient interest. Livers are then individually homogenized as described in the SOP. The supernatant collected is then used for measuring VTG with a homologous ELISA technique (see ANNEX 6 for an example of quantification in zebrafish). Following this approach, it is also possible to have individual fish data on both VTG and gonad histology.

*Blood plasma method for VTG analysis*

49. Blood is collected from the anaesthetized fish by cardiac puncture, caudal vein or tail cutting and centrifuged at 4° C for plasma collection. The plasma is stored at -70° C or less until use. The whole fish is euthanized and fixed for histology. Both plasma samples and fish are numbered individually to relate VTG levels to the sex of the fish.

![Figure 1: How to cut a fish for measurement of VTG in head/tail homogenate and histological evaluation of the mid section](image-url)

Genetic sex determination
50. A biological sample for the determination of the genetic sex is taken from individual fish in species possessing appropriate markers. For Japanese medaka, the anal fin or dorsal fin is collected. A detailed description is given in Annex 9 including tissue sampling and sex determination by a PCR-method.

**VTG measurement**

51. The measurement of VTG should be based upon a quantitative and analytical validated method. Information should be available upon the intra-assay and inter-assay variability of the method used in a given laboratory. The source of inter- and intra-laboratory variability is (most likely) based on the different developing stages of the fish population. Therefore, NOECs based on this endpoint alone should be treated with great care. Different methods are available to assess VTG production in the fish species considered in this assay. A measurement technique that is both relatively sensitive and specific is determination of protein concentrations via enzyme-linked immunosorbant assay (ELISA). It is recommended to use homologous antibodies (raised against VTG of the same species) and most important homologous standards. Analytical validation of a quantitative method is essential to obtain meaningful results. Ideally, an internal standard, and VTG standards should be widely available.

**Sex determination**

52. Dependent on the VTG sampling procedure, whole fish or the remaining mid-section of each fish is placed in a pre-labeled processing cassette and fixed in an appropriate fixative for histological determination of sex and evaluation of gonadal staging. Guidance on fixation and embedding is provided in Annex 7 as well as in the OECD Guidance Document No.123 on the Diagnosis of Endocrine-Related Histopathology of Fish Gonads [OECD et al. 2010]. After processing, the fish are embedded in paraffin blocks. The individuals should be placed longitudinally in the paraffin block. At least six longitudinal sections (3-5 µm in thickness) in a frontal plane including gonadal tissue from both gonads are taken from each individual. The interval between these sections should be approximately 50 µm for males and 250 µm for females. However, since each block will often contain both males and females (if more than one individual are embedded in each block), the interval between sections from these blocks should be approximately 50 µm until at least six sections of the gonads from each male are obtained. Thereafter, the interval between sections can be increased to approximately 250 µm for the females. Sections are stained with haematoxylin and eosin and examined by light-microscopy with focus on sex (male, female, intersex or undifferentiated). Intersex is defined as presence of vitellogenic ooocytes (yes/no) in testis and spermatogenic cells (yes/no) in ovaries. Histopathology and staging of ovaries and testis is optional but if investigated, the results should be analyzed and reported. It should be noted that some fish species naturally lack a fully developed pair of gonads and only one gonad may be present (e.g. Japanese medaka and occasionally zebrafish). All such observations should be recorded.

53. Genetic sex determination in individual Japanese medaka is based on the presence or absence of the medaka male-sex determining gene, DMY, which is located on the Y chromosome. The genotypic sex of medaka can be identified by sequencing the DMY gene from DNA extracted from for instance a piece of anal fin or dorsal fin. The presence of DMY indicates a XY (male) individual regardless of phenotype, while the absence of DMY indicates a XX (female) individual regardless of phenotype [Kobayashi et al. 2004]. A SOP for tissue preparation and PCR method is given in Annex 9

54. The occurrence of testicular ooocytes should be reported. Concerning interpretation of intersex determination, it should be noted that a baseline level of 5% individuals of a population with mild intersex is regarded as normal in zebrafish. In Japanese medaka, a baseline level of intersex of 3% is regarded as normal. In case these rates were found at a higher incidence or severity per individual (i.e. change>> mild), this should be weighed against historical control data and subjected to expert’s judgment. However, in the interpretation, other factors must be weighed, such as dose-responsiveness, biological plausibility, additional oestrogenic or anti-androgenic effects etc. A grading system, such as recommended in the OECD Guidance Document No.123 [OECD et al. 2010] is required.
Secondary sexual characteristics

55. Secondary sexual characters are under endocrine control in species like the Japanese medaka; therefore observations of physical appearance of the fish should if possible be made at the end of the exposure. In the Japanese medaka, the papillary formation on the posterior part of the anal fin in females is androgen sensitive. OECD Guideline 230 provides relevant photographs of male secondary sex characteristics and androgenised females.

DATA AND REPORTING

Treatment of results

56. It is important that the strongest valid statistical test must determine the endpoint. The replicate is the experimental unit. A decision flow-chart is available in ANNEX 8 to help with the most appropriate statistical test to use based on the characteristic of the data obtained from the test.

Proportions of sex and genetic sex

57. The proportions of sex should be analysed for significant effect (NOEC/LOEC approach) of exposure by Jonckheere-Terpstra (Trend test) if a monotone dose-response exists. If non-monotonicity is found then a pair wise test should be applied: Use Dunnett’s test if normality and homogenous variance can be obtained. Use Tamhane-Dunnett if heterogeneous variance is present. Otherwise use exact Mann-Whitney test with Bonferroni-Holm adjustment. A flow chart describing the statistics of the proportions of sex is placed in ANNEX 8. The proportions of sex should be presented in tables as treatment proportions ± SD of males, females, intersex and undifferentiated. Statistical significance should be highlighted. Examples are presented in the FSDT Phase 2 validation report. Genetic sex should be reported as percentage of phenotypic sex reversal of males, females, intersex and undifferentiated.

VTG concentrations

58. VTG concentrations should be analysed for significant effect (NOEC/LOEC approach). The Dunnett test is preferable to the t-test with Bonferroni correction. Where a Bonferroni correction is used, the Bonferroni-Holm correction is preferable. Allowance should be made for log-transformation of VTG to achieve normality and variance homogeneity. Next, if the concentration-response is consistent with monotonicity, then the Jonckheere test is preferable to any of the above. If T-tests or Dunnett’s test is used, there is no need for a significant ANOVA F-test in order to proceed. For details see the flow chart in ANNEX 8. Results should be reported in tables as treatment means ± SD for males, females, intersex and undifferentiated separately. Statistical significance should be highlighted. Examples are presented in the FSDT Phase 2 validation report.

Chemical water concentrations

59. The actual chamber concentrations of the exposure chemical should be analysed in frequencies described in paragraph 34. Results should be reported in tables as mean concentration ± SD on replicate basis as well as on treatment basis with information on number of samples and with outliers from the mean treatment concentration ± 20% highlighted. Examples can be found in the FSDT Phase 2 validation report.

Interpretation of results

60. The results should be interpreted with caution where measured chemical concentrations in test solutions occur at levels near the detection limit of the analytical method.
Test report

61. The test report should include the following information:

Test chemical: Relevant physical-chemical properties; chemical identification, data including purity and analytical method for quantification of the test chemical.

Test conditions: Test procedure used (e.g. semi-static/renewal, flow-through); test design including test concentrations, method of preparation of stock solutions (in an ANNEX), frequency of renewal (the solubilising agent and its concentration should be given, when used); the nominal test concentrations, the means of the measured values and their standard deviations in the test chambers and the method by which these were attained (the analytical method used, should be presented in an ANNEX). Evidence that the measurements refer to the concentrations of the test chemical in true solution; Water quality within test chambers: pH, hardness, temperature and dissolved oxygen concentration; detailed information on feeding (e.g. type of food(s), source, amount given and frequency and analyses for contaminants (e.g. PCBs, PAHs and organochlorine pesticides) if relevant.

Results: Evidence that controls met the validity criteria: Data on hatching rate should be presented in tables as percentage per replicate and per treatment. Outliers from the validity criteria (in controls) should be highlighted. Survival should be presented as percentage per replicate and per treatment. Outliers from the validity criteria (in controls) should be highlighted.

Clear indication of the results obtained on the different endpoints observed: embryo survival and hatching success; external abnormalities; VTG measurements (ng/g fish, ng/ml blood or ng/mg liver); gonadal histology, sex ratio, genetic sex data. Incidence of any unusual reactions by the fish and any visible effects produced by the test chemical.

62. The results should be presented as mean values ± standard deviation (SD) or standard error (SE). Statistics should be reported as a minimum as NOEC/LOEC and confidence intervals. The statistical flow chart (Annex 8) should be followed.
References


Ref Type: Serial (Book,Monograph)


Ref Type: Generic


Ref Type: Generic


Ref Type: Report


ANNEX 1

ABBREVIATIONS & DEFINITIONS

FSDT: Fish Sexual Development Test
ED: Endocrine Disrupter
TG: Test Guideline
SOP: Standard Operational Procedure
GLP: Good Laboratory Practice
ELISA: Enzyme-Linked Immunosorbent Assay
Fish weight: Fish wet weight (blotted dry)
Loading rate: The wet weight of fish per volume of water.
Stocking density: The number of fish per volume of water.
VTG: Vitellogenin
HPG axis: Hypothalamic-pituitary-gonadal axis
ASV: Air saturation value
DMY: Y-specific DM-domain gene required for male development in the medaka fish
RT-PCR: Reverse Transcriptase Polymerase Chain-Reaction
Undifferentiated fish: Fish with gonads exhibiting no discernible germ cells.
Intersex fish: Fish with oocytes in testis or spermatogenetic cells in ovaries
ANNEX 2

EXPERIMENTAL CONDITIONS FOR THE FSDT (FRESHWATER SPECIES)

1. Recommended species
   Japanese medaka (*Oryzias latipes*)  
   Zebrafish (*Danio rerio*)

2. Test type
   Flow-through or semi-static

3. Water temperature
   25 ± 2°C  
   27 ± 2°C

4. Illumination quality
   Fluorescent bulbs (wide spectrum)

5. Light intensity
   10-20 µE/M²/s, 540-1080 lux, or 50-100 ft-c (ambient laboratory levels)

6. Photoperiod
   12-16 h light, 8-12 h dark

7. Loading rate
   Individual chambers should contain a minimum of 7 L water volume

8. Volume exchanges of test solutions
   Minimum of 5 daily

9. Age of test organisms at start of exposure
   Newly fertilised eggs

10. No. of eggs per treatment
    Minimum 160

11. No. of treatments
    Minimum 3 (plus appropriate controls)

12. No. replicates per treatment
    4 (minimum)

13. Feeding regime
    Live *Artemia*, frozen adult brine shrimp, flake food, etc. It is recommended to feed twice daily

14. Aeration
    None unless DO concentration falls below 60% saturation

15. Dilution water
    Clean surface, well or reconstituted water

16. Chemical exposure duration
    60-dph

17. Biological endpoints
    Hatching success, Survival
    Gross- morphology, VTG
    gonadal histology, Genetic sex
    Sex ratio

18. Test acceptability
    - Hatching success > 80%
    - Post hatch survival ≥ 70% of fish in the controls
    - Hatching success > 80%
    - Post hatch survival ≥ 70% of fish in the controls
## ANNEX 3

**SOME CHEMICAL CHARACTERISTICS OF AN ACCEPTABLE DILUTION WATER**

<table>
<thead>
<tr>
<th>SUBSTANCE</th>
<th>CONCENTRATIONS</th>
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</thead>
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<tr>
<td>Particular matter</td>
<td>&lt; 20 mg/l</td>
</tr>
<tr>
<td>Total organic carbon</td>
<td>&lt; 2 mg/l</td>
</tr>
<tr>
<td>Unionised ammonia</td>
<td>&lt; 1 ug/l</td>
</tr>
<tr>
<td>Residual chlorine</td>
<td>&lt; 10 ug/l</td>
</tr>
<tr>
<td>Total organophosphorus pesticides</td>
<td>&lt; 50 ng/l</td>
</tr>
<tr>
<td>Total organochlorine pesticides plus polychlorinated biphenyls</td>
<td>&lt; 50 ng/l</td>
</tr>
<tr>
<td>Total organic chlorine</td>
<td>&lt; 25 ng/l</td>
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</table>
ANNEX 4  (Might go out)

FROM TG 215/GUIDANCE ON TEST CONCENTRATIONS

<table>
<thead>
<tr>
<th>Column (Number of concentrations 100 and 10, or between 10 and 1)*</th>
<th>1</th>
<th>2</th>
<th>3</th>
<th>4</th>
<th>5</th>
<th>6</th>
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</tr>
</tbody>
</table>

* A series of three (or more) successive concentrations may be chosen from a column. Mid-points between concentrations in column (x) are found in column (2x + 1). The values listed can represent concentrations expressed as percentage per volume or weight (mg/l or μg/l). Values can be multiplied or divided by any power of 10 as appropriate. Column 1 might be used if there was considerable uncertainty on the toxicity level.

ANNEX 5

GUIDANCE: HOMOGENISATION OF HEAD & TAIL FROM JUVENILE ZEBRAFISH, FATHEAD MINNOW, THREE SPINED STICKLEBACK AND JAPANESE MEDAKA

The purpose of this section is to describe the procedures that occur prior to the quantification of the VTG concentration. Other procedures that result in comparable VTG quantification can be used.

It is an option to determine the VTG concentration in blood plasma or liver instead of head/tail homogenate

Procedure

1. The fish are anaesthetised and euthanised in accordance with the test description.
2. The head and tail are cut off the fish in accordance with the test description. Important: All dissection instruments, and the cutting board should be rinsed and cleaned properly (e.g. with 96% ethanol) between handling of each single fish to prevent “VTG pollution” from females or induced males to un-induced males.
3. The weight of the pooled head and tail from each fish is measured to the nearest mg.
4. After being weighed, the parts are placed in appropriate tubes (e.g. 1.5 ml eppendorf) and frozen at –80 ºC until homogenisation or directly homogenised on ice with two plastic pistils. (Other methods can be used if they are performed on ice and the result is a homogenous mass). Important: The tubes should be numbered properly so that the head and tail from the fish can be related to their respective body-section used for gonad histology.
5. When a homogenous mass is achieved an amount of 4-10 x the tissue weight of ice-cold homogenisation buffer* is added (note the dilution). Keep working with the pistils until the mixture is homogeneous. Important note: New pistils are used for each fish.
6. The samples are placed on ice until centrifugation at 4ºC at 50000 x g for 30 min.
7. Use a pipette to dispense portions of 20 to 50 µl (note the amount) supernatant into at least two tubes by dipping the tip of the pipette below the fat layer on the surface and carefully sucking up the supernatant without fat- or pellet fractions.
8. The tubes are stored at -80ºC until use.

*Homogenisation buffer:

(50 mM Tris-HCl pH 7.4; 1% Protease inhibitor cocktail (Sigma)): 12 ml Tris-HCl pH 7.4 + 120 µl Protease inhibitor cocktail.

TRIS: TRIS-ULTRA PURE (ICN) e.g. from Bie & Berntsen, Denmark.

Protease inhibitor cocktail: From Sigma (for mammalian tissue) Product number P 8340.

NOTE: The homogenisation buffer has to be used the same day as manufactured. Place on ice during use
ANNEX 6

GUIDANCE: QUANTIFICATION OF HEAD & TAIL HOMOGENATE VITELLOGENIN IN ZEBRAFISH (DANIO RERIO) (MODIFIED FROM HOLBECH ET AL., 2001)

1. Microtiterplates (certified Maxisorp F96, Nunc, Roskilde Denmark) previously coated with 5 µg/ml anti zebrafish lipovitellin-IgG are thawed and washed 3 times with washing buffer*.

2. Battelle zebrafish Standard AP4.6.04 (1.18 mg/ml (AAA)) is serially diluted to 0.2, 0.5, 1, 2, 5, 10 and 20 ng/ml in dilution buffer** and samples are diluted at least 200 times (to prevent matrix effect) in dilution buffer and applied to the plates. An assay control is applied in duplicate. 150 µl are applied to each well. Standards are applied in duplicate and samples in triplicate. Incubate over night at 4°C on a shaker.

3. The plates are washed 5 times with washing buffer*.

4. AMDEX (HRP coupled to a dextran chain (AMDEX A/S, Denmark)) conjugated antibodies are diluted in washing buffer; Actual dilution differs by batch and age. 150 µl are applied to each well and the plates are incubated for 1 hour at room temperature on a shaker.

5. The plates are washed 5 times with washing buffer* and the bottom of the plates is carefully cleaned with ethanol.

6. 150 µl TMB plus*** are applied to each well. Protect the plate against light with tinfoil, and watch the colour development on a shaker.

7. When the standard curve is fully developed the enzyme activity is stopped by adding 150 µl 0.2 M H₂SO₄ to each well.

8. The absorbance is measured at 450 nm (e.g. on a Molecular Devices Thermomax plate reader). Data are analysed on the associated software (e.g. Softmax).

* Washing buffer:
PBS-stock**** 500.0 ml
BSA 5.0 g
TWEEN 20 5.0 ml
Adjust pH to 7.3 and fill to 5 l with millipore H₂O. Store at 4°C.

** Dilution buffer
PBS-Stock*** 100.0 ml
BSA 3.0 g
TWEEN 20 1.0 ml
Adjust pH to 7.3 and fill to 1 l with millipore H₂O. Store at 4°C.

*** TMB plus is a "ready-to-use" substrate produced by KemEnTec (Denmark). It is sensitive to light. Store at 4°C.

**** PBS stock
NaCl 160.0 g
KH₂PO₄ 4.0 g
Na₂HPO₄,2H₂O 26.6 g
KCl 4.0 g
Adjust pH to 6.8 and fill with millipore H₂O to 2 l. Store at room temperature.
ANNEX 7

GUIDANCE: PREPARATION OF TISSUE SECTIONS FOR SEX DETERMINATION AND STAGING OF GONADS

The purpose of this section is to describe the procedures that occur prior to the evaluation of histological sections. Other procedures that result in similar sex determination and gonadal staging can be used.

With a few exceptions these procedures are similar for fathead minnow (FHM), Japanese medaka (JMD), zebrafish (ZF),)

EUTHANASIA, NECROPSY, AND TISSUE FIXATION

Objectives:

1. Provide for the humane sacrifice of fish.
2. Obtain necessary body weights and measurements.
3. Evaluate secondary sex characteristics.
4. Dissect tissues for VTG analysis.
5. Fixation of the gonads.

Procedures:

1. Fish should be sacrificed immediately prior to necropsy. Therefore, unless multiple prosectors are available, multiple fish should not be sacrificed simultaneously.
2. Using the small dip net, a fish is removed from the experimental chamber and transported to the necropsy area in the transport container.
3. The fish is placed in the euthanasia solution. The fish is removed from the solution when there is cessation of respiration and the fish is unresponsive to external stimuli.
4. The fish is wet weighed.
5. For preparation of tissues for VTG analysis the fish can be placed on a corkboard on the stage of a dissecting microscope.
   a. For FHM and ZF the head is cut right behind the pectoral fin and tail is cut right behind the dorsal fin.
   b. For JM the abdomen is opened via a carefully made incision that extends along the ventral midline from the pectoral girdle to a point just cranial to the anus. Using the small forceps and small scissors, the liver is carefully removed.
6. Specimen for VTG analysis are placed in eppendorf tubes and immediately frozen in liquid nitrogen.
7. The carcass including the gonads is placed into a pre-labelled plastic tissue cassette, which is transferred into Davidson’s or Bouin’s fixative. The volume of fixative should be at least 10 times the approximated volume of the tissues. The fixative container is gently agitated for five seconds to dislodge air bubbles from the cassette.
8. a. All tissues remain in Davidson’s fixative overnight, followed by transfer to individual containers of 10% neutral buffered formalin the next day. Containers with cassettes are gently agitated for 5 seconds to ensure adequate penetration of formalin into cassettes.

b. Tissues remain in Bouins fixative for 24 h, followed by transfer to 70% ethanol.

**TISSUE PROCESSING**

**Objectives:**

1. Dehydrate tissue for adequate penetration of paraffin.
2. Impregnate the tissue with paraffin to maintain tissue integrity and create a firm surface for microtomy.

**Procedures:**

1. Labelled tissue cassettes are removed from formalin/ethanol storage and the cassettes are placed in the processing basket(s). The processing basket is loaded in the tissue processor.
2. The processing schedule is selected.
3. After the tissue processor has completed the processing cycle, the basket(s) may be transferred to the embedded station.

**EMBEDDING**

**Objective:**

Properly orient the specimen in solidified paraffin for microtomy.

**Procedures:**

1. The basket(s) of cassettes is/are removed from the processor and immersed in the paraffin-filled front chamber of the embedding station thermal console or the cassettes are moved to a separate paraffin heater.
2. The first cassette to be embedded is removed from the front chamber of the thermal console or the paraffin heater. The cassette lid is removed and discarded, and the cassette label is checked against the animal records to resolve potential discrepancies prior to embedding.
3. An appropriately sized embedding mould is selected.
4. The mould is held under the spout of the dispensing console and filled with molten paraffin.
5. The specimen is removed from the cassette and placed in the molten paraffin in the mould. This is repeated with 4-8 specimens for each paraffin mould. The position of individual fish is marked by putting fish no 1 in 180 degrees to fish 2-4/8.
6. Additional paraffin is added to cover the specimen.
7. The mould with the cassette base is placed on the cooling plate of the cryo console.
8. After the paraffin has solidified, the block (i.e., the hardened paraffin containing the tissues and the cassette base) is removed from the mould.

MICROTOMY

Objective:
Cut and mount histological sections for staining.

Procedures:

1. The initial phase of microtomy termed “facing” is conducted as follows:
   a. The paraffin block is placed in the chuck of the microtome.
   b. The chuck is advanced by rotating the microtome wheel and thick sections are cut from the paraffin surface of the block until the knife reaches the embedded tissues.
   c. The section thickness on the microtome is set between 4 – 10 microns. The chuck is advanced and multiple sections are cut from the block to remove any artefacts created on the cut surface of the tissue during rough trimming.
   d. The block can be removed from the chuck and placed facedown on ice to soak the tissue.

2. The next phase of microtomy is final sectioning and mounting of tissue sections on slides. These procedures are conducted as follows:
   a. If the block has been placed on ice, the block is removed from the ice and replaced in the chuck of the microtome.
   b. With the section thickness on the microtome set to 4 – 5 microns, the chuck is advanced by rotating the microtome wheel. Sections are cut from the block until a “ribbon” containing at least one acceptable section including the gonads has been produced. (As necessary during sectioning, the block may be removed from the chuck, placed on ice to soak the tissue, and replaced in the chuck.)
   c. The sections are floated flat on the surface of the water in the water bath. An attempt is made to obtain at least one section that contains no wrinkles and has no air bubbles trapped beneath it.
   d. A microscope slide is immersed beneath the best section, which is lifted out of the water using the slide. This process is referred to as “mounting” the section on the slide.
   e. Three sections are prepared for a set of fish. The second and third sections are taken at 50 micron intervals following the first section. If the fish are not embedded with their gonads in the same sectioning level, more sections are to be made to ensure that at least three sections including the gonads are obtained from each fish.
   f. With a slide-marking pen, the block number from which the slide was produced is recorded on the slide.
   g. The slide is placed in a staining rack.
   h. The block is removed from the chuck and placed facedown for storage.
STAINING, COVER SLIPPING, AND SLIDE LABELLING

Objectives:

1. Stain the sections for histopathological examination
2. Permanently seal mounted and stained tissues.
3. Permanently identify stained sections in a manner that allows complete trace ability.

Procedures:

1. Staining
   a. Slides are air-dried overnight before staining.
   b. The sections are stained by Hematoxylin-Eosin.
2. Cover slipping
   a. Cover slips can be applied manually or automatically.
   b. A slide is dipped in xylene or TissueClear, and the excess xylene/TissueClear is gently knocked off the slide.
   c. Approximately 0.1 ml of mounting medium is applied near the end of the slide opposite to the frosted end or on the cover slip.
   d. The cover slip is tilted at a shallow angle as it is applied to the slide.
3. Labelling
   a. Each slide label should contain the following information.
      i. Laboratory name
      ii. Species
      iii. Specimen No. / Slide No.
      iv. Chemical / Treatment group
      v. Date
Both solvent control and non-solvent control are present.

Yes

- Compare controls using Wilcoxon or T-test
- Do controls differ?

No

- Drop water control
- Combine controls, retain subgroups

Determine whether Dose-Response is monotone

Monotone

- Step-down trend test on replicate means

Rep means normal & homogeneous

Step-down Jonckheere or Williams test

Rep means not normal or not homogeneous

Step-Down Jonckheere Test

Not monotone

Rep means normally distributed

- Variance equal
  - Dunnett test
- Variance unequal
  - variance stabilizing transform?
    - Yes
      - Normalizing transform?
        - Yes
          - Nested ANOVA normal
          - Dunn or Mann-Whitney test
        - No
          - Nested ANOVA not normal
          - Dunn Test on rep means
    - No
      - Dunn or Mann-Whitney test on rep means

Rep means not normally distributed

- Normalizing transform?
  - Yes
    - Nested ANOVA normal
    - Dunn or Mann-Whitney test
  - No
    - Dunn Test on rep means

Variance stabilizing transform?
Version of 21th December 2010

Statistical Flow Chart for sex ratio analysis

Statistics Flow-Diagram for Sex Ratio Response-NOEC

Is solvent used?

Yes

Compare controls using t-test.
Do controls differ?

Yes

Drop water control†

No

Combine controls†

No

Are data consistent with monotone dose-response?

Yes

Apply step-down Jonckheere-Terpstra test * to determine NOEC

No

Are data normally distributed?*

Yes

Use Dunnett test if homogeneous variances*, Tamhane-Dunnett (T3) test otherwise, to determine NOEC

No

Dunn or Mann-Whitney U-test w/ Bonferroni- Holm adjustment to determine NOEC

† Or other agreed control selection

* After arcsin square-root transform

+ With fewer than 5 experimental units per treatment, exact J-T or M-W tests should be used if available.
Standard Operation Procedure (SOP)
for
TISSUE SAMPLING, PREPARATION AND STORAGE BEFORE DETERMINATION
OF GENETIC SEX
(BY PCR-METHOD)
IN MEDAKA

Prepared Nov 14, 2008
Prepared by the Laboratory for Aquatic Organisms of Bayer CropScience AG
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9. With fine scissors the anal or the dorsal fin will be cut off in each individual fish and placed into a tube filled with 100 µL of extraction-buffer 1 (details on buffer preparation see below). The scissors will be cleaned after each single fish in a beaker filled up with dest. H2O and dried with a paper tissue.

10. Now the fin-tissues will be homogenized by a micro tube teflon pistol for the lysis of cells. For each tube a new pistol will be used to prevent any contaminations. The pistils will be placed overnight in 0.5 M NaOH, rinse for 5 minutes in dest. H2O and stored in ethanol or sterile after autoclave until use.

11. It is also possible to store the fin tissue without any extraction-buffer 1 on dry-ice and then at -80°C refrigerator to prevent any degeneration of the DNA. But the extraction runs better, if you extract the DNA at the same time (handling see above; samples must be thawed on ice after storing at -80°C before the buffer will be filled in the tubes).

12. After homogenizing all tubes will be placed in a water bath and boiled for 15 minutes at 100°C.

13. Then 100 µL of the extraction buffer 2 (details on buffer preparation see below) will be pipetted into each tube. The samples will be stored at room temperature for 15 minutes and in the meantime they will be sometimes gently shaken by hand.

14. Afterwards all tubes will be placed in the water bath again and boiled for another 15 minutes at 100°C.
15. Until further analysis the tubes will be frozen at -20°C.

16. Important: Before further use of the sample, the thawed tubes have to be centrifuged in an Eppendorf centrifuge (for 30 sec at max. speed). For PCR use the clear supernatant. It has to be absolutely avoided that any traces of Chelex are transferred to the PCR reaction, because this will interfere with the Taq polymerase activity.

**Buffer preparation:**

1. PCR-buffer 1:
   a. 500 mg N-Lauroylsarcosine (Merck KGaA, Darmstadt, GE)
   b. 2 mL 5M NaCl (Carl Roth GmbH + Co. KG, Karlsruhe, GE)
   c. ad 100 mL dest. H₂O → autoclave

2. PCR-buffer 2:
   a. 20 g Chelex (Biorad, Munich, GE)
   b. To swell in 100 mL dest. H₂O → autoclave
Standard Operation Procedure (SOP) for 
DETERMINATION OF GENETIC SEX (BY PCR-METHOD) 
IN MEDAKA

Prepared April 30, 2009

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The prepared and frozen tubes (described in the SOP “TISSUE SAMPLING, PREPARATION AND STORAGE BEFORE DETERMINATION OF GENETIC SEX (BY PCR-METHOD) IN MEDAKA”) will be thawed on ice. After that they will be centrifuged using an Eppendorf centrifuge (30sec at max. speed, at room temperature). For the PCR the clear supernatant separated from the precipitate will be used. It has absolutely to be avoided that any traces of Chelex (localized in the precipitate) are transferred to the PCR reaction, because this will interfere with the “Taq”-polymerase activity. The supernatant will be used directly or can be stored frozen (at -20 °C) and rethawed again in several cycles without negative impact on the DNA for later analyses.

1. **Preparation of the “Reaction Mix” (25 μL per sample):**

<table>
<thead>
<tr>
<th>Component</th>
<th>Volume</th>
<th>Final Concentration</th>
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<tbody>
<tr>
<td>Template DNA</td>
<td>0.5μl-2μl</td>
<td></td>
</tr>
<tr>
<td>10xPCR-buffer with MgCl2</td>
<td>2.5μl</td>
<td>1x</td>
</tr>
<tr>
<td>Nucleotides (each of dATP, dCTP, dGTP, dTTP)</td>
<td>4μl (5mM)</td>
<td>200μM</td>
</tr>
<tr>
<td>Forward Primer (10μM) (see below 3-5)</td>
<td>0.5μl</td>
<td>200nM</td>
</tr>
<tr>
<td>Reverse Primer (10μM) (see below 3-5)</td>
<td>0.5μl</td>
<td>200nM</td>
</tr>
<tr>
<td>DMSO</td>
<td>1.25μl</td>
<td>5%</td>
</tr>
<tr>
<td>Water (PCR grade)</td>
<td>up to 25μl</td>
<td></td>
</tr>
<tr>
<td>Taq E- Polymerase</td>
<td>0.3μl</td>
<td>1.5U</td>
</tr>
</tbody>
</table>

10xPCR-buffer with MgCl2: 670mM Tris/HCl (pH8.8 at 25°C), 160mM (NH₄)₂SO₄, 25mM MgCl2, 0.1% Tween 20

For each PCR (see below 3-5) the special primer as a new combination of “Reaction-Mix” and the adequate needed amount of template DNA for each sample (see above) is needed. The respective volumes will be transferred into new tubes using pipettes. After that all tubes will be closed, stirred (ca. 10 sec) and centrifuged (10 sec, at room temperature). Now the respective PCR-programs can be started. Additionally a positive control (exemplary DNA sample with known activity and clear results) and a negative control (1 μL dest. H₂O) will be used in each PCR-program.

2. **Preparation of the agarose gel (1 %) – During running PCR-programs:**

- Solve 3 g agarose in 300mL 1 x TAE-buffer (1% agarose gel)
- This solution has to be boiled using an microwave (ca. 2-3 min)
- Transfer the hot solution into a special casting box, which lies on ice
- After ca. 20 min the agarose gel is ready to use
- Storage the agarose gel in 1x TAE-buffer until the end of the PCR-programs
3. **Actin-PCR-program:**

This PCR-reaction is aimed to demonstrate that the DNA in the sample is not harmed.

- **Special primer:**
  - “M act 1(upper/forward)” → TTC AAC AGC CCT GCC ATG TA
  - “M act 2(lower/reverse)” → GCA GCT CAT AGC TCT TCT CCA GGG AG

- **Program:**
  - 5 min 95 °C
  - Cycle (35-times):
    - Denaturation → 45 sec at 95 °C
    - Annealing → 45 sec at 56 °C
    - Elongation → 1 min at 68 °C
  - 15 min 68 °C

4. **X- and Y-Gene-PCR-program:**

The samples with intact DNA will be used in this PCR-program to detect the X- and Y-Genes. Male DNA should show one double-band and female DNA should show one single band (after staining and gel-electrophoresis). For this program-run one positive control for males (XY-sample) and one for females (XX-sample) has to be included.

- **Special primer:**
  - “PG 17.5” (upper/forward) → CCG GGT GCC CAA GTG CTC CCG CTG
  - “PG 17.6” (lower/reverse) → GAT CGT CCC TCC ACA GAG AAG AGA

- **Program:**
  - 5 min 95 °C
  - Cycle (40-times):
    - Denaturation → 45 sec at 95 °C
    - Annealing → 45 sec at 55 °C
    - Elongation → 1 min 30 sec at 68 °C
  - 15 min 68 °C

5. **Y-Gene-PCR-program as “control” for X- and Y-Gene-PCR-program:**

This PCR-program verifies the results of the “X- and Y-Gene-PCR-program”. The “male-samples” should show one band and the “female-samples” shouldn’t show any band (after staining and gel-electrophoresis).

- **Special primer:**
  - “DMTYa (upper/forward)” → GGC CGG GTC CCC GGG TG
  - “DMTYd (lower/reverse)” → TTT GGG TGA ACT CAC ATG G
Program:
- 5 min 95 °C
- Cycle (40-times):
  - Denaturation ➝ 45 sec at 95 °C
  - Annealing ➝ 45 sec at 56 °C
  - Elongation ➝ 1 min at 68 °C
- 15 min 68 °C

6. Staining of the PCR-samples:
- Staining solution:
  - 50 % Glycerin
  - 100 mM EDTA
  - 1 % SDS
  - 0.25 % Bromphenolblue
  - 0.25 % Xylenxyanol
- Pipette 1 µL of the staining solution into each single tube

7. Start of the Gel-Electrophoresis:
- The prepared 1 % agarose gel will be transferred into a gel-electrophoresis-chamber filled with 1 x TAE-Puffer
- 10 - 15 µL of each stained PCR-sample will be pipetted into an agarose gel slot
- Also 5 - 15 µL of the 1kb-“Ladder”(Invitrogen) will be pipetted into a separate slot
- Start the electrophoresis by 200 V
- Stop after 30-45 min

8. Determination of the bands:
- Clean the agarose gel in dest. H₂O
- Now transfer the agarose gel into Ethidiumbromid for 15 - 30 min
- After that a picture of the agarose gel has to be taken in an UV-light-box
- Finally the samples are analyzed in comparison to the positive control-band (or bands) and the ladder