Section 2
Effects on Biotic Systems

Test Guideline No. 250

EASZY assay: Detection of Endocrine Active Substances, acting through estrogen receptors, using transgenic tg(cyp19a1b:GFP) Zebrafish embrYos

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OECD Guidelines for the Testing of Chemicals

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EASZY assay: Detection of Endocrine Active Substances, acting through estrogen receptors, using transgenic tg(cyp19a1b:GFP) Zebrafish embrYos

INTRODUCTION

1. The EASZY assay is a mechanism-based *in vivo* screening assay designed to detect endocrine active chemicals acting as agonist through estrogen receptors (ERs), by inducing the expression of the green fluorescent protein (GFP) driven by the cyp19a1b promoter. The EASZY assay allows for the detection of estrogenic activity of chemicals on transgenic *tg(cyp19a1b:GFP)* Zebrafish embryos exposed for 96 hours during the embryonic stages of development. Annex 1 describes the genetic construct used to establish the transgenic line (1).

2. The cyp19a1b gene codes for the brain aromatase, the enzyme responsible for the synthesis of estrogens from androgens. In fish the expression of the cyp19a1b gene is restricted to radial glial cells, which are progenitors' cells giving birth to new neurons from the embryonic stages to adulthood (2)(3). The transcriptional regulation of the cyp19a1b gene requires functional estrogen receptors (ERs) and the binding of liganded-ER on estrogen response elements (EREs) and half EREs located in the promoter region of the cyp19a1b gene (2). The cyp19a1b regulation also requires glial specific factor(s) that bind(s) to a glial x-responsive element (GxRE) acting synergistically with the ERE sequences (4). The GxRE, plays an important role in the cell-specific regulation of the cyp19a1b gene and its regulation by estrogens (4).

3. In addition to estrogens, steroidal androgens can regulate the transcriptional activity of the cyp19a1b gene. For aromatizable androgens (*e.g.* testosterone), this regulation implies their aromatization into estrogens that will subsequently bind to ERs and induce cyp19a1b expression. While 11 ketotestosterone, a non-aromatizable androgen, is ineffective to induce cyp19a1b expression, 5α-dihydrotestosterone (DHT), another non aromatizable androgen, has been shown to elicit an estrogenic activity by inducing cyp19a1b expression in a ER-dependant manner. This may be due to its conversion into β-diol, a known estrogenic steroid, through 3β-hydroxysteroid dehydrogenase activity (5).

4. Zebrafish embryos express detectable ERβ1 and ERβ2 mRNA in the telencephalon, the preoptic area and the hypothalamus but at the developmental stages used in this assay, transcripts for ERα are not detected in the brain (5)(6). This suggests that ERβ1 and/or ERβ2 would drive the aromatase B expression Zebrafish ERβ2 isoform presents higher affinity than Zebrafish ERα for natural and synthetic estrogens unlike what has been described on human ERs subtypes affinity for these substances (7).

5. In transgenic *tg(cyp19a1b:GFP)* Zebrafish, GFP perfectly mimics the expression of the endogenous cyp19a1b in the brain of Zebrafish (1)(8)(9). Therefore, measurement of the reporter gene GFP in *tg(cyp19a1b:GFP)* embryos enables the assessment of chemicals inducing expression of the cyp19a1b gene.

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6. The EASZY assay results from research and validation studies during which EASZY was successfully applied to a panel of chemicals - including natural and synthetic hormones, pharmaceuticals, pesticides, industrial chemicals - for assessing their capacity to induce GFP in *tg(cyp19a1b:GFP)* Zebrafish embryos (10)(11)(12)(13)(14)(15).

7. The Revised Guidance Document 150 on Standardised Test Guidelines for Evaluating Chemicals for Endocrine Disruption (16) identifies EASZY as a level 3 fish screening assay of the OECD Conceptual Framework for Endocrine Disruption, *i.e.* an *in vivo* assay providing data about selected endocrine mechanism(s)/ pathway(s). As a screening assay of level 3, EASZY allows identifying active and inactive test substances on the ER-signalling pathway to induce GFP driven by the ER-regulated cyp19a1b promoter. Data are not intended to be used for risk assessment of chemicals.

DEFINITIONS

8. The definitions, relevant for the purpose of this guideline, are given in ANNEX 2

INITIAL CONSIDERATIONS AND LIMITATIONS

9. Annex 3 illustrates the pathways through which chemicals can elicit estrogenic activity as measured by induction of GFP driven by the Zebrafish ER-regulated cyp19ab promoter in the EASZY assay.

10. EASZY enables the detection of chemicals acting as agonist ligands of ERs to subsequently activate ER signaling pathways in the brain. It detects and distinguishes chemicals showing estrogenic activity from the low ng/l (nM) to the mg/L (µM) range (10)(11)(13). This includes for instance natural and synthetic steroidal estrogens, some bisphenols and alkylphenolic compounds. 17β-trenbolone, an androgenic steroid, is also active in EASZY reflecting its ER agonist activities (17)(18).

11. Zebrafish embryos have some biotransformation capacities catalyzing both Phase I and Phase II enzymatic reactions (19)(20)(21)(22)(23); therefore, the EASZY assay may also be able to provide some information on the estrogenic activity of pro-estrogenic chemicals, *i.e.* that require metabolic activation prior to elicit an estrogenic response (10)(11). Comparative analysis of metabolic profiles of some estrogenic chemicals in Zebrafish embryos and adults further demonstrated the metabolic competence of the Zebrafish embryo (24). The similar metabolic profiles found in Zebrafish embryos with adult fish supports the relevance of the Zebrafish embryos as a suitable model for assessing the estrogenic activity of chemicals requiring metabolic activation (24). Nevertheless, care should be taken as the metabolic activity in embryonic fish is not always similar to that of juveniles and adults which can lead to a lack of activity for some pro-estrogenic chemicals (22).

12. Beyond substances acting as ER agonist ligands, either directly or after metabolization, some steroidal androgens have been shown to induce *cyp19a1b* expression (see paragraph 3) and are active in EASZY leading to induction of GFP. The responses are strictly mediated through ERs without involving either androgen receptor (AR) or androgen response element (5). Even if steroidal androgens have been shown to be active in EASZY, the assay is not intended to screen androgen active substances.

13. EASZY may not detect chemicals showing an *in vitro* estrogenic activity if the range of concentrations eliciting estrogenic activity is close to the one inducing toxicity on Zebrafish embryos.

14. Negative results may be obtained with certain types of chemicals either rapidly excreted or chemicals that do not pass the chorion. For substances with a molecular weight ≥ 3kDa, a very bulky molecular structure, and substances causing delayed hatch which might preclude or reduce the post-

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hatch exposure, embryos are not expected to be sensitive because of limited bioavailability of the substance, and other tests might be more appropriate (25).

15. When considering testing of mixtures, difficult-to-test chemicals (*e.g.* unstable), or test chemicals not clearly within the applicability domain described in this Guideline, consideration should be given upfront as to whether the results of such testing will yield results that are scientifically meaningful. Recommendations about the testing of such chemicals (*e.g.*, mixtures, UVCB or multi-constituent substances) are given in Guidance Document No. 23 (26). Finally, the test design is not suitable to test volatile chemicals.

16. During the validation of the EASZY assay, only single chemicals were used and the applicability of the EASZY assay for evaluating binary or multi-component mixtures was not addressed. Nevertheless, experience showed that the EASZY assay could be appropriate for assessing binary or multi-component mixtures (10)(12)(27)(28).

PRINCIPLE OF THE TEST

17. Newly fertilized Zebrafish eggs (up to 4 hours post-fertilization) are exposed to the test chemical for 96 hours under semi-static conditions with a total renewal of the test medium every 24 hours. At the end of the experiment, the fluorescence of each newly hatched eleutheroembryo is measured using a fluorescence microscope (see Annex 4 for the general scheme of the EASZY assay).

18. The measurement of GFP is performed using fluorescence microscope *in vivo* imaging of transgenic *tg(cyp19a1b:GFP)* Zebrafish embryos. Because the skull of early developmental stages of Zebrafish is transparent, GFP is observed, imaged and quantified *in vivo*. The intensity of fluorescence is then quantified using image analysis software.

INFORMATION ON THE TEST CHEMICAL

19. Useful information about chemical-specific properties include the structural formula, molecular weight, purity, stability in light and under test conditions, acid dissociation constant (pKa), organic carbon partition coefficient (Koc) and n-octanol water partition coefficient (Kow), water solubility and vapour pressure, as well as results of a test for ready biodegradability OECD TG 301 (29) or OECD TG 310 (30). Solubility and vapour pressure can be used to calculate Henry's law constant, which will indicate whether losses due to evaporation of the test chemical may occur.

20. A validated analytical method, of known accuracy, precision, and sensitivity, for the quantification of the test chemical in the test solution should be available (31), where technically feasible. Performance parameters should be reported (*e.g.* accuracy, precision, Limit of Detection, Limit of Quantification, specificity, working range).

DEMONSTRATION OF LABORATORY PROFICIENCY

21. Prior to routine use of the EASZY assay, each laboratory should demonstrate technical proficiency, using the proficiency chemicals listed in Table 1. Selected chemicals cover active substances in EASZY as well as inactive chemicals. Active chemicals exhibit a suitable range of estrogenic activity and cover different pathways of induction of GFP (*e.g.*, ER agonist ligand, pro-estrogenic, aromatizable and non-aromatizable chemicals). Table 1 shows appropriate sensitivity ranges determined during the validation study (15).

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REFERENCE CHEMICALS

22. In laboratories where the assay has been successfully implemented, the testing of a reference chemical should be carried out regularly to verify the responsiveness of the transgenic embryos. This should be conducted once per year by performing a full test with 17α-ethinylestradiol (EE2) or 17β-estradiol to derive EC50 (see Table 1 for appropriate test concentration and EC50 value ranges). Test on the reference chemical is also recommended when significant changes in the breeding conditions occur (*e.g.* test medium, environmental conditions, new generation of genitors).

23. In addition, each EASZY assay must include a positive control, *i.e.* EE2 at a final concentration of 14.8 ng/L (0.05 nM), the lowest concentration of EE2 leading to a maximum fold induction of GFP. In each EASZY assay, the mean measured fold induction of GFP induced by the positive control should be ≥ 9 as compared to solvent control.

24. An induction of GFP with EE2 lower than 9-fold invalidates the overall results of the test. In that case, the following actions should be considered: (i) technical problems as a reason for low fold induction in the positive control, such as using fluorescence light source beyond its life-time resulting in weakened intensity, (ii) renew the stock solution (if relevant), (iii) verify the concentration of the stock solution to ensure that the conditions were appropriate, (iv) carry out a full test allowing the determination of a concentration-response curve with EE2 on embryos from new genitors. The EC50 should be in the range indicated in Table 1.

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Table 1. List of proficiency chemicals including positive and negative chemicals. The suitable test concentration range and the mean, minimum and maximum EC50 values for each proficiency chemical are indicated in mass concentrations (mol (M)) and (ng/L or µg/L).

Substance	CAS number	Mode of action	Expected response	Test concentrations range (M) (ng/L or µg/L)	EC50 (M) (ng/L or µg/L)	
					Mean	Min - Max
17α-Ethinylestradiol	57-63-6	ER agonist	POS	6.2×10^{-13}– 1.0×10^{-10} (0.18 – 29.6 ng/L)	1.5×10^{-11} (4.4 ng/L)	8.00×10^{-12} – 2.8×10^{-11} (2.4 – 8.3 ng/L)
17β-Estradiol	50-28-2	ER agonist	POS	1.2×10^{-10} – 1.0×10^{-8} (32.7 – 2723 ng/L)	1.48×10^{-9} (404 ng/L)	5.7×10^{-10} – 2.87×10^{-9} (153 – 781 ng/L)
Bisphenol A	80-05-7	ER agonist	POS	1.2×10^{-7} – 1.0×10^{-5} (27.3 – 2282 µg/L)	3.75×10^{-6} (856 µg/L)	2.5×10^{-6} – 5.10×10^{-6} (571 – 1141 µg/L)
4-tert-Octylphenol	140-66-9	ER agonist	POS	1.2×10^{-8} – 1.0×10^{-6} (2.5 – 206 µg/L)	2.48×10^{-7} (51 µg/L)	1.80×10^{-7} – 4.40×10^{-7} (37 – 85 µg/L)
Norethindrone	68-22-4	ER agonist (pro-estrogen)	POS	1×10^{-9} – 1.0×10^{-7} (0.3 – 29.8 µg/L)	8.4×10^{-9} (2.5 µg/L)	4×10^{-9} – 1.40×10^{-8} (1.2 – 4.2 µg/L)
Testosterone	58-22-0	Aromatizable androgen	POS	1.2×10^{-7} – 1.0×10^{-5} (34.6 – 2884 µg/L)	7.39×10^{-7} (213 µg/L)	4.6×10^{-7} – 1.35×10^{-6} (118 – 389 µg/L)
11-Ketotestosterone	564-35-2	Non-aromatizable androgen	NEG	1.2×10^{-7} – 1.0×10^{-5} (36 – 3024 µg/L)	-	-
Dexamethasone	50-02-2	Glucocorticoid receptor agonist	NEG	1.2×10^{-7} – 1.0×10^{-5} (47 – 3924 µg/L)	-	-

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VALIDITY OF THE TEST

25. For the test to be valid, the following criteria apply:

- The fertilization rate of the eggs collected from the batches should be ≥ 70%;
- Mortality in controls (test medium and solvent (if used) should not exceed one embryo per replicate at the end of the test;
- The hatching rate in controls (test medium and/or solvent) should be ≥ 90% at the end of the test;
- The mean measured fold induction of GFP induced by the positive control (EE2 14.8 ng/L) should be ≥ 9 as compared to solvent control.

DESCRIPTION OF THE METHOD

Apparatus

26. The following equipment is required:

a) Binocular microscope;
b) Fluorescence microscope equipped with a 10X objective, GFP filter (excitation filter 470 nm [Band Path 450-490]; emission filter 525 nm [BP 500-550]) and a fluorescence camera for fluorescence imaging;
c) Computer with Image Analysis Software;
d) Exposure vessels (*e.g.* crystallizing dish, Petri dish) made of chemically inert material (*e.g.* glass) in which the Zebrafish are exposed. Exposure vessels should be of suitable capacity in relation to the recommended loading (15 to 25 mL of test medium);
e) Incubator with controlled temperature of 27°C ± 2°C;
f) Glass plates or lids to cover exposure vessels;
g) Automatic pipette (25 mL);
h) Pipettes: P10, P200, P1000;
i) Glass vessels to prepare stock solutions and the different test concentrations;
j) Tanks with large volume of test medium for maintaining sexually mature transgenic fish used as breeding stocks;
k) Spawn trap: glass trays covered by a mesh (*e.g.* green plastic mesh);
l) Sterile Pasteur Pipette for collecting eggs from the spawning tray, for transferring eggs into exposure vessels;
m) Glass vessels to collect Zebrafish embryos;
n) Multi-well Teflon printed immunofluorescence slides for GFP measurement in live embryos using a fluorescence microscope;
o) Equipment for conductivity, pH, oxygen measurement.

Reagents

a) Benzocaine (*e.g.* stock solution: 100 g/L in absolute ethanol) or tricaine (MS-222) (*e.g.* stock solution: 1.5 g/L in test medium, buffered with calcium carbonate to reach pH 7.0 – 7.8) as

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anaesthetic; the use of other molecules for that purpose should be carefully evaluated before their use while considering their absence of effect on GFP;

b) Concentrated bleach solution to euthanize the Zebrafish embryos (sodium hypochlorite 6.15%; 1 volume of bleach to 5 volume of water).

Test preparation

Test organisms

27. The transgenic *tg(cyp19a1b:GFP)* Zebrafish is a homozygous line that expresses GFP across generations with a stable rate of transmission of the transgene to progeny without any modification of expression of the transgene and responsiveness of transgenic embryos to reference agonist chemicals across generations. The transgenic line is available upon request to the laboratory in charge of maintaining the line[1].

Holding of animals

28. The culture conditions used for the transgenic homozygous *tg(cyp19a1b:GFP)* line are identical to those for wild-type Zebrafish (25). Transgenic adults are in-crossed regularly to obtain homozygous embryos. To ensure diversity of the genetic background of the *tg(cyp19a1b:GFP)* strain, transgenic males or females are outcrossed with wild-type fish every five generations. By observing the constitutive expression of GFP in offspring carrying the transgene, transgenic embryos are selected and used to maintain the strain.

Egg production and selection

29. For egg production, a breeding stock of unexposed transgenic Zebrafish is used. Fish should be free of macroscopically discernible symptoms of infection and disease and should not have undergone any pharmaceutical (acute or prophylactic) treatment for 2 months before spawning. Breeding fish are maintained at 27°C ± 2°C in aquaria with a photoperiod ranging from 12 – 16h light and a recommended loading capacity of 1L per fish. Fish are maintained in charcoal filtered water or in any water of sufficient quality to promote fish health and reproduction (25).

30. Transgenic Zebrafish eggs are produced via mass spawning in large tanks. In the breeding tank, 60 to 100 transgenic adult males and females are continuously held together (the male:female sex ratio varies from 1:1 to 2:1). Alternatively, eggs can be collected from different spawns obtained by pair-crossing adult fish rather than mass-spawning. In that case, the rate of fertilization should be ≥ 70% for each individual spawn before pooling them. Otherwise, the spawn is not used for test purpose.

31. The following feeding regime is appropriate: dry flake food (max. 3% fish weight per day) 3 - 5 times daily; additionally, brine shrimp (Artemia spec.) nauplii and / or small daphnids of appropriate size obtained from an uncontaminated source. Feeding live food provides a source of environmental enrichment and therefore live food should be given wherever possible. The day prior to the assay, a glass tray covered with a green mesh (made of inert material and of appropriate mesh size to protect eggs from predation by their parents) is placed in the tank. The next morning, spawning is stimulated by the onset of light. The glass tray containing eggs is carefully removed from the tank, between 90 and 120 minutes after the onset of light.

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32. Eggs are transferred from the glass tray to a glass vessel containing test medium and the excess of organic matter (food, faeces) is removed.

33. Using a binocular microscope, fertilized eggs with normal development are selected according to Kimmel et al., 1995 (32) and transferred into glass vessels with a loading rate of one embryo for 2 mL.

Test chambers / Equipment

34. Incubation is carried out in exposure vessels made of chemically inert material (*e.g.* glass). Exposure vessels should be of suitable capacity in relation to the recommended loading (15 to 25 mL of test medium).

Test medium

35. A suitable test medium should be used, such as reconstituted water (*e.g.* ISO 6341 water (33)) or well-characterized surface water or well water. The test medium used is aerated, prior to the test, to achieve maximum oxygen saturation and should be in acceptable range for pH (6.5-8.5), conductivity (300- 700 µS/cm), temperature (27°C ± 2°C).

Test Solutions

36. Test solutions of the selected concentrations are usually prepared by dilution of a stock solution. The stock solution should preferably be prepared by mixing the test chemical in the test medium by mechanical means (*e.g.* stirring and/or ultra-sonication). The use of organic solvents or dispersants may be required in some cases in order to produce a suitably concentrated stock solution, but every effort should be made to avoid the use of such chemicals.

37. If the use of solvent is required, its concentration in the final test solutions shall be lower or equal to 0.1 mL/L and should be the same in all test vessels, excluding the test medium control. Dimethyl sulfoxide (DMSO) is an example of a suitable solvent. Results of the validation study demonstrated the lack of significant difference on GFP expression between negative and DMSO controls at 0.1 mL/L.

38. For the testing of chemicals classified as "difficult to test" chemicals, suitable solvents and approaches, described in the OECD Guidance Document No. 23 should be followed (26). The choice of solvent is determined by the chemical properties of the test chemical. The data should demonstrate that the solvent does not affect the GFP expression.

TEST PROCEDURE

39. The EASZY assay begins with fertilized embryos of up to 4 hours post-fertilization and is terminated after 96 hours of exposure. Detailed description of the normal developmental stage of Zebrafish embryos can be found in Annex 3 of OECD Fish Embryo Acute Toxicity Test (25).

Conditions of exposure

Loading

40. Seven fertilized embryos are placed in an exposure vessel containing 15 to 25 mL of test medium (test concentrations and controls). The test design should include three replicates for each test concentration and controls. The fertilized embryos should be randomly distributed among test conditions.

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Each exposure vessel is appropriately and clearly labelled and covered with a glass lid to avoid evaporation of the test medium and reduce the risk of cross-contamination.

Test conditions

41. Exposure vessels are incubated in a humidified climatic chamber at a temperature of 27°C ± 2°C, under a controlled photoperiod (12 to 16 hours light : 12 to 8 hours dark). At the beginning of the test and at each renewal, the dissolved oxygen concentration should be ≥ 80% of saturation.

42. Test and control solutions are entirely renewed every 24 hours within the exposure period.

Feeding

43. No feeding is required for the duration of the test.

Test concentrations

44. The maximum test concentration should be set by the solubility limit of the test chemical in the test medium, the maximum tolerated concentration (MTC), or a maximum concentration of 100 mg/L, whichever is lowest. The MTC is defined as the highest test concentration of the chemical for which mortality does not exceed one embryo per replicate at the end of the test.

45. If toxicity data are already available (*e.g.* acute toxicity data from other aquatic species, including fish embryos, QSAR data or possibilities for read-across), then expert judgment could be used to inform the determination of the maximum test concentration.

46. If no relevant acute toxicity data is available on the test chemical, a range-finding study should be performed on Zebrafish *tg(cyp19a1b:GFP)* embryos to evaluate possible toxicity. For that purpose, at least three test concentrations should be used. They should be arranged in a geometric series with a separation factor not exceeding 10. Only one replicate with 10 embryos is prepared per test condition (test concentrations and control). For the range finding study, each exposure vessel contains 25 mL of test medium.

47. A minimum of three test concentrations should be used in the main study with a separation factor preferably ranging from 3 to 10. Testing higher number of concentrations could allow to establish a full concentration-response relationship which can be used for determining effective concentrations (ECx).

48. The highest concentration should result in a maximal effect on GFP expression for the test chemical and the lowest concentration should result in no significant effect on fluorescence as compared with the control. The maximal effect on GFP expression for the test chemical can be comparable or lower than that of the positive control. The concentrations of test chemical should not induce a mortality exceeding one embryo per replicate. If the aforementioned criteria are not fulfilled, the data of test concentration(s) are not considered for studying the concentration-response relationship.

Controls

49. Controls are required and include:

- Negative control: Zebrafish embryos are exposed to the test medium control;
- Positive control: Zebrafish embryos are exposed to EE2 at a final concentration of 14.8 ng/L (0.05 nM,) *i.e.* the lowest concentration of EE2 leading to a maximum fold induction of GFP. This test solution is prepared from a 10^3 X concentrated stock solution of EE2 solubilised in DMSO and stored at -20°C in the dark. To ensure reproducible results among experiments, the shelf life of EE2 stock solution should not exceed one month;

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- Solvent control (if appropriate): When a solvent control is required, the maximum solvent level should not exceed 0.1 ml/L or 100 mg/L (27) and the effect of the solvent must be checked prior to the running of the test to ensure that it does not significantly induce GFP expression. No statistically significant difference should be observed between solvent and negative control regarding GFP expression. If a statistically significant difference is detected the test should be repeated. Nevertheless, the dilution water control can be omitted, and the test conducted and evaluated with a solvent control only, provided it is appropriate when considering the needs for these data and the requirements of the relevant regulatory authorities.

Analytical measurements

50. As a semi-static renewal exposure method is used, the stability of the test chemical concentration should be documented. Analysis of the concentrations of the test chemical should be measured as a minimum in the highest and lowest test concentrations, but preferably in all test concentrations. Concentrations of test chemical should be measured in freshly prepared test solutions (T0) and immediately prior to renewal of the test solution in one of the three replicates (T24 ± 2 hours), at least once during the exposure period. The stability of the test chemical should allow the exposure concentration to remain within 20% of the nominal concentration in a 24 h time frame. Preconditioning of the test vessel with the test solutions for at least 24 h before initiation of the test to minimize test chemical loss by adsorption could be considered. If concentrations cannot be maintained within ± 20% of the nominal concentrations, the results should be expressed relative to the geometric mean of the measured concentrations.

Observations and renewal of test/control solutions

51. The test solutions (control and treatment groups) are renewed daily. For each test condition, freshly prepared test solution is added immediately in the exposure vessel after the test solution is removed. Upon renewal, care should be taken to avoid drying of embryos. pH, dissolved oxygen concentration, conductivity and temperature are measured before renewal and in freshly prepared test solutions and controls.

52. During the exposure period, mortality of embryos is checked every day and the dead embryos are removed. At the end of exposure, the cumulative mortality rate is calculated for control and treatment groups and reported (see paragraph 66). Hatching is recorded in treatment and control groups on a daily basis starting from 48 hours. Observations are recorded every 24 hours, until the end of the test. These observations, carried out according to OECD TG 236 (26), might help in the data interpretation. Notably a delay in hatching rate can reduce exposure of fish to the test chemical limiting the potential of the EASZY assay to detect an effect.

53. Any other relevant observations of the development of the fish such as oedema, tail malformations, should be reported on a quantitative basis.

Fluorescence measurement

54. At the end of the exposure, hatched Zebrafish embryos are carefully transferred (one per well) from each exposure vessel to a multi-well fluorescence hydrophobic glass slide using a Pasteur pipette. A 24-well fluorescence slide allows for the transfer of all the embryos collected from the same exposure group. This step can be carried out with or without anesthetized embryos.

55. If anaesthesia of Zebrafish is retained, it is performed directly in the test chambers using benzocaine at a final concentration of 50 mg/L or tricaine at final concentration of 150 mg/L. After 5 minutes of exposure to the anaesthetic, hatched embryos are transferred to the observation slide. This mild

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anaesthesia is not lethal for this duration of exposure and embryos recover quickly. Each well of the fluorescence slide containing live Zebrafish embryos is observed.

56. Each Zebrafish is then photographed dorsally. A proper positioning of the fish is mandatory to ensure comparison of GFP expression from a fish to another (see Annex 5 and Annex 6). Improper positioning of the fish will result in incorrect fluorescence measurement. Therefore, in case of incorrect position (ventral or lateral view), the operator must re-orientate the embryo using a pipette tip to observe it dorsally. Mounting medium (*e.g.* methyl cellulose) could be used during this process. At this developmental stage, Zebrafish embryos present pigmentation that varies between individuals. Nevertheless, it does not prevent GFP measurement and does not affect the test response. In case of severe malformations (*e.g.* severe peri-vitellin and/or pericardial oedema, severe malformations of the head), a correct orientation is sometimes difficult and can preclude correct fluorescence measurement. When appropriately orientated, the head of each embryo is photographed. The parameters of the microscope for fluorescence measurement shall be determined before testing any test chemical and kept identical from one experiment to another to allow comparison of the data.

57. The setting of the fluorescence parameters and the quantification of GFP by image analysis are performed according to Annex 7 and 8, respectively.

58. At the end of the test, Zebrafish embryos are euthanized using a protocol combining anaesthesia induced by benzocaine and a hypothermal shock to induce death (34) or the addition of bleach solution (sodium hypochlorite 6.15%; 1 volume of bleach to 5 volumes of water).

DATA AND REPORTING

Analysis of data / Evaluation of test results

59. After image analysis of each individual embryo, the results are reported in a worksheet. For each exposure condition, the quantification of the fluorescence in individuals, measured as integrated density in transgenic *tg(cyp19a1b:GFP)* Zebrafish (see Annex 8), is expressed as fold induction compared with test medium control or solvent control, if solvent control is used. Annex 9 provides an example of data reporting and analysis.

60. To identify potential activity of a chemical, responses are compared between treatments and control groups using analysis of variance (ANOVA) of means of each replicate. An appropriate statistical test should be performed between the test medium and solvent controls for GFP. Guidance on how to handle test medium and solvent control data in the subsequent statistical analysis can be found in the OECD Document on Current Approaches to Statistical Analysis of Ecotoxicity Data (35). If the required assumptions for parametric methods are not met - non-normal distribution (*e.g.* Shapiro-Wilk's test) or heterogeneous variance (Bartlett's test or Levene's test), consideration should be given to transform the data to homogenize variances prior to performing the ANOVA. Log-transformation of fold inductions is the first transformation to consider. The choice of a post-hoc test after the ANOVA depends on the monotonicity of the concentration-response relationship. Dunnett's test (parametric) on multiple pair-wise comparisons or either a Dunn test or a Mann-Whitney with Holm-Bonferroni adjustment (non-parametric) may be used for non-monotonous concentration-response. Other statistical tests may be used (*e.g.* Jonckheere-Terpstra test or Williams test) if the concentration-response is approximately monotone. A flowchart is provided in Annex 10. Additional information can also be obtained from the OECD Document on Current Approaches to Statistical Analysis of Ecotoxicity Data (35).

61. In addition to the determination of a statistically significant effect, magnitude of effects should be considered to identify if a test chemical is either inactive (the chemical has no estrogenic activity) or active

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(the test chemical is estrogenic). This combination will help to avoid potential false positive results in the EASZY assay. A minimum 2-fold induction threshold was established based on the validation studies during which some inactive test chemicals could occasionally lead to significant effects but with fold-induction rates below or equal to 2.

62. Annex 11 provides examples of concentration-response curves combining both statistical analysis and the 2-fold induction threshold as well as their interpretation in regard to the activity of the test chemical in the EASZY assay.

63. If a test chemical leads to a statistically significant induction as compared to control with mean GFP induction below 2, then the test chemical cannot be considered as active in EASZY (see annex 11, example (i)). In such case, it is highly recommended to repeat the assay. If, at the occasion of a new test, the test chemical is still eliciting a significant response with a mean fold induction below or equal to 2, then the test chemical cannot be classified as active. Conversely, if a test chemical leads to a non-significant induction with mean GFP induction fold greater than 2, then the test should be repeated (see annex 11, example (j)). If similar data are obtained, no conclusion can be drawn based solely on the results of EASZY assays.

64. For active test chemicals in the EASZY assay, the lowest concentration of test chemical leading to a significant effect can be determined as well as the concentration leading to the maximum induction level of GFP (see Annex 9).

65. The two phases of the validation study provided evidence of the reliability, accuracy and robustness of the EASZY assay. It has been shown that conclusion regarding the estrogenic activity of a test chemical can be made based on one single assay providing that the validity criteria (see paragraph 25) and the decision criteria are fulfilled (see paragraph 61).

Test report

66. The test report should include the following information:

Test chemical:

- physical appearance, water solubility and additional relevant physicochemical properties;
- chemical identification, such as CAS registry number or CAS name, IUPAC name, CSMILES or InChI code, structural formula, purity and chemical identity of impurities as appropriate.

Test organisms:

- scientific name, strain, source and method for producing, collecting the eggs and the subsequent handling of the fertilized eggs.

Test conditions:

- test design: types of controls, number of concentrations, type of test vessels;
- photoperiod, light intensity;
- characteristics of water used for fish maintenance (*e.g.* pH, temperature, conductivity, dissolved oxygen, etc);
- dissolved oxygen concentration, pH, temperature and conductivity of the test solutions at the start of the experiment and before the renewal of the test medium;
- method of preparation of stock solutions and test solutions as well as frequency of renewal;

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- justification for use of solvent and justification for choice of solvent, if appropriate;
- the presence of any visible non-dissolved test chemical, if appropriate;
- the nominal test concentrations and the result of all analyses to determine the concentration of the test chemical in the test vessels; the recovery efficiency of the method and the limit of detection should also be reported.

Results:

- the fertilization rate of the eggs;
- the mortality rate in control and treatment groups (individual replicates and mean value);
- the hatching rate in control and treatment groups (individual replicates and mean value);
- the mean measured GFP expression in the positive control;
- the individual GFP expression in all treatment and control groups;
- the statistical analysis and treatment of GFP data;
- the maximum concentration tested causing no effect on GFP within the duration of the test;
- the lowest concentration for which a statistically significant effect has been determined;
- the concentration and the fold induction of the test chemical at which its maximum response is observed;
- a graph of the concentration-response curve for the GFP expression;
- incidence and description of morphological and physiological abnormalities, if any;
- incidents in the course of the test which might have influenced the results.

67. Any deviation from the Guideline and relevant explanations should be reported. The results should be interpreted with respect to the potential estrogenic activity of the test substance in light of the validity and decision criteria. Results should be discussed. If the number of concentrations tested allowed to establish a full concentration response curve, effective concentration leading 50% of the maximal effect of the test chemical (EC50) can be derived with 95% confidence limits and indicated in the discussion section. Results of the intercalibration study have shown that EASZY reliably quantifies the EC50 of estrogenic chemicals.

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ANNEX 1 Genetic construction used to produce the tg(cyp19a1b:GFP) founders.

The transgenic *tg(cyp19a1b:GFP)* Zebrafish line was developed and characterized by Tong et *al.*, (2009) (1). In this model, green fluorescent protein (GFP) expression is controlled by 3.4kb of the Zebrafish *cyp19a1b* proximal promoter followed by the first exon, the first intron and the beginning of the second exon comprising the natural translation start site (see Figure 1 for the genetic construction).

Figure 1: Schema showing the *tg(cyp19a1b:GFP)* construct used to produce transgenic founders. The construct contains 3,424 bp of the 5' flanking region, 54 bp of exon I (untranslated), 1,698 bp of intron I, and 20 bp of untranslated exon II followed by the natural translation start site. The GFP gene with the SV40 polyA sequence (poly A) was fused to the first 10 amino acids of CYP19A1B with a 9-aa linker. GxRE: Glial X Response Element, ATG: translation start site (from Tong et al., 2009) (1).

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ANNEX 2 Definitions and abbreviations

aa: amino acid

Agonist: A chemical/ligand that binds to and activates a specific nuclear receptor leading to the transcriptional activity of gene(s) regulated by the nuclear receptor.

Antagonist: A chemical/ligand that binds to and blocks or inhibits the response mediated by agonist ligand.

Anti-estrogenic activity: the capability of a chemical to suppress the action of 17β-estradiol mediated through estrogen receptors.

Active test chemical: any test chemical that significantly induces the GFP expression with a minimum mean measured fold induction of GFP > 2 as compared to control.

Bp: base pair

Brain aromatase: the enzyme encoded by the cyp19a1b gene. The enzyme is responsible for the endogenous synthesis of estrogens.

Cyp19a1b: the name of the gene that codes for the brain form of aromatase.

tg(cyp19ab:GFP): it refers to the transgenic Zebrafish stably expressing a reporter gene (the Green Fluorescent Protein, GFP) under the control of the Zebrafish cyp19a1b promoter.

DMSO: Dimethyl sulfoxide.

Dpf: days post fertilization

Estrogenic activity: The capability of a test substance to mimic the action of 17β-estradiol in its ability to activate, in an ER-specific manner, the endogenous cyp19a1b and/or the GFP driven by the cyp19a1b promoter.

EE2: 17α-ethinylestradiol.

E2: 17β-estradiol.

ER: Estrogen receptor.

ERE: Estrogen response element.

Fold induction of GFP: is the way the fluorescence of individual embryos is expressed.

GFP: Green Fluorescent Protein.

Hpf: hours post fertilization.

Inactive test chemical: any substance for which no significant effect is measured on GFP expression as compared to control with a mean measured GFP expression ≤ 2.

Integrated density: is the sum of all the pixel intensities in the Region of Interest (ROI).

Proficiency chemicals: a subset of the reference chemicals that can be used by laboratories to demonstrate their technical competence with a standardized assay.

Relevance: Description of relationship of the test to the effect of interest and whether it is meaningful and useful for a particular purpose. It is the extent to which the test correctly measures or predicts the biological effect of interest. Relevance incorporates consideration of the accuracy of a test method.

Reliability: Measure of the extent to which a test method can be performed reproducibly within and between laboratories over time, when performed using the same protocol. It is assessed by calculating intra- and inter-laboratory reproducibility.

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ROI: Region of Interest

Sensitivity: The proportion of all positive/active chemicals that are correctly classified by the test. It is a measure of accuracy for a test method that produces categorical results and is an important consideration in assessing the relevance of a test method (36).

Specificity: The proportion of all negative/inactive chemicals that are correctly classified by the test. It is a measure of accuracy for a test method that produces categorical results and is an important consideration in assessing the relevance of a test method (36).

UVCB: Unknown or Variable Composition (UVCB), Complex Reaction Products or Biological materials

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ANNEX 3 Pathways through which chemicals can elicit estrogenic activity as measured by induction of GFP driven by the Zebrafish cyp19a1b promoter

The figure 1 illustrates the pathway through which chemicals can elicit estrogenic activity as measured by induction of GFP driven by the Zebrafish cyp19a1b promoter.

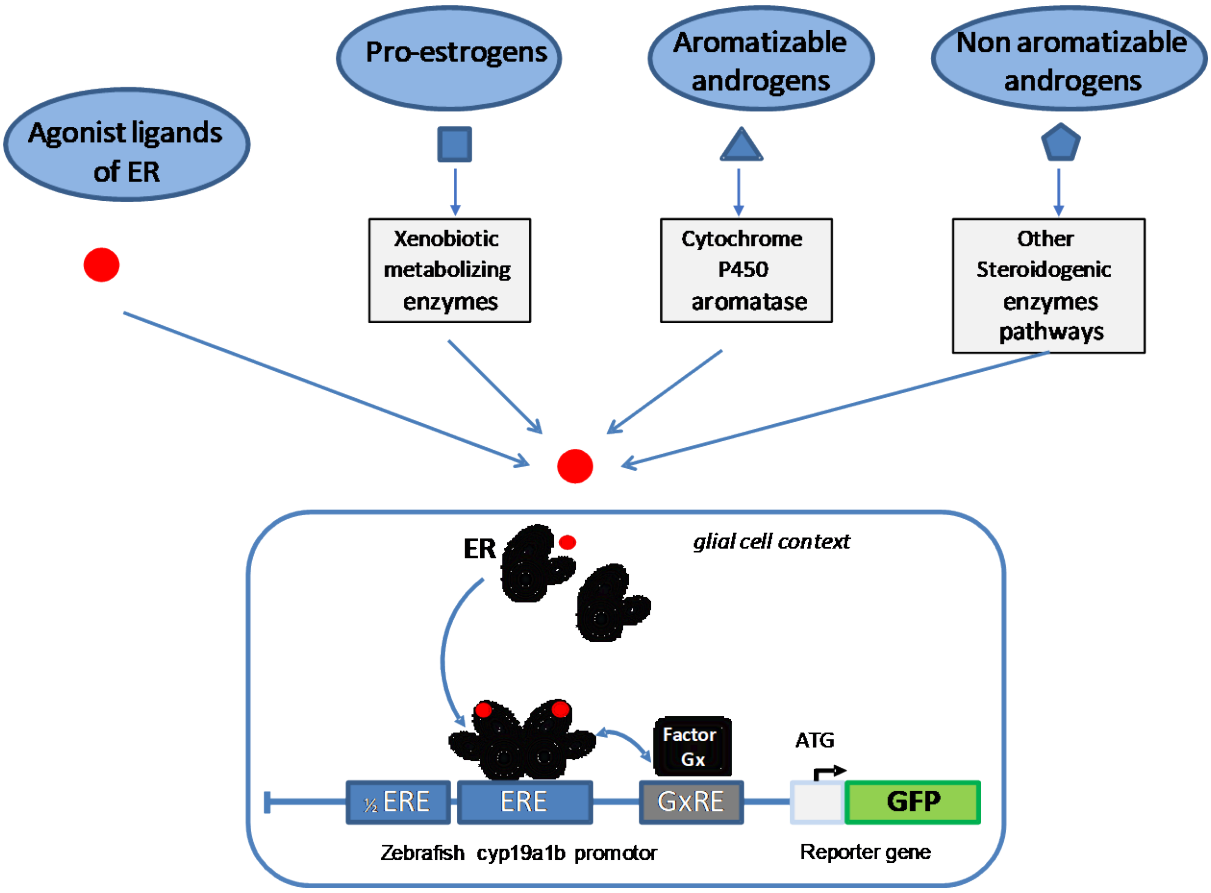

Figure 1: Figure illustrating how the test chemicals elicit an estrogenic activity as revealed by their capacity to induce GFP expression driven by the Zebrafish ER-regulated cyp19a1b promoter. It includes direct binding of chemicals to ERs as agonists or binding to ERs after metabolization (pro-estrogens). Some aromatizable androgens have been shown to be active in EASZY through their aromatization into estrogens as well as dihydrotestosterone (DHT), a non-aromatizable androgen, likely reflecting its conversion, through other steroidogenic pathway, into 5alpha-androstane-3beta,17beta-diol (β-diol), a metabolite of DHT with known estrogenic activity.

A panel of natural and synthetics hormones as well as chemicals belonging to various chemical families have been tested for their capacity to induce GFP in the transgenic tg(cyp19a1b:GFP) embryo model (Brion et al., 2012, Cano-Nicolau et al., 2016, Le Fol et al., 2017a, Neale et al., 2017, Serra et al., 2018).

Based on these studies and the interlaboratory experiments realized for the validation of the EASZY assay (EASZY validation report), it can be concluded that GFP is induced in an ER-specific manner by compounds that bind directly to estrogen receptors as agonists. The assay detects and distinguishes ER agonist compounds acting from the low ng/L (e.g., synthetic and natural steroidal estrogens) range to the high mg/L per liter range (e.g., bisphenols). Among them, it is noteworthy that some non-aromatizable androgens such as 17β-trenbolone, were also active. For 17β-trenbolone, its estrogenic activity likely reflects its capacity to bind to and activate ER at high concentrations. Indeed, in several in silico and in

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vitro ER transactivation assays, 17β-trenbolone has been identified as positive substance (Brown et al., 2015, OECD TG 455). Furthermore, in vivo, in rat, 17β-trenbolone has been shown to act at the brain level to alter expressions of proteins through both AR and ER-mediated process (Fucui Ma & Daicheng Liu, 2015). These in vitro and in vivo data show that 17β-trenbolone binds to ER as agonist to induce ER response in vitro, in vivo in different vertebrate models including Zebrafish embryos.

In EASZY, compounds that need to be bio-transformed prior to elicit an induction of GFP are also detected. Several compounds that require metabolic activation into estrogenic metabolites have been shown to induce GFP in an ER-dependent manner. This was exemplified by methoxychlor whose estrogenic activity is due to estrogenic metabolites such as 2,2-bis(p-hydroxyphenyl)-1,1,1-trichloroethane HPTE. Furthermore, several 19 nortestosterone synthetic progestins have been shown to induced GFP in ER-dependent manner in the developing brain of zebrafish (Cano-Nicolau et al., 2016). While the metabolic profiles are not known in the Zebrafish model, these compounds are known to be biotransformed into estrogenic derivatives in mammals.

In EASZY, aromatizable androgens such as testosterone and methyltestosterone have been shown to induce GFP. This androgenic regulation of the cyp19a1b is strictly mediated through ER and does not involve neither androgen receptor (AR) nor androgen response element (Mouriec et al., 2009) but is due to aromatization of testosterone and methyltestosterone into estradiol and methyltestradiol respectively. Furthermore, some non aromatizable androgens, but not all, have been shown to induce GFP in an ER-dependent manner. It is the case of 5α-dihydrotestosterone (DHT). In vitro, it has been shown that the transcriptional stimulation of the cyp19a1b by DHT occurred in the presence of the Zebrafish ERs but not in the presence of Zebrafish AR. In contrast, 11 Keto-testosterone (11-KT), another non aromatizable androgen failed to induce the transcriptional activity of the cyp19a1b gene (Mouriec et al., 2009). In vivo, DHT is also an efficient inducer of the cyp19a1b expression in the Zebrafish brain but not 11-KT. In vivo, the estrogenic activity of DHT can be blocked by ICI 182 780 (an antagonist of ER) but not Flutamide (an antagonist of AR). The estrogenic activity of DHT may be due to its conversion into βdiol through 3β-hydroxysteroid dehydrogenase activity. βdiol induce the transcriptional activity of the brain aromatase expression in vitro in the presence of Zebrafish ERs. In vivo, βdiol induce brain aromatase expression, an effect blocked by ICI 182 780, again suggesting the involvement of functional ER (Mouriec et al., 2009). Overall, 11-KT is totally ineffective in stimulating aromatase B expression, but DHT can induce estrogenic effects most likely following biotransformation into βdiol through 3β-hydroxysteroid dehydrogenase activity (Mouriec et al., 2009).

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ANNEX 4 Overview of the EASZY assay

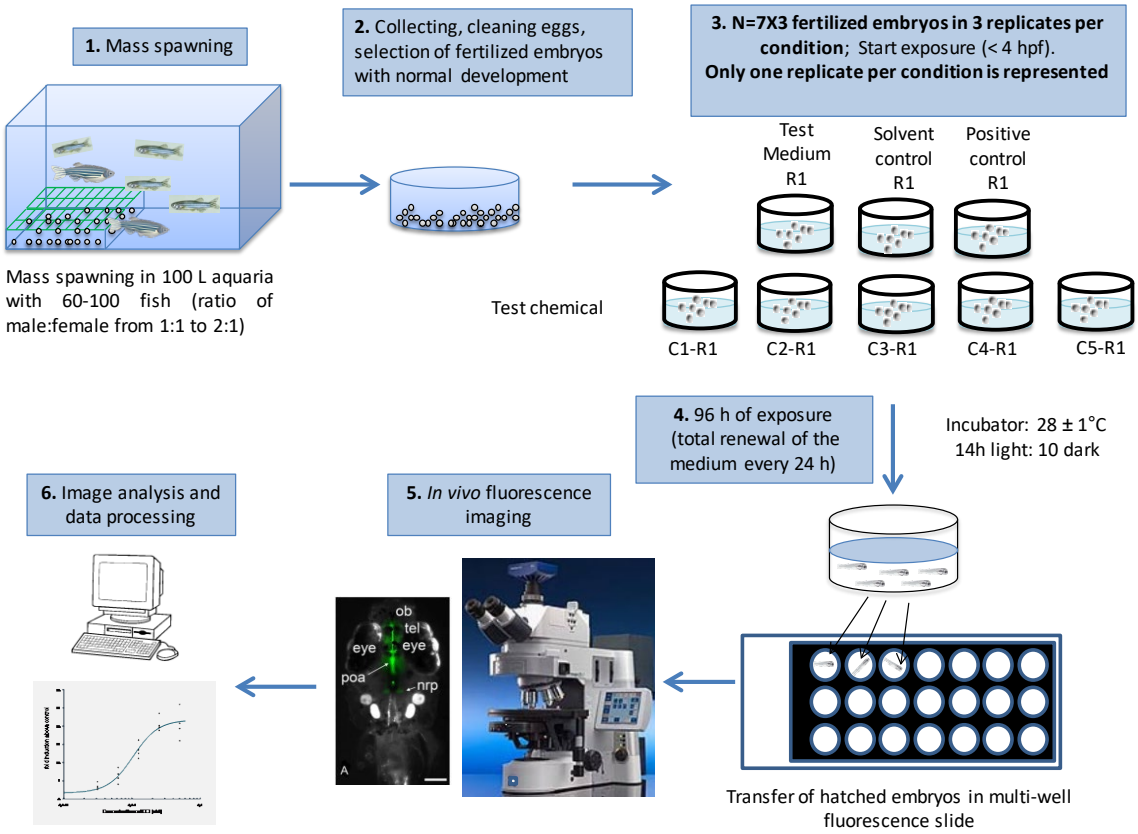

Figure 1: Scheme of the EASZY assay illustrating the main steps for its accomplishment.

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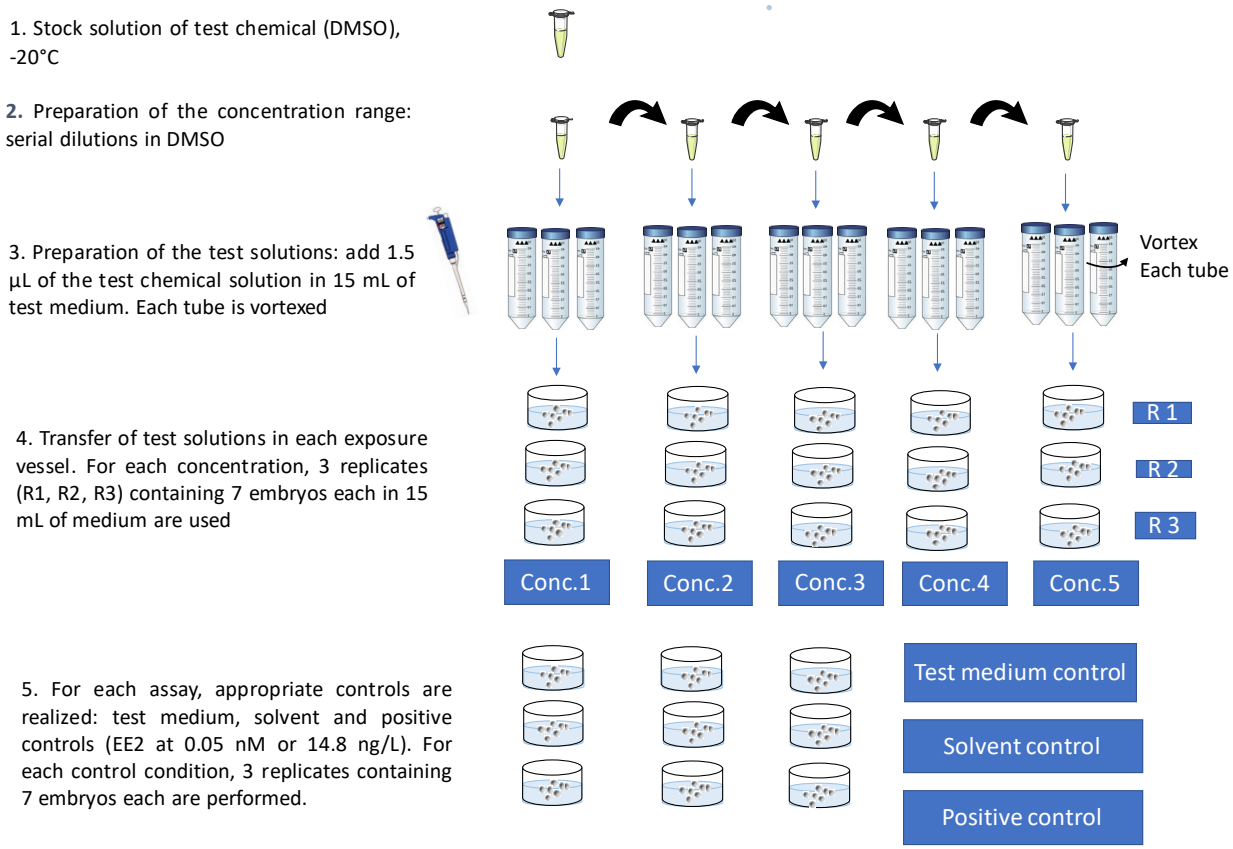

Figure 2: Detailed preparation of the test concentrations when solvent is required.

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ANNEX 5 Image analysis: detailed views of a fluorescence slide containing transgenic Zebrafish embryos after 96 hours exposure

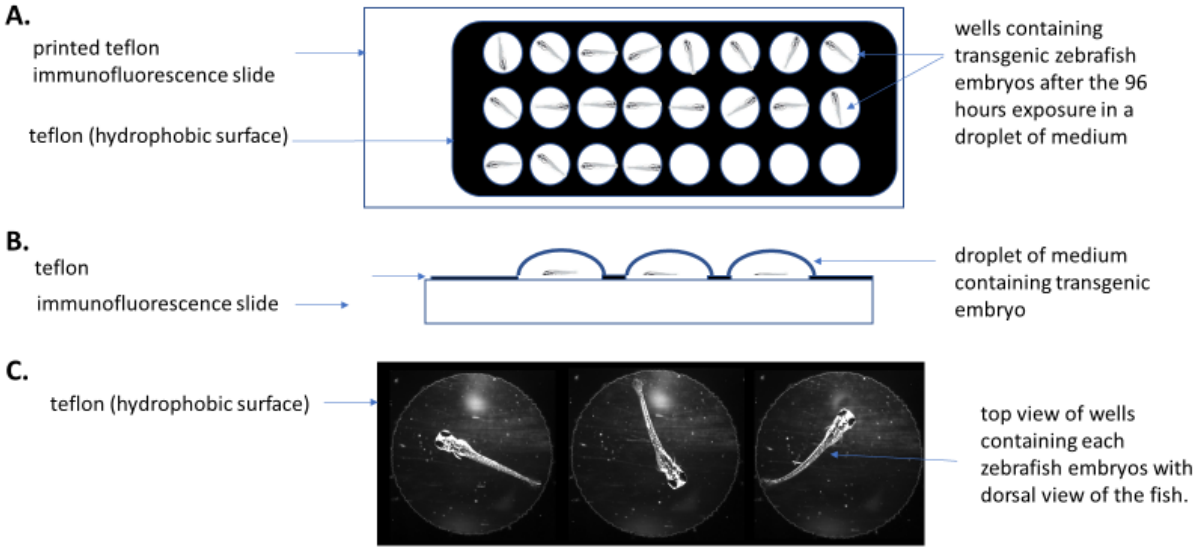

Figure 1: **A** Top view of a 24-well Teflon printed immunofluorescence slide (*e.g.* 24-w Immuno-cell Teflon printed diagnostic slide). **B.** Lateral view of a fluorescence slide showing that each transgenic Zebrafish embryo is placed within a droplet of medium. **C.** Top view of wells each containing a transgenic Zebrafish embryo with a dorsal view of the head. Each embryo must be viewed dorsally to correctly observe the GFP expression within the brain.

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ANNEX 6 *In vivo* fluorescence imaging of transgenic Zebrafish embryos after 96 hours exposure

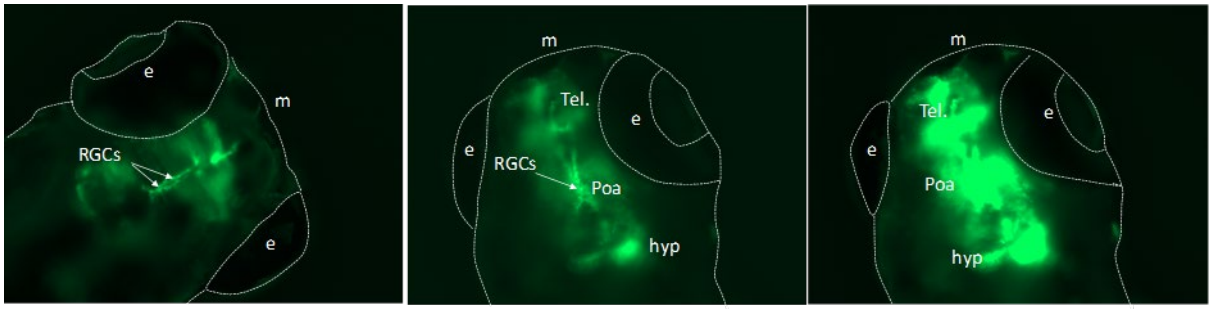

Figure 1: *In vivo* fluorescence imaging of live transgenic *tg(cyp19a1b:GFP)* Zebrafish expressing different levels of GFP (from low level on the left picture to high level on the right picture). Dorsal views (anterior to the top). e: eye, m: mouth, RGCs: radial glial cells. The arrows indicate the cellular bodies of RGCs along the ventricle. On these images, the GFP can be easily observed in various brain regions: the telencephalon (Tel), preoptic area (Poa), and the hypothalamus (hyp). Different "exposure times" were used to take these images and were not used to quantify the GFP to compare GFP intensity between these individuals.

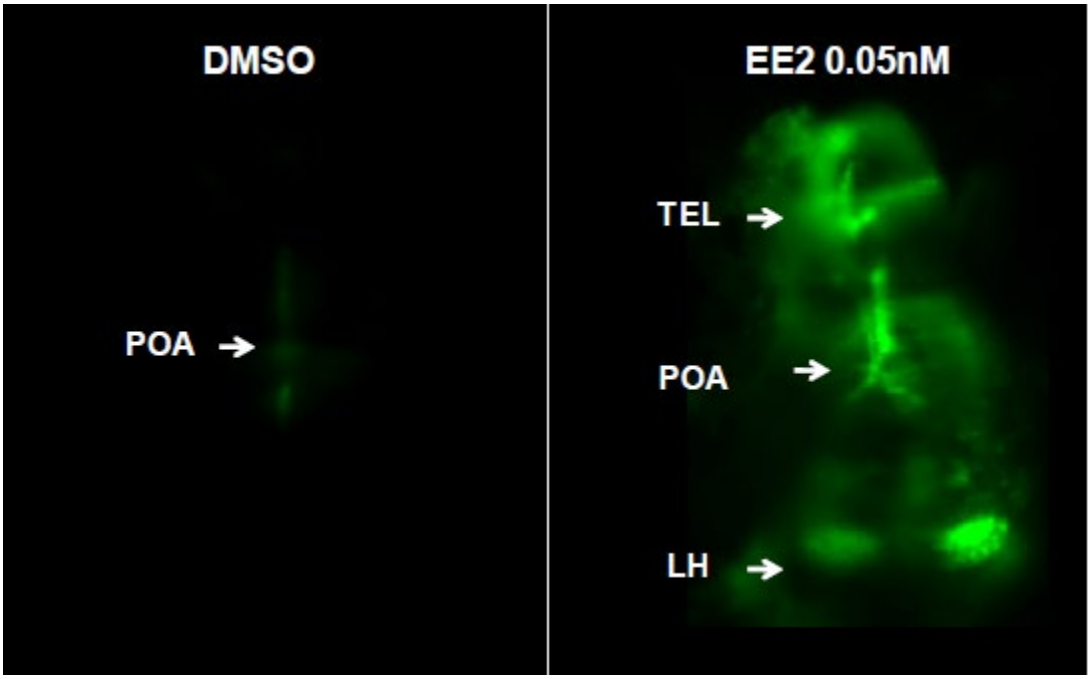

Figure 2: *In vivo* imaging of live transgenic *tg(cyp19a1b:GFP)* Zebrafish embryos exposed to solvent alone (DMSO) and the reference chemical 17α ethinylestradiol (EE2) at a final concentration of 14.8 ng/L (0.05 nM). Dorsal views (anterior to the top) of the telencephalon (TEL), preoptic area (POA), and the inferior lobe of the hypothalamus (LH). The images were taken using the same parameters for fluorescence analysis. In this specific example, a 25-fold induction was measured for the EE2-exposed embryos as compared to the solvent control

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ANNEX 7 *In vivo* imaging of *tg(cyp19a1b:GFP)* Zebrafish: Wide-field fluorescence microscopy.

Setting the parameters for the fluorescence microscope.

Fluorescence imaging is realized using a fluorescence microscope equipped with a 10X objective, a GFP filter, an external light source (*e.g.* HBO lamp) and a fluorescence camera. Upright or inverted microscopes can be used.

Setting the parameters for fluorescence acquisition is critical and depends on the main technical characteristics of each microscope. Once these parameters are set, they must be kept for all experiments.

Objective: a 10X magnification objective allows the head of the embryo (defined as the region of interest or ROI) to be photographed hence avoiding to photograph the yolk sac that fluoresces at similar wavelengths as GFP. The use of a specific objective for fluorescence applications (*e.g.* Fluar) is highly recommended.

GFP filter: the following characteristics are highly recommended for the GFP filter: excitation wavelength 470 nm [Band Path 450-490]; emission wavelength 525 nm [BP 505-545]. For each excitation and emission wavelength the band path is low with no overlap between the excitation and emission spectra.

External light source: various light sources can be used. Very often, the light source is composed of an HBO lamp. During the lifetime of the lamp (which does not exceed 500 hours), the intensity should be constant. However, the stability of the external light source can be assessed before and after a series of measurements using a fluorescence calibration slide.

Fluorescence camera: the camera should have a high resolution and a highly sensitive charge-couple device (CCD) sensor. A monochrome (black and white) (CCD) digital camera is recommended as it increases the sensitivity and reduces the time of exposure as compared to color camera.

Time of exposure: the time of exposure must be optimized for each microscope to obtain the best signal-to-noise ratio.

To set this parameter, it is recommended to vary this setting and to evaluate its influence on the fluorescence emitted by unexposed and exposed embryos. For that purpose, it is recommended to perform an assay with at least two groups: one test medium control and one positive control (EE2, 14.8 ng/L). At the end of the experiment, each fish is photographed at different times of exposure (*e.g.* from 50 ms to 300 ms with a regular space of interval) and the images are then subsequently analysed to determine the optimal time of exposure. When properly set, it must allow detection of the fluorescence in both control and exposed fish. If the time of exposure is too short, the basal fluorescence cannot be detected properly. In contrast, if time of exposure is too high, it will saturate the signal for embryos with high expression levels of GFP. The optimal time of exposure should result in an optimal signal to noise ratio.

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ANNEX 8 *In vivo* imaging of *tg(cyp19a1b:GFP)* Zebrafish: Image analysis using the ImageJ EASZY_FAST macro

In the EASZY test, the quantification of the fluorescence emitted by the fish embryos is performed from the images taken under a fluorescence microscope (see Annex 7). The image analysis tasks have been fully automated to allow the analysis of a large number of images in a short period of time. These analyses are performed using a freely available ImagJ macro that has been specifically developed for the EASZY assay (EASZY_FAST macro). The macro can be found at the following address https://imagej.net/FAST

The following information explains how the macro works. A parameter in the image analysis step corresponds to a threshold value which allows the autofluorescence of the embryos to be distinguished from the fluorescence of the reporter gene. This parameter must be determined prior to quantifying GFP in fish exposed to reference or unknown substances.

1. Determining the threshold level of grey level

A critical criterion for image analysis is the threshold grey level. The threshold grey level corresponds to a grey value which allows the fluorescence due to the reporter fluorescent protein to be distinguished from the natural auto-fluorescence of fish (background fluorescence). To determine this parameter, the following procedure was established.

a) Non-transgenic Zebrafish are selected and photographed using the same parameters as previously defined (see figure 1).
b) Photographs are imported in the image analysis software.
c) A region of interest (ROI) is manually defined. The ROI corresponds to brain regions where expression of GFP is normally observed in transgenic *tg(cyp19a1b:GFP)* Zebrafish.
d) Run the analysis for each ROI to obtain grey level of each pixel within the selected area.
e) The threshold is defined as the maximal grey level found in the ROI of non-transgenic fish. To refine the threshold grey level, it is recommended to analyze several non-transgenic fish.

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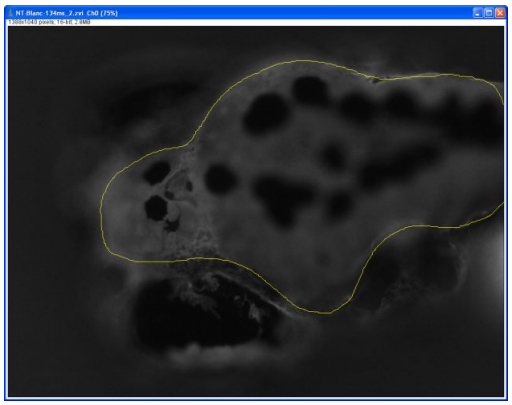

Figure 1: Fluorescence imaging of a non-transgenic Zebrafish embryo to measure to background level of fluorescence (threshold level of grey value).

Once defined, only pixels having a grey level above the threshold will be analysed. This threshold value is specific to each fluorescence microscope device as grey scale varies according to the camera used to capture images and the resolution of the image (*e.g.* 8, 12, 16 bit/pixel). This threshold grey level is stable over time but may be refined when changes on the fluorescence device occur such as installation of a new light source.

2. General description of the ImageJ EASZY_FAST macro

Minimum system requirements

Software resources needed to use EASZY_FAST macro are:

- ImageJ version 1.53 or higher - https://imagej.net/Download or https://imagej.net/Fiji
- Java 8 (or later) runtime - www.java.com
- Bio-Formats 6.6.1 or higher http://www.openmicroscopy.org/bio-formats/ (library for reading and writing life sciences image file formats)
- FAST_Analyze.ijm file saved to the Plugins directory within ImageJ (available on the following address https://imagej.net/FAST see details for automatic installation)

More information on other operating systems can be found on the above-mentioned links.

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Description of the Image processing pipeline of “EASZY_FAST” macro (figure 2):

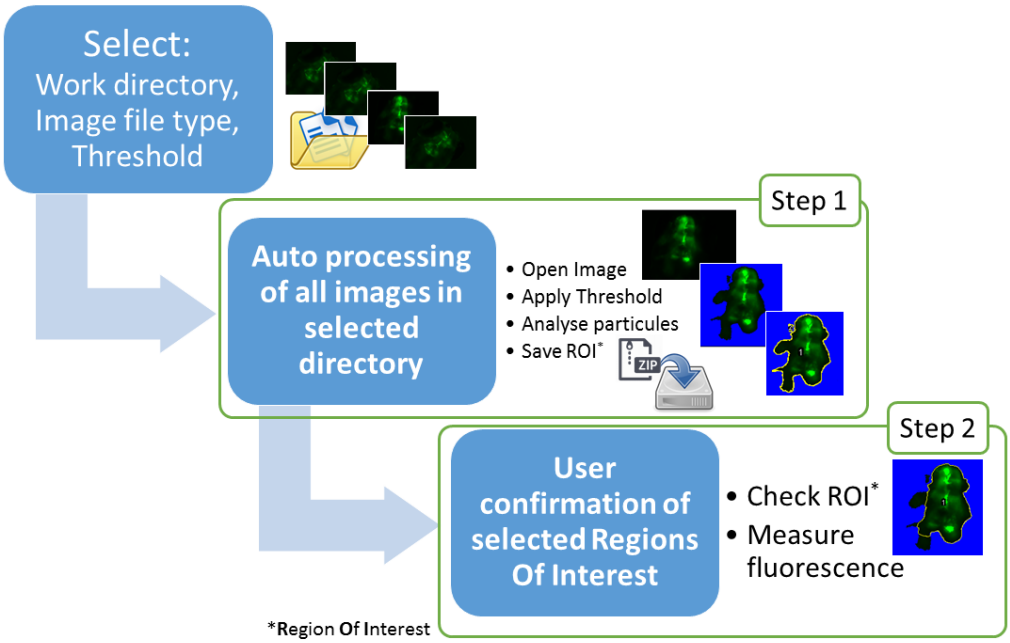

Figure 2: Image Analysis Processing

This ImageJ macro automates several tasks (open, set threshold, save, measure) to process a large number of images.

- Step 1 - Batch processing of selected directory
  - List all files containing user-defined extension (.czi - .zvi - .tif - .nd2) within selected directory and sub-directories.
  - Apply the user-defined threshold (default 290) then analyse particles above the threshold.
  - Group pixels above the threshold in one Region Of Interest and save this ROI in a zip file in the image directory.
- Step 2 – Check selected Region Of Interest and measure
  - Open images one by one to check the auto-selected ROI.
  - User can confirm the ROI, modify it directly or remove image from further analysis.

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Detailed description of image analysis steps

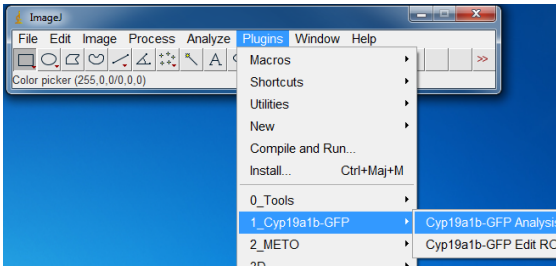
Run **ImageJ.exe**.

Launch the macro in ImageJ toolbar menu:

Plugins

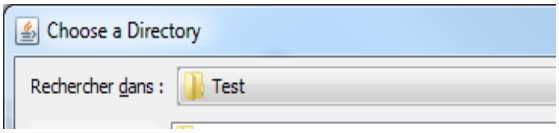
FAST

FAST_Analyze



The macro prompts to select the directory containing image files.

The selected directory <u>and all subdirectories</u> will be processed for analysis.



The macro displays a dialog box to set analysis options:

Threshold value – set lower threshold value for pixel intensity, segmenting the image into features of interest (above threshold) and background.

<u>Threshold value has to be defined for each acquisition system</u> (microscope + camera + exposure time).

File type – select image type between CZI, ZVI (Zeiss Vision Image), ND2 (Nikon) or TIFF (Tagged Image File Format).

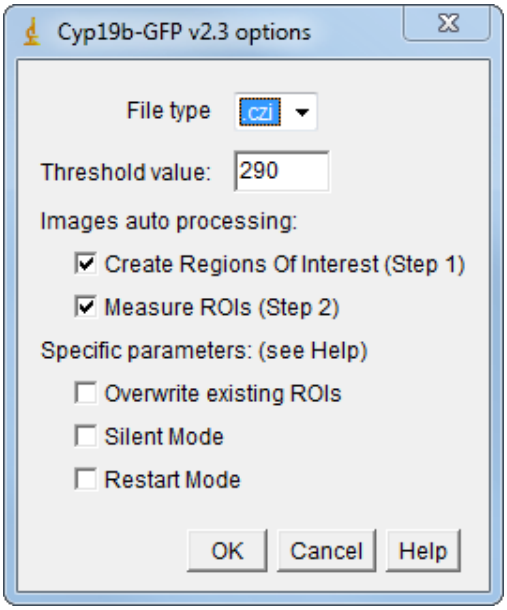
Create ROIs (Regions Of Interests) – Step 1, analyses pixels above the user-defined threshold value and automatically saves ROIs zip file in the image directory (Figure).

Measure ROIs – Step 2 of image analysis to check individually and measure previously created ROIs. With Step 1 not checked and Step 2 checked user can reanalyze previous data.

Overwrite existing ROIs – if selected, the macro will overwrite ROIs zip files without prompting the user many times.

Silent Mode - if selected, the macro will measure all images with their associated ROI without prompting the user many times. Useful to reanalyze previous data.

Restart Mode: when auto processing is cancelled accidentally, this mode checks whether a ROI was created and creates one if needed.



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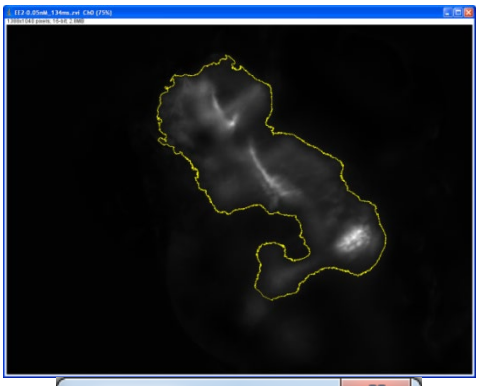
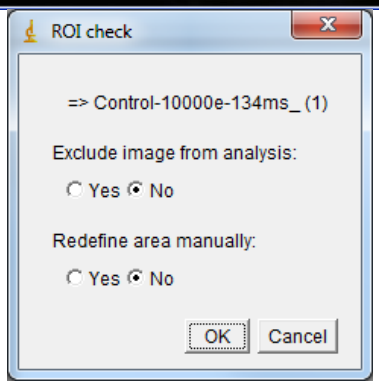
After completion of Step 1 of image processing, images and their corresponding ROIs are checked by the user.

A dialog box is displayed to validate ROI. If the area is correct the user must click OK to proceed with next image.
The dialog box allows to exclude current image from analysis (*e.g.* blurred image) **OR** to manually redefine selected area.
Just select Yes on the radio button option if needed then click OK or **ENTER**↵ to continue

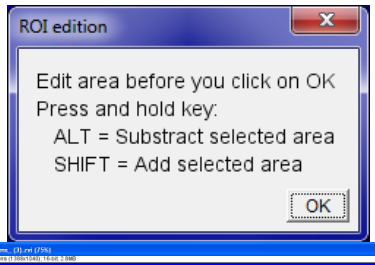


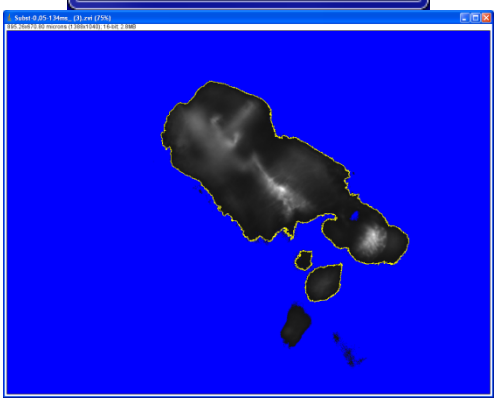


If you selected to redefine area manually, a new dialog box is displayed to get access to the ImageJ toolbar and modify the yellow selection. The user-defined threshold is then automatically applied.

Select the appropriate tool in ImageJ toolbar:
Freehand (default) OR Wand tool
To remove non-specific fluorescence (*e.g.* vitellus), use Freehand tool user and hold Alt key while selecting area to remove from ROI.
To add area to the previously selected ROI, hold Shift key while selecting new area to add.

Click OK to validate the new ROI: image is then measured and zip file is automatically updated.





In case the selection is empty after manual correction the macro discard image from analysis.

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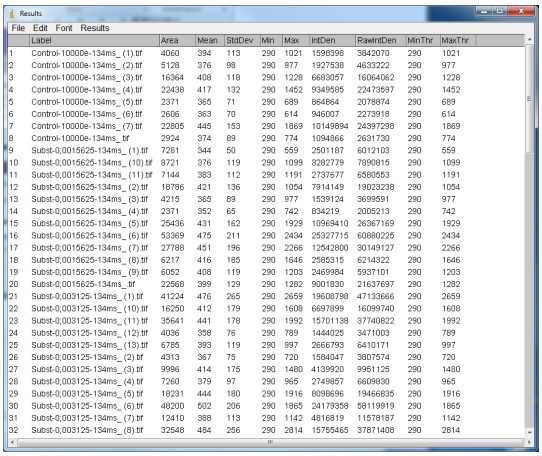
After completion of Step 2, a list of all measured images with filename is displayed in the Result Table window of ImageJ.
This table can be automatically saved as a csv or xls file in the work directory selected at startup.



Results

File Edit Font Results

	Label	Area	Mean	StdDev	Min	Max	IntDen	RawIntDen	MinThr	MaxThr
1	Control-10000e-134ms_ (1).tif	4060	394	113	290	1021	1598398	3842070	290	1021
2	Control-10000e-134ms_ (2).tif	5128	376	98	290	977	1927538	4633222	290	977
3	Control-10000e-134ms_ (3).tif	16364	408	118	290	1228	6683057	16064062	290	1228
4	Control-10000e-134ms_ (4).tif	22438	417	132	290	1452	9349585	22473597	290	1452
5	Control-10000e-134ms_ (5).tif	2371	365	71	290	689	864864	2078874	290	689
6	Control-10000e-134ms_ (6).tif	2606	363	70	290	614	946007	2273918	290	614
7	Control-10000e-134ms_ (7).tif	22805	445	153	290	1869	10149894	24397298	290	1869
8	Control-10000e-134ms_.tif	2924	374	89	290	774	1094866	2631730	290	774
9	Subst-0,0015625-134ms_ (1).tif	7281	344	50	290	559	2501187	6012103	290	559
10	Subst-0,0015625-134ms_ (10).tif	8721	376	119	290	1099	3282779	7890815	290	1099
11	Subst-0,0015625-134ms_ (11).tif	7144	383	112	290	1191	2737677	6580553	290	1191
12	Subst-0,0015625-134ms_ (2).tif	18786	421	136	290	1054	7914149	19023238	290	1054
13	Subst-0,0015625-134ms_ (3).tif	4215	365	89	290	977	1539124	3699591	290	977
14	Subst-0,0015625-134ms_ (4).tif	2371	352	65	290	742	834219	2005213	290	742
15	Subst-0,0015625-134ms_ (5).tif	25436	431	162	290	1929	10969410	26367169	290	1929
16	Subst-0,0015625-134ms_ (6).tif	53369	475	211	290	2434	25327715	60880225	290	2434
17	Subst-0,0015625-134ms_ (7).tif	27788	451	196	290	2266	12542800	30149127	290	2266
18	Subst-0,0015625-134ms_ (8).tif	6217	416	185	290	1646	2585315	6214322	290	1646
19	Subst-0,0015625-134ms_ (9).tif	6052	408	119	290	1203	2469984	5937101	290	1203
20	Subst-0,0015625-134ms_.tif	22568	399	129	290	1282	9001830	21637697	290	1282
21	Subst-0,003125-134ms_ (1).tif	41224	476	265	290	2659	19608798	47133666	290	2659
22	Subst-0,003125-134ms_ (10).tif	16250	412	179	290	1608	6697899	16099740	290	1608
23	Subst-0,003125-134ms_ (11).tif	35641	441	178	290	1992	15701138	37740622	290	1992
24	Subst-0,003125-134ms_ (12).tif	4036	358	76	290	789	1444025	3471003	290	789
25	Subst-0,003125-134ms_ (13).tif	6785	393	119	290	997	2666793	6410171	290	997
26	Subst-0,003125-134ms_ (2).tif	4313	367	75	290	720	1584047	3807574	290	720
27	Subst-0,003125-134ms_ (3).tif	9996	414	175	290	1480	4139920	9951125	290	1480
28	Subst-0,003125-134ms_ (4).tif	7260	379	97	290	965	2749857	6609830	290	965
29	Subst-0,003125-134ms_ (5).tif	18231	444	180	290	1916	8098696	19466835	290	1916
30	Subst-0,003125-134ms_ (6).tif	48200	502	206	290	1865	24179358	58119919	290	1865
31	Subst-0,003125-134ms_ (7).tif	12410	388	113	290	1142	4816819	11578187	290	1142
32	Subst-0,003125-134ms_ (8).tif	32548	484	256	290	2814	15755465	37871408	290	2814

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ANNEX 9 Data reporting and analysis

In Annex 9, typical data obtained with the EASZY assay are reported.

1. Observed variability of GFP measurements and responses in solvent, test medium and positive control obtained by independent laboratories involved in the intercalibration study.

The table 1 reports GFP measurement (expressed as fold-induction above solvent) in control groups from independent EASZY assays performed in four laboratories. These data illustrate the variability of GFP measurement in controls groups which is consistent among assays and laboratories. The coefficient of variation (CV %) in solvent and test medium are high, likely reflecting the variability of the expression of the brain aromatase gene during Zebrafish development and the variability of the method to measure GFP *in vivo* by fluorescence imaging. Nonetheless, the statistical power of the EASZY assay is high reflecting its capacity to detect a difference between control and exposed groups.

Table 1. Example of variability of GFP measurement in different laboratories in three independent assays. For each laboratory, the GFP (expressed as fold-induction above solvent) ± standard deviation (SD) is reported for the solvent, the test medium and the positive control (EE2 14.8 ng/L). The coefficient of variation (CV expressed as %) is indicated.

	Lab A			Lab B			Lab C			Lab D		
	Assay 1	Assay 2	Assay 3	Assay 1	Assay 2	Assay 3	Assay 1	Assay 2	Assay 3	Assay 1	Assay 2	Assay 3
Solvent control												
Mean	1.0	1.0	1.0	1.0	1.0	1.0	1.0	1.0	1.0	1.0	1.0	1.0
SD	0.4	0.6	0.5	0.6	0.5	0.6	0.7	0.7	0.7	0.4	0.5	0.6
CV (%)	42.6	58.2	45.8	64.7	48.5	60.0	67.1	66.9	73.9	44.1	45.2	64.4
Test medium control												
Mean	1.0	0.8	0.9	1.4	1.4	0.9	1.0	1.4	1.3	0.9	1.0	1.2
SD	0.5	0.5	0.6	1.2	0.9	0.7	0.7	0.9	1.1	0.3	0.6	0.6
CV (%)	55.1	60.2	68.8	89.7	65.7	74.3	69.2	61.1	83.6	33.7	59.8	46.4
Positive control												
Mean	17.2	24.1	20.5	13.9	9.4	9.6	29.4	15.0	22.3	10.3	9.9	17.0
SD	4.9	8.4	7.7	2.1	2.2	3.2	9.4	4.2	7.8	4.0	2.9	4.7
CV (%)	28.3	35.0	37.4	15.1	23.6	33.0	31.8	27.8	35.0	38.7	29.2	27.5

2. Typical example of data collected from an EASZY assay

In this example, a substance “A” was tested at 5 different concentrations (from the lowest concentration Conc. 1 to the highest Conc. 5).

Three different control groups were realized: solvent control, test medium control (ISO reconstituted water), positive control (EE2 14.8 ng/L).

In each treatment group, the survival rate and the hatching rate are reported as well as the number of embryos analyzed by fluorescence image analysis.

In this example, the test is valid as:

I. The control survival and hatching rates met the validity criteria defined for these parameters (see paragraph 25)

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II. The mean measured GFP induction in the positive control is > 9 meaning that the positive control is valid

III. The mortality rates in exposed groups agree with the acceptability criterion defined in paragraph 48. All the concentrations are used to analyze the effect of the test chemical.

RawintDen = fluorescence intensity of an embryo (based on image analysis; see Annex 8)

$$GFP\ (fold) = \frac{\textbf{\textit{Fluorescence intensity measured in an embryo}}}{mean\ fluorescence\ intensity\ measured\ in\ the\ control\ group}$$

$$Survival\ rate\ (\%) = \frac{Number\ of\ live\ embryo\ at\ 96\ hpf}{Total\ number\ of\ embryos\ in\ the\ replicate} \text{ x } 100$$

$$Hatching\ rate\ (\%) = \frac{\text{number of hatched embryos at 96 hpf}}{\text{total number of live embryos at 96 hfp}} \text{ x } 100$$

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Table 2: Example of data collected from the EASZY assay

Condition	Replicate # (R#)	# individual	Label	RawIntDen	GFP (fold)	Mean GFP (fold)	SD	n /replicate	Survival rate (%)	Hatching rate (%)	Mean (RawIntDen R1+R2+R3)	Mean GFP (fold) (R1-R2-R3)	Standard Deviation
Solvent group	1	1	DMSO_R1_	4 874 116	0,8	0,74	0,34	7	100	100	5 980 768	1,00	0,41
		2	DMSO_R1_	6 654 906	1,1								
		3	DMSO_R1_	622 207	0,1								
		4	DMSO_R1_	4 076 376	0,7								
		5	DMSO_R1_	6 779 792	1,1								
		6	DMSO_R1_	3 912 076	0,7								
		7	DMSO_R1_	4 281 717	0,7								
	2	1	DMSO_R2	11 635 508	1,9	1,27	0,51	6	86	100			
		2	DMSO_R2	9 900 998	1,7								
		3	DMSO_R2	4 210 012	0,7								
		4	DMSO_R2	5 674 354	0,9								
		5	DMSO_R2	5 865 124	1,0								
		6	DMSO_R2	6 687 435	1,1								
	3	1	DMSO_R3	4 940 098	0,8	1,07	0,31	6	86	100			
		2	DMSO_R3	6 396 897	1,1								
		3	DMSO_R3	7 158 886	1,2								
		4	DMSO_R3	7 239 720	1,2								
		5	DMSO_R3	8 961 822	1,5								
		6	DMSO_R3	3 762 552	0,6								
water control (ISO water)	1	1	ISO_Water_R1	2 666 553	0,4	1,24	0,42	7	100	100	6 986 430	1,17	0,42
		2	ISO_Water_R1	6 351 281	1,1								
		3	ISO_Water_R1	9 904 374	1,7								
		4	ISO_Water_R1	7 550 167	1,3								
		5	ISO_Water_R1	6 793 603	1,1								
		6	ISO_Water_R1	9 486 469	1,6								
		7	ISO_Water_R1	9 148 253	1,5								
	2	1	ISO_Water_R2	5 156 341	0,9	1,18	0,53	7	100	100			
		2	ISO_Water_R2	10 897 349	1,8								
		3	ISO_Water_R2	7 533 429	1,3								
		4	ISO_Water_R2	5 025 053	0,8								
		5	ISO_Water_R2	4 776 223	0,8								
		6	ISO_Water_R2	3 997 141	0,7								
		7	ISO_Water_R2	11 819 339	2,0								
	3	1	ISO_Water_R3	5 842 230	1,0	1,08	0,36	6	86	100			
		2	ISO_Water_R3	9 431 310	1,6								
		3	ISO_Water_R3	5 757 365	1,0								
		4	ISO_Water_R3	7 308 139	1,2								
		5	ISO_Water_R3	7 338 579	1,2								
		6	ISO_Water_R3	2 945 410	0,5								
positive control (EE2 14.8 ng/L)	1	1	EE2_0.05nM_R1	71 930 735	12,0	11,69	2,78	7	100	100	84 271 975	14,09	4,68
		2	EE2_0.05nM_R1	105 122 998	17,6								
		3	EE2_0.05nM_R1	53 386 070	8,9								
		4	EE2_0.05nM_R1	64 083 240	10,7								
		5	EE2_0.05nM_R1	60 552 970	10,1								
		6	EE2_0.05nM_R1	68 295 748	11,4								
		7	EE2_0.05nM_R1	66 149 186	11,1								
	2	1	EE2_0.05nM_R2	102 759 393	17,2	14,24	4,98	7	100	100			
		2	EE2_0.05nM_R2	58 198 318	9,7								
		3	EE2_0.05nM_R2	124 051 865	20,7								
		4	EE2_0.05nM_R2	58 021 914	9,7								
		5	EE2_0.05nM_R2	120 673 117	20,2								
		6	EE2_0.05nM_R2	72 252 446	12,1								
		7	EE2_0.05nM_R2	60 313 370	10,1								
	3	1	EE2_0.05nM_R3	112 369 410	18,8	16,34	5,26	7	100	100			
		2	EE2_0.05nM_R3	77 830 991	13,0								
		3	EE2_0.05nM_R3	139 233 203	23,3								
		4	EE2_0.05nM_R3	137 034 544	22,9								
		5	EE2_0.05nM_R3	64 271 974	10,7								
		6	EE2_0.05nM_R3	71 380 413	11,9								
		7	EE2_0.05nM_R3	81 799 575	13,7								

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Table 2: (continued)

Condition	Replicate #	# individual	Label	RawIntDen	GFP (fold)	mean GFP	SD	n /replicate	Survival rate	Hatching rate	Mean RawIntDen (R1+R2+R3)	Mean GFP (fold) (R1-R2-R3)	Standard Deviation
Substance A Concentration 1	1	1	subtance_C1_R1	13 507 593	2,3	1,89	0,40	7	100	100	10 816 778	1,81	0,66
		2	subtance_C1_R1	11 836 562	2,0								
		3	subtance_C1_R1	14 976 217	2,5								
		4	subtance_C1_R1	10 908 321	1,8								
		5	subtance_C1_R1	10 010 384	1,7								
		6	subtance_C1_R1	10 036 746	1,7								
		7	subtance_C1_R1	7 756 360	1,3								
	2	1	subtance_C1_R2	16 527 601	2,8	2,02	0,81	7	100	100			
		2	subtance_C1_R2	6 310 398	1,1								
		3	subtance_C1_R2	9 783 991	1,6								
		4	subtance_C1_R2	14 343 054	2,4								
		5	subtance_C1_R2	17 064 048	2,9								
		6	subtance_C1_R2	5 417 556	0,9								
		7	subtance_C1_R2	14 930 672	2,5								
	3	1	subtance_C1_R3	5 615 174	0,9	1,52	0,71	7	100	100			
		2	subtance_C1_R3	10 448 075	1,7								
		3	subtance_C1_R3	8 869 381	1,5								
		4	subtance_C1_R3	14 725 626	2,5								
		5	subtance_C1_R3	14 252 784	2,4								
		6	subtance_C1_R3	5 568 572	0,9								
		7	subtance_C1_R3	4 263 219	0,7								
Substance A Concentration 2	1	1	subtance_C2_R1	6 775 729	1,1	1,40	0,54	7	100	100	8 459 415	1,41	0,46
		2	subtance_C2_R1	12 434 825	2,1								
		3	subtance_C2_R1	4 407 107	0,7								
		4	subtance_C2_R1	12 624 433	2,1								
		5	subtance_C2_R1	9 470 396	1,6								
		6	subtance_C2_R1	7 305 193	1,2								
		7	subtance_C2_R1	5 678 952	0,9								
	2	1	subtance_C2_R2	4 496 991	0,8	1,32	0,48	7	100	100			
		2	subtance_C2_R2	6 987 774	1,2								
		3	subtance_C2_R2	7 257 037	1,2								
		4	subtance_C2_R2	5 504 582	0,9								
		5	subtance_C2_R2	7 767 892	1,3								
		6	subtance_C2_R2	12 667 120	2,1								
		7	subtance_C2_R2	10 757 400	1,8								
	3	1	subtance_C2_R3	9 308 406	1,6	1,52	0,41	7	100	100			
		2	subtance_C2_R3	12 899 089	2,2								
		3	subtance_C2_R3	9 951 068	1,7								
		4	subtance_C2_R3	8 467 905	1,4								
		5	subtance_C2_R3	4 795 936	0,8								
		6	subtance_C2_R3	7 996 179	1,3								
		7	subtance_C2_R3	10 093 706	1,7								
Substance A (concentration 3)	1	1	subtance_C3_R1	14 600 703	2,4	1,84	0,49	7	100	100	12 292 910	2,06	0,55
		2	subtance_C3_R1	10 101 759	1,7								
		3	subtance_C3_R1	7 281 076	1,2								
		4	subtance_C3_R1	8 902 999	1,5								
		5	subtance_C3_R1	15 269 349	2,6								
		6	subtance_C3_R1	10 509 714	1,8								
		7	subtance_C3_R1	10 398 688	1,7								
	2	1	subtance_C3_R2	14 534 037	2,4	2,14	0,41	6	86	100			
		2	subtance_C3_R2	11 519 299	1,9								
		3	subtance_C3_R2	8 960 455	1,5								
		4	subtance_C3_R2	11 719 652	2,0								
		5	subtance_C3_R2	14 867 058	2,5								
		6	subtance_C3_R2	15 082 903	2,5								
	3	1	subtance_C3_R3	12 646 133	2,1	2,20	0,70	7	100	100			
		2	subtance_C3_R3	15 363 363	2,6								
		3	subtance_C3_R3	16 852 074	2,8								
		4	subtance_C3_R3	13 450 406	2,2								
		5	subtance_C3_R3	8 519 588	1,4								
		6	subtance_C3_R3	18 274 318	3,1								
		7	subtance_C3_R3	7 004 622	1,2								
substance A (concentration 4)	1	1	subtance_C4_R1	38 287 375	6,4	5,31	1,60	7	100	100	25 458 397	4,26	1,54
		2	subtance_C4_R1	24 544 667	4,1								
		3	subtance_C4_R1	29 107 967	4,9								
		4	subtance_C4_R1	36 096 743	6,0								
		5	subtance_C4_R1	23 646 007	4,0								
		6	subtance_C4_R1	22 236 473	3,7								
		7	subtance_C4_R1	48 271 083	8,1								
	2	1	subtance_C4_R2	18 592 109	3,1	3,80	1,07	7	100	100			
		2	subtance_C4_R2	25 768 398	4,3								
		3	subtance_C4_R2	13 015 362	2,2								
		4	subtance_C4_R2	32 592 848	5,4								
		5	subtance_C4_R2	19 489 317	3,3								
		6	subtance_C4_R2	22 684 485	3,8								
		7	subtance_C4_R2	26 854 149	4,5								
	3	1	subtance_C4_R3	12 260 474	2,0	3,67	1,49	7	100	100			
		2	subtance_C4_R3	23 377 756	3,9								
		3	subtance_C4_R3	19 131 382	3,2								
		4	subtance_C4_R3	39 511 233	6,6								
		5	subtance_C4_R3	25 386 701	4,2								
		6	subtance_C4_R3	15 465 375	2,6								
		7	subtance_C4_R3	18 306 430	3,1								
Subtance A (concentration 5)	1	1	subtance_C5_R1	130 323 975	21,8	17,59	3,71	7	100	100	25 458 397	19,00	4,59
		2	subtance_C5_R1	111 652 396	18,7								
		3	subtance_C5_R1	117 792 473	19,7								
		4	subtance_C5_R1	112 146 114	18,8								
		5	subtance_C5_R1	82 246 955	13,8								
		6	subtance_C5_R1	67 096 120	11,2								
		7	subtance_C5_R1	114 946 079	19,2								
	2	2	subtance_C5_R2	108 883 032	18,2	19,37	6,16	5	86	100			
		3	subtance_C5_R2	144 832 092	24,2								
		4	subtance_C5_R2	131 283 589	22,0								
		5	subtance_C5_R2	54 632 538	9,1								
		6	subtance_C5_R2	139 580 958	23,3								
	3	1	subtance_C5_R3	159 768 777	26,7	20,35	4,41	6	86	100			
		2	subtance_C5_R3	104 079 657	17,4								
		3	subtance_C5_R3	125 300 941	21,0								
		4	subtance_C5_R3	92 586 729	15,5								
		5	subtance_C5_R3	103 730 320	17,3								
		6	subtance_C5_R3	144 902 198	24,2								

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Graphical representation of the data and analysis

To identify potential activity of a chemical, responses are compared between treatments and control groups using appropriate statistical tests (see paragraph 60).

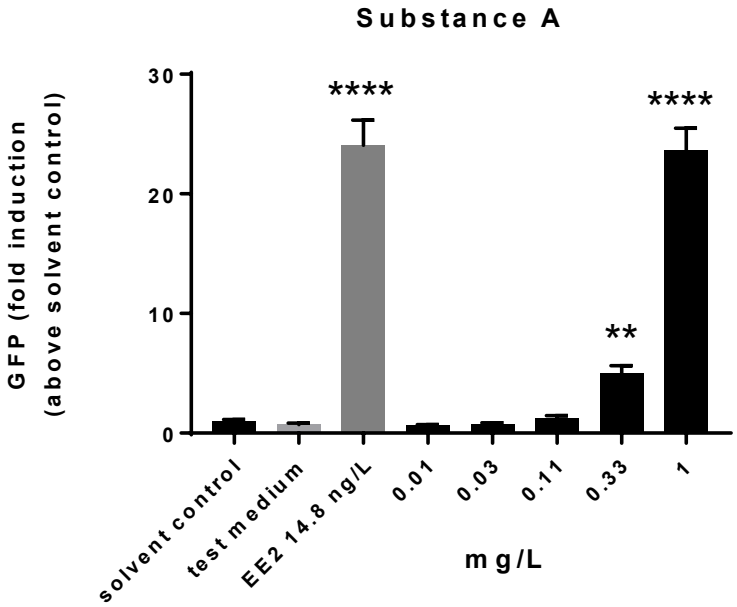

Figure 1: GFP expression (expressed as mean fold induction) ± standard deviation. **** (p=0. 0001) and ** (p=0.0042) denote significant differences as compared to the appropriate control group. The lowest concentration of the test chemical inducing a significant effect is reported as well as the concentration leading to the maximal induction of GFP and the fold induction. The maximal induction level is 23.5-fold as compared to the appropriate control group and the concentration inducing this maximal effect is 1 mg/L.

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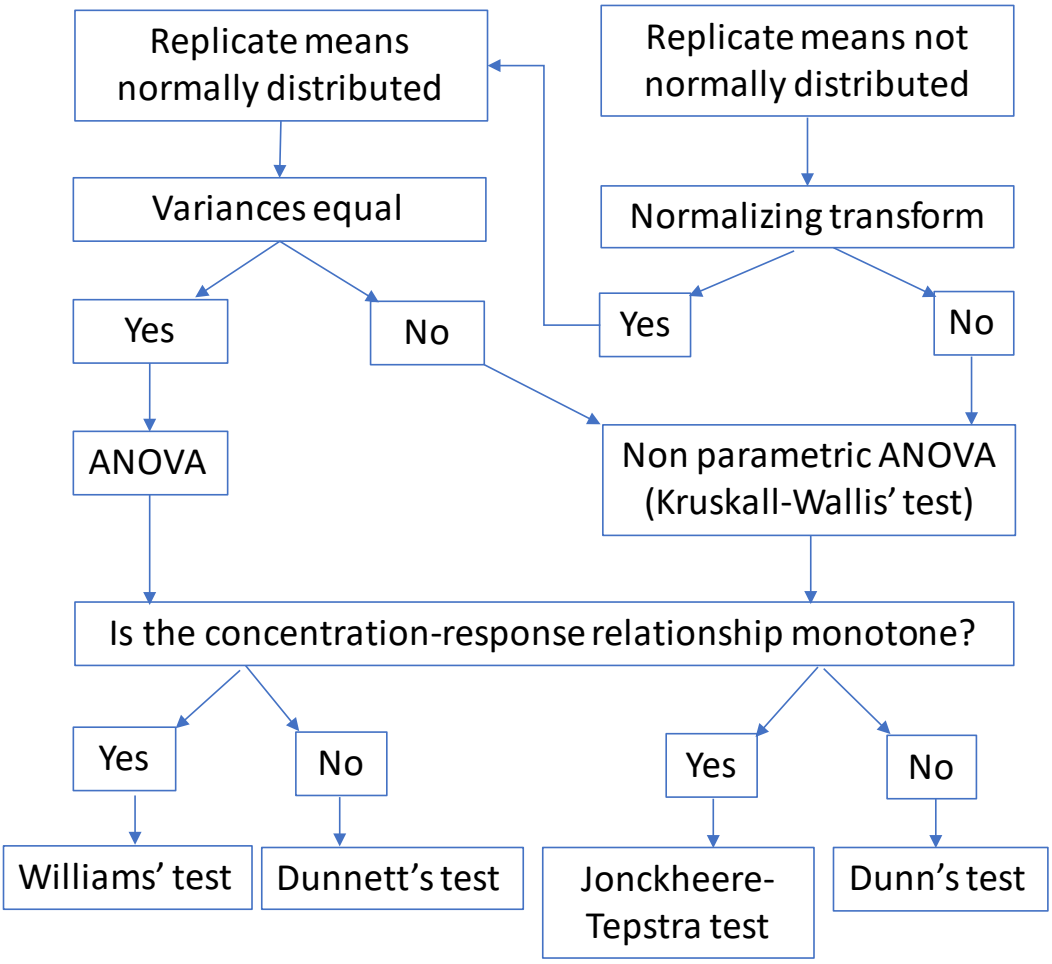
ANNEX 10 Decision flow chart for the statistical analysis



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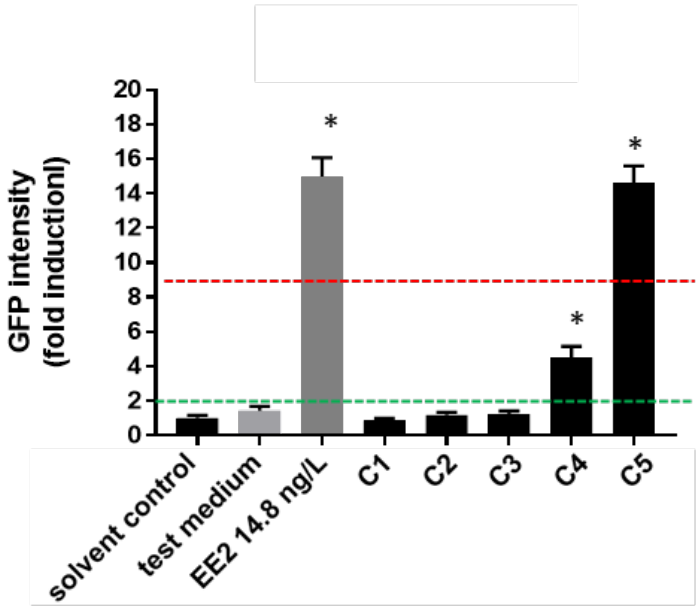
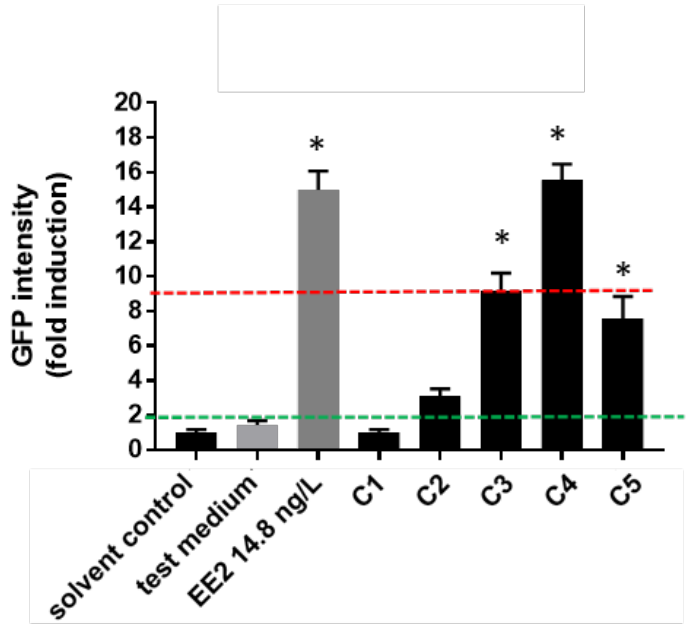
ANNEX 11 Examples of concentration-response curves that can be obtained in the EASZY: Interpretation and recommendations

(a)



- No significant difference between GFP expression in test medium and solvent controls.
- The mean measured GFP induction in the positive control is ≥ 9.
- The test chemical leads to a concentration-dependent induction of GFP. As compared to the solvent control, the effect is significant, and the fold induction is > 2.
- The test chemical is positive in the assay.

(b)



- No significant difference between GFP expression in test medium and solvent controls.
- The mean measured GFP induction in the positive control is ≥ 9.
- The test chemical leads to a concentration-dependent induction of GFP. As compared to the solvent control, the effect is significant, and the mean measured fold induction of GFP is > 2.
- The test chemical is positive.
- The fold induction rate measured in the highest concentration (conc. 5) is lower as compared to conc. 3 and conc. 4 which could reflect a sub-toxic effect of the test chemical This concentration should be excluded if the intent is to quantify the estrogenic activity by deriving an EC50. The hatching and mortality rate for the conc. 5 should be examined carefully.

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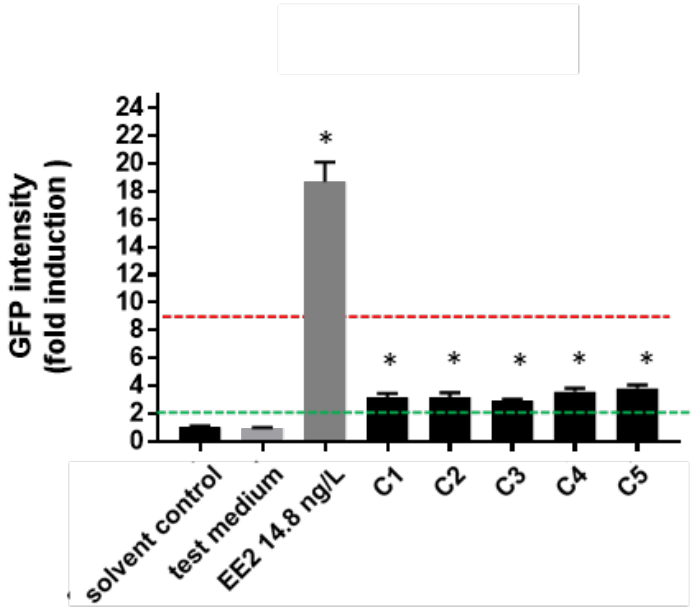
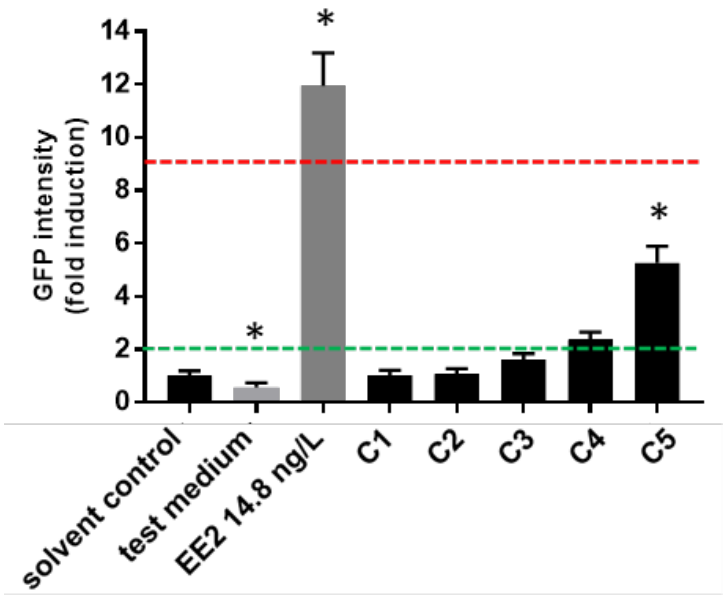
(c)



- No significant difference between GFP expression in test medium and solvent controls.
- The mean measured GFP induction in the positive control is ≥ 9.
- A significant difference is measured for all the concentrations tested and the mean measured GFP expression is > 2.
- The test chemical is identified as active.

(d)



- The mean measured GFP induction in the positive control is ≥ 9.
- A significant induction of the GFP is observed for the highest concentration of the test chemical.
- A significant difference between GFP expression in test medium and solvent controls is measured.
- The test chemical is active in the assay.
- Performing another assay could be recommended as a solvent effect is observed. The use of a new solvent solution is highly recommended.

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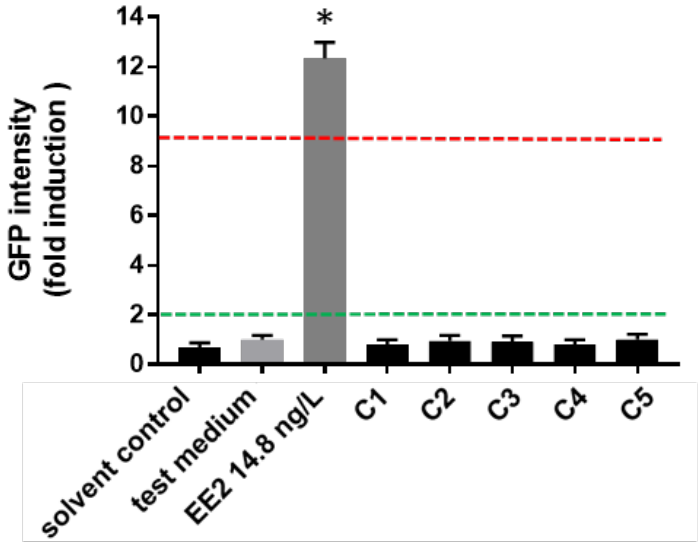
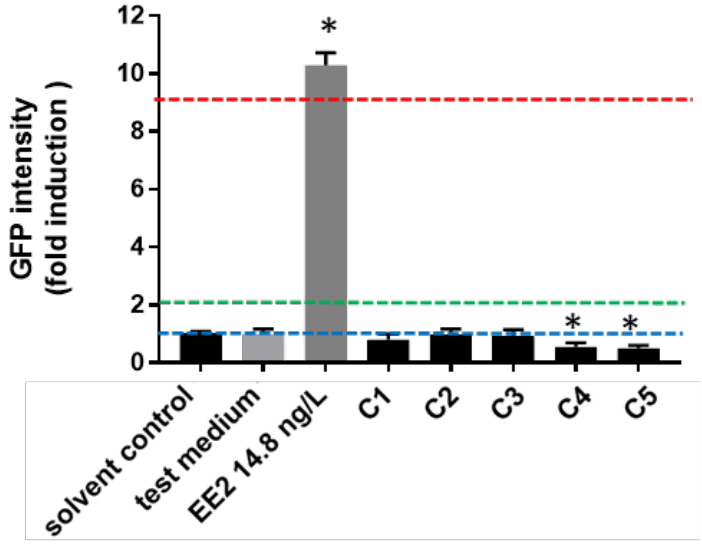
(e)



- No significant difference between GFP expression in water and solvent control.
- The mean measured GFP induction in the positive control is ≥ 9.
- As compared to the solvent control, no significant difference is noticed, and the mean measured GFP expression are below 2.
- The test chemical is negative in the assay.

(f)



- No significant difference between GFP expression in test medium and solvent controls.
- The mean measured GFP induction in the positive control is ≥ 9.
- As compared to the solvent control, a significant inhibition is noticed for conc. 4 and conc 5 of the tested chemical.
- The test chemical is inactive in EASZY which intends to identify substances leading to an up-regulation of the brain aromatase expression.

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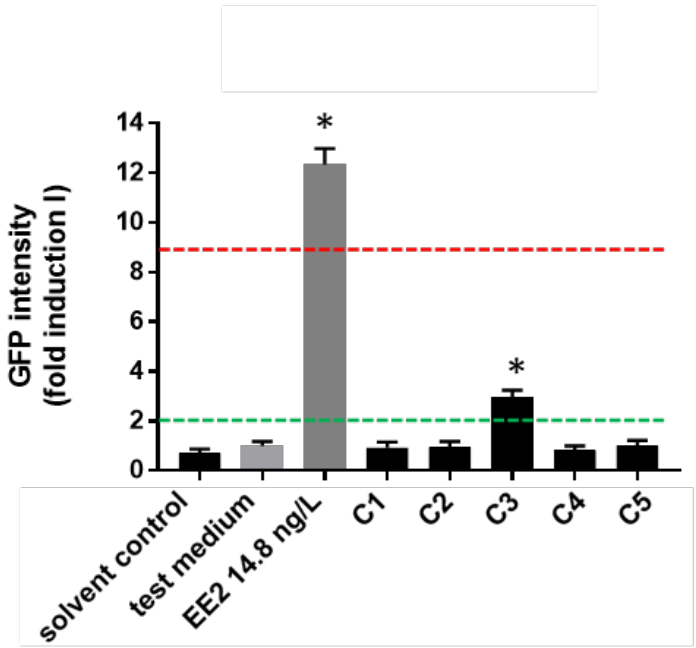
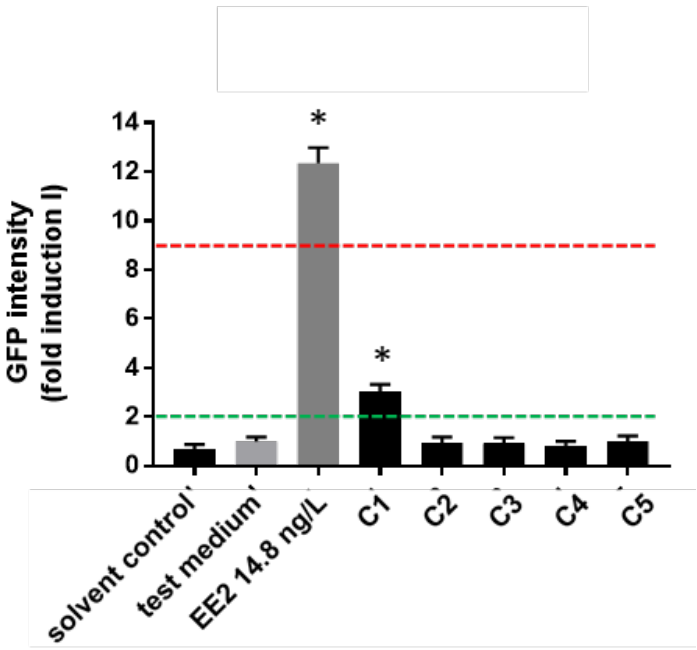
(g)



- No significant difference between GFP expression in test medium and solvent controls.
- The mean measured GFP induction in the positive control is ≥ 9.
- A significant difference is measured at an intermediate concentration (conc. 3) and the mean measured GFP induction is > 2.
- No formal conclusion can be drawn.
- The test should be repeated given the atypical concentration response curve.
- The hatching and/or mortality rates in the two highest concentrations should be examined carefully as it may explain the lower GFP induction in the two highest exposure concentrations.

(h)



- No significant difference between GFP expression in test medium and solvent controls.
- The mean measured GFP induction in the positive control is ≥ 9.
- A significant difference is measured at the lowest concentration with a mean measured fold induction of GFP > 2.
- As the concentration-response curve is atypical, it would be appropriate to repeat that assay with a new range of concentrations including concentrations lower than Conc. 1.

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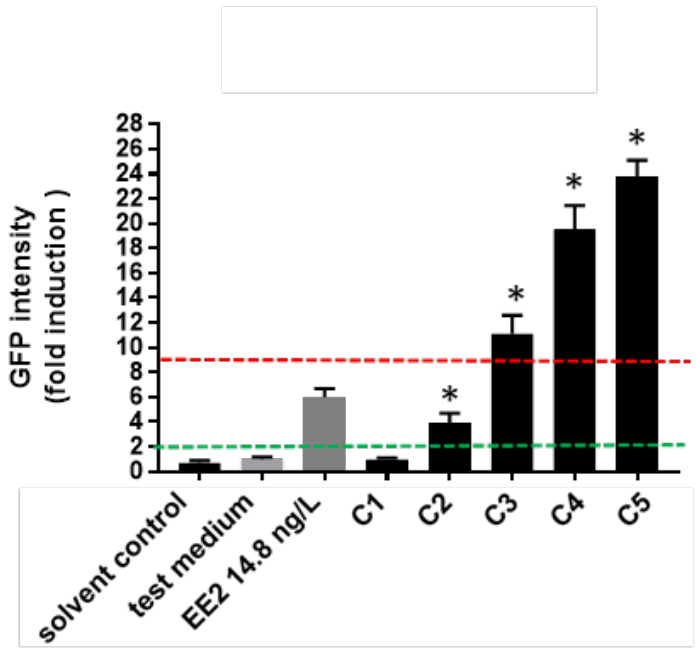
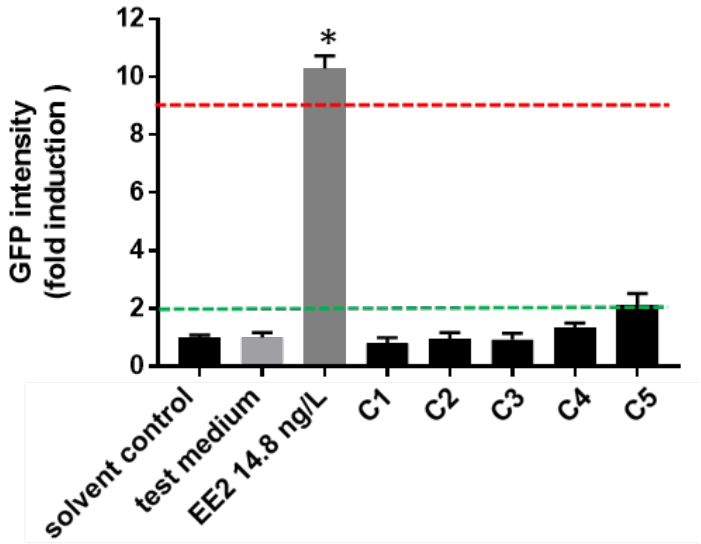
(i)



- No significant difference between GFP expression in test medium and solvent controls.
- The test chemical leads to a concentration-dependent induction of the GFP with mean measured GFP > 2-fold.
- However, the mean measured GFP induction in the positive control is < 9.
- As the validity criterion for the positive control is not meet, the test should be repeated.
- In that case, the tested chemical is highly suspected to be active, but the results of a valid test are needed.

(j)



- No significant difference between GFP expression in test medium and solvent controls.
- The mean measured GFP induction in the positive control is ≥ 9.
- The test chemical lead to a significant GFP induction at the highest concentration tested.
- However, the mean measured GFP induction for the conc. 5 is ≤ 2.
- It is recommended to perform another assay by testing higher concentration(s) than C5 while considering the solubility limit and the MTC.
- If similar results are obtained for the second assay, the test chemical cannot be identified as active in EASZY.
- Other relevant assays could be performed.

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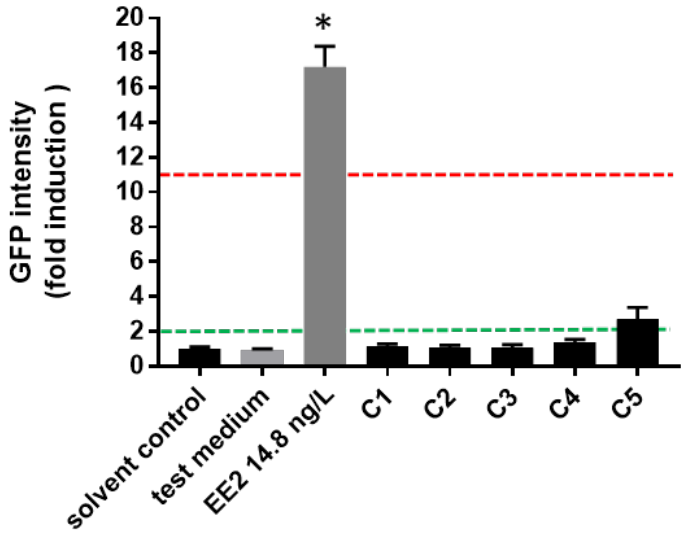
(k)



- No significant difference between GFP expression in test medium and solvent controls
- The mean measured GFP induction in the positive control is ≥ 9
- The mean measured GFP induction for the highest concentration of the tested chemical is above >2.
- However, the induction is non-significant as compared to the solvent group.
- The test should be repeated.
- If similar data are obtained, no formal conclusion can be drawn based solely on the results of EASZY assays.

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