Section 2
Effects on Biotic Systems

Test Guideline No. 251
Rapid Androgen Disruption Activity Reporter (RADAR) assay

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OECD Guidelines for the Testing of Chemicals

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OECD GUIDELINE FOR THE TESTING OF CHEMICALS

Rapid Androgen Disruption Activity Reporter Assay (RADAR)

1. INTRODUCTION

1. The Rapid Androgen Disruption Activity Reporter (RADAR) test guideline describes an aquatic assay that utilizes transgenic *Oryzias latipes (O. latipes*, Japanese medaka) eleutheroembryos at day post hatch zero (DPH0; see Annex 1 for abbreviations), in a multi-well format to detect chemicals active on the androgen axis. The RADAR assay was designed as a screening tool to provide a short-term assay to measure the response of eleutheroembryos to chemicals potentially active on the androgen axis (Muschket et al., 2018; Ogino et al., 2020; Sébillot et al., 2014). The RADAR assay is intended to be a fish screen classifying the chemicals into potentially active or inactive on the androgen axis, but the RADAR assay is not intended to determine toxicity values for risk assessment (e.g., no observable effect concentration [NOEC] or ECX [see Annex 1]). The RADAR assay is placed at level 3 of the OECD conceptual framework for the testing of endocrine disrupters (OECD, 2018). The OECD GD 150 provides further guidance on the interpretation and extrapolation between taxa of the results of the RADAR assay (OECD, 2018).

2. The Japanese medaka fish, *O. latipes*, is the test species selected for the RADAR assay. Japanese medaka estrogen receptors (ER) and androgen receptors (AR) show conformational conservation when compared to human receptors (Cui et al., 2009). This species is also utilized in a number of validated OECD Test Guidelines including: OECD TG 203 (Fish Acute Toxicity Test; OECD, 2019a), OECD TG 210 (Fish Early Life Stage Toxicity Test; OECD, 2013), OECD TG 212 (Fish Short Term Toxicity Test on Embryo and Sac-fry Stages; OECD, 1998), OECD TG 229 (Fish Short-Term Reproduction Assay; OECD, 2012), OECD TG 230 (21-day Fish Assay; OECD, 2009), OECD TG 234 (Fish Sexual Development Test; OECD, 2011b) and OECD TG 240 (Medaka Extended One Generation Reproduction Test; OECD, 2015).

3. The RADAR assay is transcription-based and uses a transgenic Japanese medaka line harbouring the *spg1-gfp* genetic construct. This genetic construct comprises the promoter of the spiggin 1 gene coupled to a reporter gene for Green Fluorescent Protein (GFP). Spiggin is a glycoprotein (mucin-like glue protein) produced in the kidney of sexually mature male three-spined stickleback (*Gasterosteus aculeatus*) in order to build a nest (Jakobsson et al., 1999). Borg et al. [1993] established that this process was controlled by androgens, the most potent compound being 11-ketotestosterone (11KT), following injecting castrated males with a range of steroids. An ELISA for spiggin has been developed, demonstrating that female sticklebacks are capable of synthesising spiggin when challenged with exogenous androgens, but under normal conditions their circulating spiggin levels are close to detection limits both *in vivo* (Katsiadaki et al, 2002a; 2002b;

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Hahlbeck et al., 2004) and *ex vivo* (Jolly et al., 2009), demonstrating the value of spiggin as a biomarker for androgens. Subsequently, an assay was developed for detecting antiandrogens (Katsiadaki et al, 2006), which was validated (OECD, 2010a) and resulted in OECD guidance document 148 (OECD, 2011a). The spiggin 1 gene is stickleback specific. A single homologue of the stickleback spiggin genes is present in at least some teleost genomes including Japanese medaka, although very little is known regarding these homologues. A single orthologue of the stickleback spiggin gene family, muc19, has also been identified in mammals (Kawahara and Nishida, 2007).

4. The *spg1-gfp* transgenic line used in the RADAR assay harbours 4.159 kb of the three spined stickleback spiggin 1 gene promoter immediately upstream of the start codon driving expression of GFP coding sequence. The *spg1-gfp* transgene faithfully replicates the tissue specificity of the spiggin 1 gene in Japanese medaka as observed in three-spined stickleback, with expression of GFP strictly limited to the developing kidney (mesonephros). The expression of the transgene has also been shown to be significantly inhibited in response to estrogens (OECD, 2022). Using spiggin as a biomarker, it had previously been noted both *in vitro* and *in vivo* (OECD, 2011a; Jolly et al., 2009; Katsiadaki et al., 2006) that estrogens exert an anti-androgenic effect. The strong antiandrogenic effect of estrogens is supported by additional *in vitro* assays (e.g. Sohoni and Sumpter, 1998) and was also observed during the characterisation of the RADAR assay (Sébillot et al., 2014). As activation of the spiggin 1 promoter is a terminal step in androgen axis signalling, the quantity of spiggin 1 protein produced in a three-spined stickleback or the quantity of GFP produced in the *spg1-gfp* Japanese medaka model represents the overall or net effects of both endogenous and exogenous factors altering androgen axis signalling (alterations in production, transport, metabolism and excretion of hormones as well as activation and inhibition of AR).

5. Before performing the RADAR assay, the laboratory should verify that it has the certifications that may be required by local regulations on the use of transgenic organisms. The RADAR assay should only be performed using the *spg1-gfp* transgenic line used for the test guideline development, which is commercially available (OECD, 2022). The use of another transgenic line based on the spiggin 1 promoter driving the expression of GFP or another reporter gene requires a complete OECD validation to adapt the validity criteria, the statistical analysis and the fluorescence thresholds used in the decision logic. Therefore, other transgenic lines could not be considered as appropriate for the implementation of the RADAR assay.

6. This guideline proposal is based on an international interlaboratory validation study conducted between 2019 and 2020 (OECD, 2022). The test has been validated in five laboratories with eight mono-constituent test chemicals, in addition to 17α-methyl testosterone (17MT) and flutamide concentration ranges which were included in every experiment performed in the validation exercise. It should be noted that one of the five laboratories only tested five of the eight test chemicals.

7. The measurement endpoint is induction of fluorescence in eleutheroembryos. When transcription of the genetic construct is activated or inhibited following chemical exposure, eleutheroembryos express more or less GFP and, therefore, emit more or less fluorescence compared to the relevant control group.

8. The test chemical is tested in the presence and absence of 3 µg/L of 17MT as an AR agonist. As circulating androgen levels remain very low at this eleutheroembryonic life stage, adding 17MT to the test medium allows the detection of substances affecting 17MT availability or antagonising AR. The concentration of 17MT used for the co-treatment was determined empirically after testing different concentrations of 17MT (3 µg/L and 5 µg/L).

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The chosen concentration (3 µg/L) gave the highest sensitivity to both pro- and anti-androgenic reference chemicals. The differential gene expression induced by the combination of 17MT and the tested chemical is, therefore, a laboratory induced phenomenon, not observed in the absence of exogenous 17MT at this developmental stage, and thus not relevant to natural fish individuals and populations in the field. It does, however, allow identification of active test chemicals acting via modes of action that would not be identified as such in the absence of an aromatisable androgen, such as, alterations in aromatase or 5α-reductase activity or AR antagonism.

2. INITIAL CONSIDERATIONS AND LIMITATIONS

9. The assay measures the ability of a chemical to activate or inhibit transcription of the spg1-gfp genetic construct, whether directly through binding to AR or modifying the binding of androgens to the AR, or indirectly by modifying the amount of androgen available to activate the AR and thereby transcription of the spg1-gfp construct. To date the RADAR assay has been shown to detect chemicals acting through various mechanisms of action including: AR agonists (e.g., 17MT, 17α-methyl-5α-dihydrotestosterone [mDHT]); antagonists of the AR (e.g., flutamide, linuron, fenitrothion); modulators of androgen clearance including aromatase enzyme inhibitors (e.g., anastrozole and fadrozole), aromatase transcriptional modulators (e.g., prochloraz) and the inhibitory action of estrogens on the androgen axis (e.g., via induction of aromatase expression or antagonism of AR by estrogens); modulators of androgen metabolism, including 5α-reductase inhibitors (e.g., dutasteride) and chemicals requiring metabolic activation (e.g., vinclozolin, M1 and M2 metabolites are AR antagonists) (OECD, TBD; Sébillot et al., 2014). In addition, it is possible that modulators of androgen transport via interaction with plasma binding proteins could contribute to the overall results of the RADAR assay. The RADAR assay does not distinguish between the different modes of action but provides information on whether a chemical acts as a global activator or inhibitor of the androgen axis in the *O. latipes* eleutheroembryos. As the transcription of the spg1-gfp construct requires the direct action of AR on the spiggin 1 promotor, chemicals affecting AR signalling through alternative signalling pathways that do not lead to an alteration in the interaction between AR and DNA (i.e., "non-genomic actions") are not expected to be detected by the RADAR assay.

10. A number of publications have supported the idea that early life stages of medaka are metabolically competent, although current data is insufficient to conclude on the full breadth of metabolic competency. Prior to liver formation it has been demonstrated that embryonic medaka could transform benzo(a)pyrene (BaP) into metabolites including BaP-3-glucuronide (Hornung et al., 2007). Strong cytochrome P450 (CYP) 1A activity has also been identified in the liver, gills and other organs in DPH1 medaka (Kashiwada et al., 2007). In addition, CYP3A40 is expressed throughout medaka development, with CYP3A38 (the post-embryonic form) being expressed from 1-day post hatch (Kullman and Hinton, 2001). Vinclozolin requires metabolic activation, with antagonism of AR occurring due to the M1 and M2 metabolites. The LOEC obtained in the RADAR assay for vinclozolin (143 µg/L; Sébillot et al., 2014) was comparable to that obtained during the interlaboratory validation of the Androgenised Female Stickleback Screen (100 µg/L; OECD, 2010a).

11. This test guideline relies on the quantification of fluorescence in the whole eleutheroembryo. A limitation of this test guideline is that it should not be used for test chemicals emitting fluorescence between 500 and 550 nm (λ_{EM} = 500–550 nm) when excited at wavelengths between 450 and 500 nm (λ_{EX} = 450–500 nm) and is fluorescent

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and fluoresces within the eleutheroembryos. Test chemicals sharing these two properties may induce a fluorescence which could be interpreted as GFP signal, leading to the test chemical being incorrectly identified as active on the androgen axis. A simple protocol to determine if the test chemical emits fluorescence is proposed in §29. This protocol requires the use of wild-type *O. latipes* eleutheroembryos.

12. The RADAR assay should not be used to test chemicals falling outside of its applicability domain. When considering testing of mixtures or difficult test chemicals, upfront consideration should be given to whether such testing will yield results that are scientifically defendable. If the test guideline is used for the testing of a mixture, a UVCB (substances of unknown or variable composition, complex reaction products or biological materials) or a multi-constituent substance, its composition should, as far as possible, be characterized, e.g., by the chemical identity of its constituents, their quantitative occurrence and their substance-specific properties. Recommendations about the testing of difficult test chemicals (e.g., mixtures, UVCB or multi-constituent substances) are given in Guidance Document No. 23 (OECD, 2019b). The test design described in this test guideline is not suitable to test volatile substances.

3. PRINCIPLE OF THE TEST

3.1. General experimental design

13. The general experimental design entails exposing DPH0 transgenic *spg1-gfp* Japanese medaka eleutheroembryos in six-well plates for 72 h to a test chemical in the presence (“spiked mode”) and absence (“unspiked mode”) of a co-treatment with 3 µg/L of 17MT. Three independent runs should be performed for each assay. It is recommended to use a minimum of five concentrations plus non-optional controls (a test medium control and/or solvent control, two 17MT controls, and a 17MT + flutamide control) per run. The test uses 20 eleutheroembryos distributed in four wells (5 organisms per well) per test condition (test concentrations and controls), under a semi-static regime. The exposure solution should be renewed daily (i.e., after 24 h and 48 h). All six wells can be used on each six-well plate. It is not problematic to have two different test or control groups occupying the same plate as volatile chemicals are excluded. With five test concentrations and the non-optional controls including either a test medium control or solvent control, performed in three runs, the RADAR assay uses 280 eleutheroembryos per run, therefore, 840 eleutheroembryos are required for all three runs constituting an experiment (see Figure 1 and §15). The assay measures GFP fluorescence in transgenic *spg1-gfp* eleutheroembryos by fluorescence imaging that transforms the fluorescence signal to a numerical format. A detailed overview of test conditions can be found in Annex 2.

3.2. Controls

14. The RADAR assay requires the following control groups, all of which shall have the same concentration of organic solvent (if one is used). This test guideline was validated using dimethylsulfoxide (DMSO) exclusively, at a final concentration of 0.2%. Where possible, no solvent or a maximum solvent concentration of 100 mg/L (0.01%) should be used as indicated in OECD Guidance Document 23 (OECD, 2019b).

- Test medium and/ or solvent control: 4 wells with 5 organisms/well are exposed to test medium. This control defines the basal fluorescence level in the test medium. If a solvent is used, then this group is exposed to test medium plus the solvent used

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at the same concentration as all other groups. In some cases, such as a solvent being used with no historical data available, both groups may be required.

- 17MT 3 µg/L: 4 wells with 5 organisms/well are exposed to 3 µg/L of 17MT. This control establishes the fluorescence level for a 17MT concentration of 3 µg/L. This control can serve as part of the 17MT standard curve if the optional controls described below are included, allowing a 17MT equivalence value to be read off the standard curve for any pro-androgenic test chemicals. The determination of 17MT equivalence values is optional.
- 17MT 10 µg/L: 4 wells with 5 organisms/well are exposed to 10 µg/L of 17MT. This control establishes the fluorescence level for a 17MT concentration of 10 µg/L. This control can serve as part of the 17MT standard curve if the optional controls described below are included, allowing a 17MT equivalence value to be read off the standard curve for any pro-androgenic test chemicals. The determination of 17MT equivalence values is optional.
- 17MT 3 µg/L + Flutamide 500 µg/L: 4 wells with 5 organisms/well are exposed to 500 µg/L of flutamide in the presence of 3 µg/L of 17MT. This control establishes the fluorescence inhibition for a flutamide concentration of 500 µg/L compared to the 17MT 3 µg/L alone control group. This control serves as part of the flutamide standard curve if the optional controls described below are included, allowing a flutamide, as an AR antagonist, equivalence value to be read off the standard curve for any test chemicals displaying anti-androgenic activity in spiked mode (presence of 3 µg/L of 17MT).

The calculation of equivalence values is not required and is for informative purposes only as the result of the assay is that the test chemical is active or inactive only. If equivalence values are to be calculated, the optional controls below should be included in each run.

The following additional control groups are optional, but are recommended for calibration of reading parameters in naïve laboratories as well as for quality control purposes.

- 17MT 1.5 µg/L: 4 wells with 5 organisms/well are exposed to 1.5 µg/L of 17MT. This control establishes the fluorescence level for a 17MT concentration of 1.5 µg/L. This control serves as part of the 17MT standard curve, allowing a 17MT equivalence value to be read off the standard curve for any chemicals inducing androgen axis signalling.
- 17MT 3 µg/L + Flutamide 167 µg/L: 4 wells with 5 organisms/well are exposed to 167 µg/L of flutamide in the presence of 3 µg/L of 17MT. This control establishes the fluorescence inhibition for a flutamide concentration of 167 µg/L compared to the 17MT 3 µg/L alone control group. This control serves as part of the flutamide standard curve.
- 17MT 3 µg/L + Flutamide 55.6 µg/L: 4 wells with 5 organisms/well are exposed to 55.6 µg/L of flutamide in the presence of 3 µg/L of 17MT. This control establishes the fluorescence inhibition for a flutamide concentration of 55.6 µg/L compared to the 17MT 3 µg/L alone control group. This control serves as part of the flutamide standard curve.
- 17MT 3 µg/L + Flutamide 18.5 µg/L: 4 wells with 5 organisms/well are exposed to 18.5 µg/L of flutamide in the presence of 3 µg/L of 17MT. This control establishes the fluorescence inhibition for a flutamide concentration of 18.5 µg/L compared to the 17MT 3 µg/L alone control group. This control serves as part of the flutamide standard curve.

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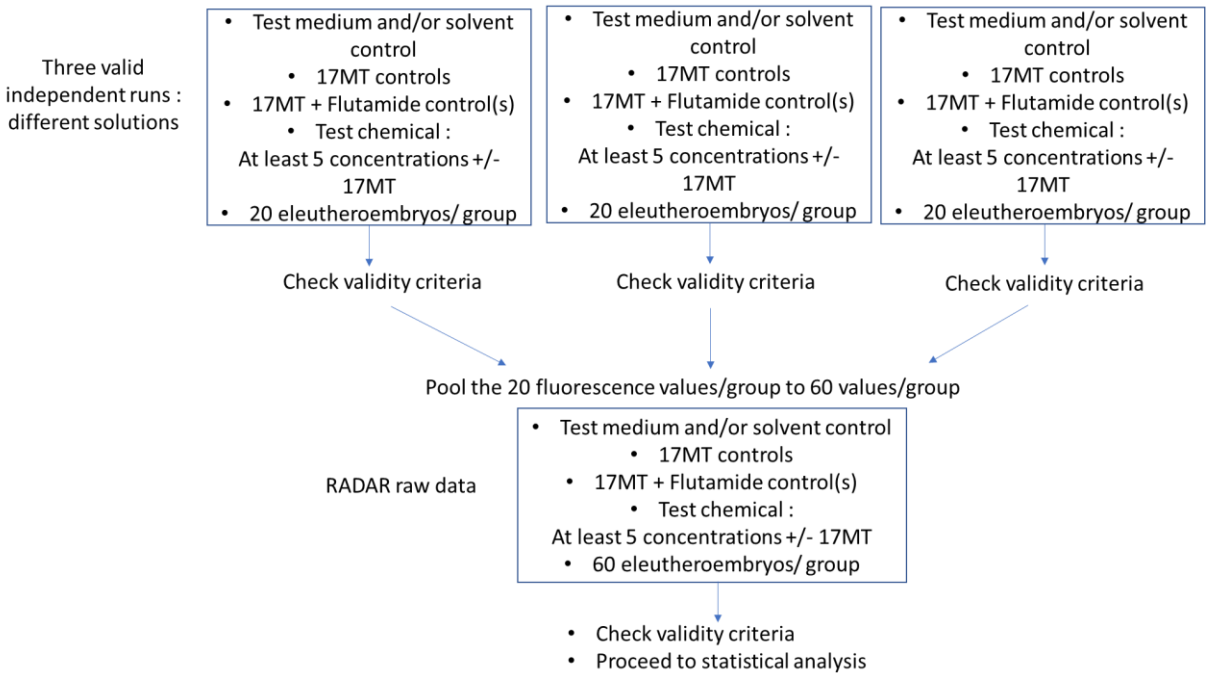
If the assay is to be performed with a solvent, all control groups and test chemical groups should receive an equal concentration of the solvent. In addition, it should be determined whether the results for the control groups pass validity criteria with the imaging system used for the readout, if not the experiment is considered invalid (see also §36).

3.3. Experimental runs

15. One test is composed of three independent and valid runs using 4 wells x 5 organisms/treatment group/run (see Figure 1). At least five concentrations of the test chemical are evaluated in the presence and absence of 17MT. The same concentrations must be evaluated in each run. Each run should be performed using independent solutions (see §40). The runs should be conducted sequentially using eleutheroembryos that have hatched on different days. The raw data for a given test chemical are obtained by pooling the data from the three runs to ideally obtain n=60 fluorescence values in each treatment group. Pooling of the data is obligatory for this test and is not performed uniquely in cases where all three runs show a positive response. It is performed to provide an improved estimate of the mean fluorescence value for each experimental group.

Figure 1: Overview of the RADAR assay. ("+/- 17MT" refers to spiked and unspiked groups). A RADAR assay is composed of three independent runs and utilises 840 eleutheroembryos in total. All non-optional controls should be performed in each run (see §14). If a solvent is being used for the first time or for the first time at a certain concentration, a test medium control should also be included (see §51).



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4. INFORMATION ON THE TEST CHEMICAL

16. Available information on the test chemical should be reported (see §57).

17. Whenever possible, the solubility of the test chemical in the test medium should be known and a validated analytical method, of known accuracy, precision, and sensitivity, should be available for the quantification of the test chemical in the test solutions with reported efficiency and limit of quantification. Guidance for the validation of quantitative analytical methods can be found in the GD 204 (OECD, 2014b). Analytical determination of the test chemical concentration should be performed as described in §41.

5. DEMONSTRATION OF PROFICIENCY

5.1. Fluorescence quantification

18. The RADAR assay relies on the quantification of the fluorescence emitted by each organism. To ensure that a proper and accurate quantification can be achieved, preliminary experiments should be conducted. These experiments are performed to calibrate the fluorescence imaging system and to ensure that a suitable dynamic range of fluorescence measurements can be read by the equipment. These experiments are detailed in Annex 3. If an alternative system for fluorescence measurement is used, it should be calibrated and validated in the same way as detailed for a fluorescence imaging system (Annex 3). However, use of a fluorescence microscope equipped with an appropriate camera is the preferred method as this allows a quality control step to be performed on the pictures to identify misplaced eleutheroembryos or fluorescence signal not related to androgen axis activation (fluorescent dust or fibres, fluorescent test chemical which fluoresces within the eleutheroembryos, abnormal fluorescent pattern).

5.2. Proficiency chemicals

19. Prior to routine use of this test guideline, laboratories should demonstrate technical proficiency by correctly categorising the four proficiency chemicals listed in Table 1.

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Table 1. Proficiency chemicals. mDHT, linuron, cefuroxime and cromolyn. The expected statistical significance limits refer to the fluorescence of the group exposed to the indicated concentration of reference chemical when compared to the relevant control. These limits were determined from the OECD RADAR assay validation exercise (OECD, 2022).

Chemical	CAS No.	Category	Concentrations to test	Expected statistical significance limit
mDHT	521-11-9	Active	16, 8, 4, 2, 1 µg/L	4 µg/L
Linuron	330-55-2	Active	2.5, 1.25, 0.625, 0.31, 0.16 mg/L	1.25 mg/L
Cefuroxime	56238-63-2	Inert	10, 1, 0.1, 0.01, 0.001 mg/L	Inert
Cromolyn	15826-37-6	Inert	1000, 100, 10, 1, 0.1 µg/L	Inert

5.3. Validity of the test

20. For the test to be valid, the following criteria should be met for each run, and if they are not, the run is considered invalid:

- A statistically significant induction of fluorescence should be measured between the solvent control group and the 17MT 10 µg/L control group. The mean fluorescence of the 17MT 10 µg/L control group should be at least 300% the mean of fluorescence of the test media control group or solvent control group if a solvent is used.
- The combined mortality and/ or malformations and invalid data due to poorly positioned eleutheroembryos (see Annex 7) should not exceed 10% in each control group and in at least five treatment groups in the presence and absence of 17MT. Groups not meeting these criteria are considered compromised.

For the test to be valid, the following criteria should be met for the pool of the three runs, and if they are not, all three runs are considered invalid:

- The mean fluorescence of the 17MT 10 µg/L control group should be at least 10% higher than the mean of fluorescence of the 17MT 3 µg/L control group. This ensures that the mean fluorescence of the 17MT 10 µg/L group is higher than that of the 3 µg/L group, which historical data has shown to be important for ensuring that the assay performed correctly. Generally, the difference is much greater than 10%.
- A statistically significant inhibition of fluorescence should be measured between the 17MT 3 µg/L control group and the 17MT 3 µg/L + flutamide 500 µg/L control group.
- For the pool of the three runs, a test should have at least five uncompromised test concentrations. A treatment group (ideally 60 individuals) is considered uncompromised if in each of the three runs (ideally 20 individuals per run) it passes validity criteria (combined mortality, and/ or malformations and invalid data due to poorly positioned eleutheroembryos (see Annex 7) should not exceed 10%).
- These validity criteria are applicable after image quality control if performed. If a minor deviation from the validity criteria is observed, the consequences should be considered in relation to the reliability of the test data and these considerations should be included in the report.

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6. DESCRIPTION OF THE METHOD

6.1. Apparatus

21. Normal laboratory equipment and in particular the following:

- laboratory incubator or any adequate apparatus for temperature and light control;
- transparent cell culture grade 6-well plates made of a chemically inert material;
- clear bottomed black 96-well plates certified for fluorescence quantification if eleutheroembryos are imaged from below or a black plastic surface suitable for fluorescence quantification if eleutheroembryos are imaged from above;
- pH meter;
- stereomicroscope equipped with a light source (for sorting fertilised/ unfertilised eggs);
- fluorescent microscope equipped for fluorescence quantification with GFP long-pass filters and a colour camera (OECD, 2022);
- image analysis software;
- analytical instrumentation appropriate for the test chemical or contracted analytical services.

6.2. Test organism

22. The test organisms for the RADAR assay are homozygous *O. latipes*, Japanese medaka eleutheroembryos of the *spg1-gfp* transgenic line. These organisms should be produced by mating two homozygous *spg1-gfp* Japanese medaka. The *spg1-gfp* transgenic line is maintained in several laboratories (Annex 10) and can be obtained upon subscribing to a license agreement. When a test chemical is shown to be fluorescent, wild type Japanese medaka eleutheroembryos could also be required to verify if a test chemical shown to be fluorescent fluoresces within the eleutheroembryos (see §29).

23. The exposure phase of the test is initiated with DPH0 eleutheroembryos (approximately 10 days post fertilisation at 26°C). Although the eleutheroembryos must be DPH0, they can have a different number of days post fertilisation (DPF). The difference should not be more than one DPF in a single run. All eleutheroembryos should be randomly selected for the different exposure groups. Eleutheroembryos should ideally be bred within the laboratory from stock animals. Alternatively, eggs could be shipped from another laboratory and received as early as possible in development to allow for the longest possible recovery period before beginning the test. Acclimation and batch acceptance criteria are outlined in Annex 4.

24. Housing, breeding and care of *O. latipes* are described in a number of sources, for example, Medaka: Biology, Management, and Experimental Protocols volumes 1 and 2 (Kinoshita et al., 2009; Murata et al., 2019) or the United States Environmental Protection Agency Guidelines for Culturing the Japanese Medaka, *Oryzias latipes*.

25. The integrity of the *spg1-gfp* transgenic line should be verified every generation by running a full set of controls including the optional controls (§14) and ensuring that all validity criteria are met and that an expected response profile is obtained for the 17MT and flutamide controls (§14).

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26. A quality control check on the developmental stage of randomly selected eleutheroembryos should be performed once a year to ensure that developmental stage of the eleutheroembryos at the end of the assay is not higher than stage 42.

6.3. Test medium

27. The test medium could be medaka medium (Annex 5), glass bottled still mineral water, spring water, well water and charcoal-filtered tap water. Because local water quality can differ substantially from one area to another, analysis of water quality should be undertaken to screen for potential contaminants (including heavy metals) and chemicals likely to interfere with the assay, particularly if historical data on the appropriateness of the water for raising *O. latipes* are not available. Special attention should be given to copper, chlorine and chloramine, all of which are toxic to *O. latipes* eleutheroembryos. Chelating agents should not be used. Results from analysis of water quality should be reported. Some chemical characteristics of an acceptable test medium suitable for *O. latipes* can be found in Annex 5. However, any medium that supports the normal growth and development of *O. latipes* and allows the test validity criteria to be met is suitable as a test medium.

7. Feeding

28. Eleutheroembryos between developmental stages DPH0 (beginning of the test) and DPH3 (end of the test) are used for this test. They are not fed before or during the test as the test is terminated at stage 40 (Iwamatsu, 2004). Yolk is still present until stage 41/42 and is used as the source of energy for the development of the eleutheroembryo.

7.1. Determining potential fluorescence of the test chemical

29. This test guideline should not be used for test chemicals emitting fluorescence between 500 and 550 nm (λ_{EM} = 500–550 nm) when excited at wavelengths between 450 and 500 nm (λ_{EX} = 450–500 nm) and able to fluoresce within the eleutheroembryos. Test chemicals sharing these two properties may induce a fluorescence which could be interpreted as GFP signal, leading to the test chemical being incorrectly identified as active on the androgen axis. A simple protocol to determine if the test chemical emits fluorescence at these wavelengths is to place 200 µL/ well of a solution of the test chemical at the highest concentration intended to be tested in the RADAR assay into ten wells of a 96-well plate. An additional ten wells of a 96-well plates should then be filled with 200 µL/ well of test medium. The fluorescence should then be quantified using the same apparatus and settings as for the quantification of eleutheroembryo fluorescence. Potential differences in fluorescence between the test medium and the test chemical should be evaluated by statistical analysis. First, a D'Agostino-Pearson normality test should be performed. If the fluorescence data for both the test medium and test chemical follow a normal distribution, a two-tailed T-test should be performed to determine whether there is a statistically significant difference in fluorescence. If one or both sets of data do not follow a normal distribution, a Mann-Whitney test should be performed. If a fluorescent chemical is identified, 20 wild type *O. latipes* eleutheroembryos should be exposed at 26 ± 1°C for 72 ± 2 h with a daily renewal of the highest concentration of the test chemical intended to be tested in the RADAR assay. The fluorescence should then be quantified and compared to the fluorescence of a group of 20 wild type eleutheroembryos exposed to test medium only in the same conditions. Statistical analysis should be performed as detailed previously in this paragraph for comparing the test medium to the test chemical. If a statistically significant difference in fluorescence is present, the chemical is fluorescent and fluoresces

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within the eleutheroembryos and should not be tested using the RADAR assay. In cases where the test chemical induces fluorescence in both unspiked and spiked modes in a RADAR assay, then it cannot be excluded that it is metabolised into a fluorescent metabolite. In these cases, the images should be examined to identify whether the fluorescence is limited to the kidneys. If this is not the case, then the procedure described above for exposing wild-type eleutheroembryos should be performed to identify whether the chemical is metabolised into a fluorescent metabolite.

7.2. Selection of test concentrations

7.2.1. Establishing the maximum test concentration

30. The maximum tolerated concentration (MTC) is defined as the highest test concentration of the chemical which results in ≤10% mortality in each of the three individual runs. The laboratory should perform a range-finding test with *O. latipes* eleutheroembryos to evaluate possible toxicity.

31. The range-finding should consist of at least three test concentrations. They should be arranged in a geometric series with a separation factor not exceeding 10. Only one run with 20 eleutheroembryos is required with the chosen test concentrations and control. The range-finding test is performed with five wild-type or *spg1-gfp O. latipes* eleutheroembryos and 8 mL of exposure solution per well, with four wells per test concentration and four wells for the control. The percentage of eleutheroembryos exhibiting mortality (and/ or malformations) is calculated combining data for all 20 eleutheroembryos exposed to the same test concentration or control. The highest concentration tested in the range-finding test must result in more than 10% combined mortality (and/ or malformations), unless the highest tested concentration is 100 mg/L or the solubility limit of the test chemical.

32. The maximum test concentration should be set by the solubility limit of the test chemical in the test medium, the MTC, or the maximum concentration inducing more than 10% combined mortality and/ or malformations in eleutheroembryos, or a maximum concentration of 100 mg/L, whichever is lowest.

7.2.2. Test concentration range

33. There is a required minimum of five test concentrations that pass validity criteria. Generally, a concentration separation (spacing factor) of 3- to 10-fold between each adjacent test concentration is recommended.

7.3. Test solutions

34. Test solutions of the chosen concentrations are usually prepared by dilution of a stock solution. The pH of each test solution should be adjusted to a pH comprised between 6.5 and 8.0. Stock solutions should be prepared by dissolving the test chemical using mechanical means if needed such as agitation, stirring or ultrasonication, or other appropriate methods. For difficult to test chemicals, the OECD Guidance Document No. 23 on aqueous-phase aquatic toxicity testing of difficult test chemicals should be consulted (OECD, 2019b).

35. Where possible, no solvent or a maximum solvent concentration of 100 mg/L (0.01%) should be used as indicated in OECD Guidance Document 23 (OECD, 2019b) if it is confirmed that the solvent and concentration of solvent used allow all validity criteria to be met. These validity criteria include eleutheroembryo survival but also the performance of the control groups (see §20).

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36. If a solvent is used, the concentration of solvent should be equal in all test concentrations and in all controls. The selection of an appropriate solvent depends on the physico-chemical properties of the test chemical and on the sensitivity of *O. latipes*, which should preferably be determined in a previous study. Possible actions of the solvent on the reproductive axis should also be considered (Hutchinson et al., 2006).

37. Control solutions should be prepared on the first day of a run and either stored at 4°C and used at each renewal of control solutions or reprepared each day. The same preparation of control or test solutions should not be used across independent runs. Solutions that have been stored at 4°C should be allowed to reach 26±1°C before being placed in contact with the eleutheroembryos to prevent thermal shock. Depending on the stability of the test chemical, exposure solutions containing test chemical should either be prepared on the first day of a run and stored at 4°C for use with that run or newly prepared immediately prior to each renewal of test solutions. Analytical measurements of renewal solutions stored at 4°C during the assay should be based on the solution as used at renewal and not at the moment it is prepared.

8. PROCEDURE

8.1. Exposure conditions

38. The organisms are exposed in chemically inert plastic cell culture grade 6-well plates (typically wells of 34 mm internal diameter and 20 mm height). Each well should contain 5 organisms in 8 mL of solution. In a run, 20 organisms are exposed to each test concentration. Each control group contains 20 organisms (see §14 for the list of control groups). If plastic well plates are not appropriate for a given test chemical, alternative glass vessels (i.e. small diameter Petri dishes) should be used.

39. Eleutheroembryos are maintained in an incubator for 72 ± 2 h at 26 ± 1°C in constant dark throughout the test. This limits the technical variability between different incubators and simplifies the requirements for performing the assay. If it was performed with a light cycle then specific wavelengths and intensities would be required to limit variability. It is also an advantage for testing photosensitive chemicals.

40. A new set of exposure solutions should be prepared for each of the three runs of the RADAR assay.

8.2. Analytical measurements

41. As a semi-static renewal method is used, the stability of the test chemical concentration should be documented. The stability of the test chemical should ideally allow the exposure concentration to remain within ±20% of the nominal concentration in a 24 h time frame. The minimum requirement for analytical measures is the minimum scientifically justifiable set of samples. OECD Guidance Document No. 23 provides guidance on issue (OECD, 2019b). Renewal periods of 24 ± 2 h are the longest periods accepted. If concentrations cannot be maintained within ± 20% in the test system, shortened renewal periods could be considered. Use of the geometric mean of measured concentrations is allowed for substances that do not remain within 80-120% of the nominal concentration; see Chapter 5 in the OECD Guidance Document No. 23 for more details (OECD, 2019b).

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8.3. Test initiation and conduct

Day 0

42. The exposure should be initiated on the day that the eleutheroembryos hatch (DPH0; approximately 10 days post fertilisation at 26°C).

43. For selection of test organisms, eleutheroembryos should be observed and those exhibiting grossly visible malformations or physical injury (e.g., damage of the tail, oedema, scoliosis) should be excluded from the assay (Annex 6). Healthy and normal looking eleutheroembryos of the stock population should be pooled in a single vessel containing an appropriate volume of test medium. The selected organisms should be homogenous in size, eleutheroembryos presenting an obvious difference in size should be removed. Batches of eleutheroembryos that contain less than 80% of normal and healthy eleutheroembryos at DPH0 should not be used for the test. This should be determined whilst removing dead and malformed eleutheroembryos from the batch prior to performing the assay.

44. To start the experiment, 5 eleutheroembryos/well should be placed into 6-well plates in drops of test medium (see §27) using a transfer pipet. The test medium should be removed and the test chemical solutions added for the first time. One should pay attention to work with one plate at a time to avoid drying out the eleutheroembryos.

Day 1 and Day 2

45. The test chemical solutions and the control solutions should be renewed at 24 ± 1 h and 48 ± 2 h. Each well is inspected for organisms with an abnormal appearance (injuries, abnormal swimming behaviour, etc.). Dead organisms or those exhibiting grossly visible malformations (Annex 6) or injuries should be removed and euthanised as described in §47. All observations should be recorded. During each run, if >10% cumulative mortality and observable sublethal effects is encountered in one of the control groups or one of the treatment groups leaving less than five uncompromised test concentrations, then the on-going independent run is stopped and the source of the mortality or abnormality should be identified.

Day 3 Fluorescence quantification

46. The fluorescence of each organism is quantified after 72 ± 2 h of exposure. The test solutions should be first renewed with 8 mL of test medium (see §27) and dead organisms or those exhibiting grossly visible malformations should be removed. All observations should be recorded. If >10% cumulative mortality and observable sub-lethal effects is encountered in one of the control groups or one of the treatment groups leaving less than five uncompromised test concentrations, then the on-going independent run is terminated and the source of the mortality or abnormality should be identified. The data of compromised groups should not be considered for analysis. If the eleutheroembryos are required to be anaesthetised for imaging, they should be anesthetized by adding 2 mL of 1 g/L buffered MS222 (tricaine methylsulfonate) into the wells of the six-well plates. Anaesthesia is recommended in all cases where the eleutheroembryos are placed in a drop of liquid for imaging. It is only not recommended if they are imaged whilst swimming freely, such as in a well of a 96-well plate. To avoid excessive anaesthesia, only the number of organisms that can be read in one series should be anaesthetised. After the onset of anaesthesia (1 to 5 min) if required, the eleutheroembryos are transferred to the support to be used for imaging such as a black plastic surface or black 96-well plates. They are then imaged with a colour camera and GFP long pass filters. An image of the dorsal region

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including the kidneys of each organism should be captured using the parameters identified during the calibration.

Terminating the experiment

47. After reading the fluorescence, each eleutheroembryo is euthanised by exposing it to 1 g/L of buffered MS222 for at least 20 minutes.

8.4. Analysis of data / Evaluation of test results

8.4.1. Data analysis considerations

48. Fluorescence measurements from images of poorly positioned eleutheroembryos (see Annex 7) should be removed from the data before analysis. Combined mortality, and/ or malformations and invalid data due to poorly positioned eleutheroembryos should not exceed 10% in each treatment group in each run (see §20).

Treatment of the colour images of the eleutheroembryos to extract a numerical value for GFP fluorescence should be performed using appropriate software. An open-source option is ImageJ or the more recent version FIJI (Schindelin et al., 2012). In order to exclude autofluorescence (non-GFP fluorescence) from the images it is recommended to separate the red, green and blue colour layers of the images. The red layer can then be subtracted from the green layer or the values of the red layer can be doubled and subtracted from the green layer. An intensity threshold can then be applied to the resulting image to reduce background caused by endogenous pigmentation. The sum of the fluorescence of all pixels in the resulting image should then be quantified. This technique is an efficient way to restrict the measurement to GFP and not endogenous (auto-) fluorescence. As GFP-related fluorescence will only appear in the green layer, but yellow fluorescence will appear in both the green and red layer. Doubling the red layer is useful depending on the imaging system if some endogenous fluorescence remains after subtracting the undoubled red layer. Other techniques to reduce the impact of endogenous pigmentation on the quantification of GFP signal can be applied depending on the imaging system and fluorescence filters used. Once an image analysis workflow has been demonstrated to allow validation criteria to be met for a given fluorescence imagery system, it should be applied for all future experiments (see §20 and Annex 3).

49. Data from the three independent runs are pooled to obtain 54 to 60 fluorescence values for each valid test concentration and control. The maximum number of values is 60 as each test condition or control is made up of 20 eleutheroembryos per run and the RADAR assays consists of three runs. The lower threshold of 54 values represents the limit of mortality and/ or malformations of 10% in each run, therefore, 18 values per run.

50. Three independent runs are performed to increase robustness of the assay. However, these three runs can give different results. If this is the case, the assay is still considered valid. The results of each of the three independent runs are only compared with each other if with the test chemical is found to be active based on the pooled data set and a non-monotonic concentration-response profile is observed (see §55).

51. If a solvent is used in the experiment, an evaluation of the potential effects of the solvent should be performed. This is done through a statistical comparison of the solvent control group and the test medium control group. If there is no statistically significant difference between the test medium control and solvent control, the pooled test medium and solvent controls should be used. If a statistically significant difference is detected between the test medium control and solvent control group for the pool of the three runs, the study is compromised. The runs should then be considered individually to determine if

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the solvent has a reproducible effect among the three runs. If it does, the study is compromised and a new RADAR assay should be performed using a new batch of solvent or another solvent. It is important to also verify that all validity criteria are met with the chosen solvent (§20, §35). If historical data exists indicating that the chosen solvent, at the chosen concentration, does not elicit a statistically significant difference when compared to the test medium control, then the test medium control may not be required.

8.4.2. Statistical analysis

52. Appropriate statistical methods should be used according to OECD Document 54 on the Current Approaches in the Statistical Analysis of Ecotoxicity Data: A Guidance to Application (OECD, 2006). In general, effects on the fluorescence of the test chemical compared to the control are investigated using two-tailed hypothesis testing at $p < 0.05$.

53. The recommended statistical approach, which was evaluated during the interlaboratory validation exercise, is to determine whether the data for each exposure group is normally distributed by performing a D'Agostino-Pearson normality test, then performing either an ANOVA test followed by a Dunnett's test if the data are normally distributed with equal variances or a Kruskal-Wallis test followed by a Dunn's test if the data does not follow a normal distribution or if the homogeneous variance assumption is violated (see Annex 8 for a more detailed description). Alternatively, a nested ANOVA test (also called mixed ANOVA) with a random effect term accounting for the variability introduced by runs or wells could be used to analyse this type of data, provided the well information is recorded during the experiment. A subsequent post-hoc test such as Dunnett's test can be applied to determine the magnitude and the significance of the difference.

8.4.3. Decision logic

54. A decision logic flowchart was developed for the RADAR assay to provide assistance in the conduct and interpretation of the results of the assay (see Figure 2). This decision logic is based on three valid runs pooled for statistical analysis (see Figure 1 and §15). A test chemical is considered to give a positive result in the RADAR assay if at least one concentration tested is active in either unspiked or 17MT spiked mode and a monotonous concentration-response relationship is observed.

- In unspiked mode an active concentration is defined as a concentration giving a statistically significant increase in fluorescence compared to the solvent control.
- In 17MT-spiked mode an active concentration is defined as a concentration giving a statistically significant increase or decrease in fluorescence compared to the 3 µg/L 17MT control.

55. If a non-monotonic concentration response is observed, the reliability of this result should be confirmed by comparing the results of the three runs of the RADAR assay. If the results are not reliable, the assay should be repeated, potentially using a different concentration range, to confirm the result (see Figure 2). If the result is reliable the chemical is considered active on the androgen axis in the RADAR assay. If the second time running the test, the same results are obtained, then, this test may not be appropriate for the test chemical and an alternative test might be necessary.

56. Fluorescence decreases in unspiked mode are not expected as the eleutheroembryos do not synthesise detectable levels of androgens at this development stage. If a statistically significant fluorescence decrease is observed in unspiked mode, it could indicate that the RADAR assay is not appropriate for the test chemical, or a potential problem with the

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organisms or the test conditions which may require further investigations. Individual runs should be considered to determine if the statistically significant fluorescence decrease is present in the three runs and best professional judgement should then be used to decide between repeating: none of the runs, only one run using a new batch of organisms; a complete RADAR assay, possibly using a lower concentration range; or performing a different androgen axis activity test.

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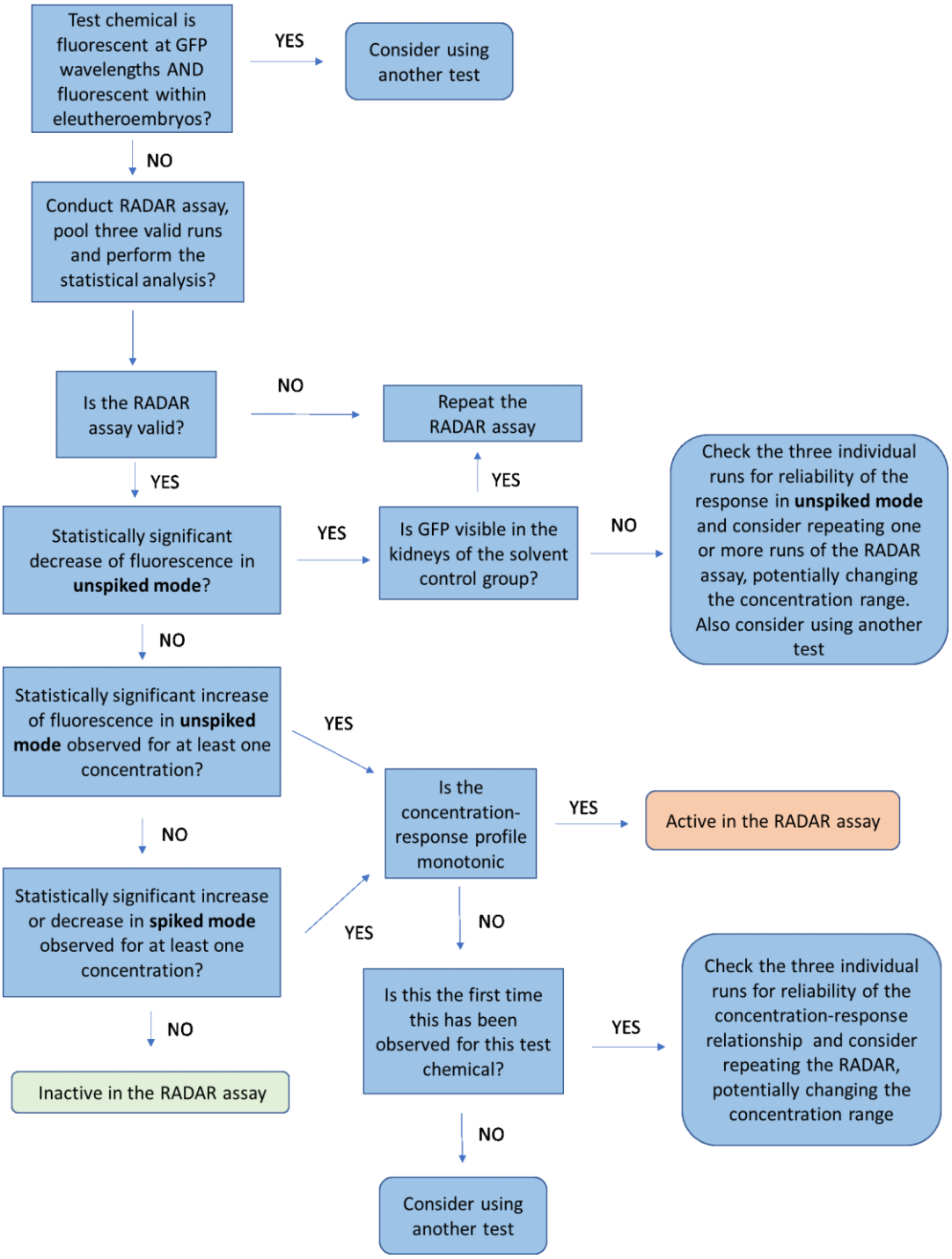
Figure 2. Decision logic for the interpretation of the result of the RADAR assay. The OECD GD 150 provides further guidance on the interpretation and extrapolation between taxa of the results of the RADAR assay (OECD, 2018). In the case of a statistically significant decrease in fluorescence in unspiked mode, refer to §56 for more detailed guidance.



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9. Test report

57. The test report should include the following information:

9.1. Test chemical

- Mono-constituent substance: physical appearance, water solubility, and additional relevant physico-chemical properties; chemical identification, such as IUPAC or CAS name, CAS number, SMILES or InChI code, structural formula, purity, chemical identity of impurities as appropriate and practically feasible, etc. (including the organic carbon content, if appropriate). Also, if available, stability in light, stability under the test conditions, pKa, Kow, information on the fate of the test chemical and its potential for being rapidly degraded in the test system e.g., results of a biodegradability test, see OECD TG 301 (OECD, 1992b) and TG 310 (OECD, 2014a).
- Multi-constituent substance, UVCBs and mixtures: characterised as far as possible by chemical identity (see above), quantitative occurrence and relevant physico-chemical properties of the constituents.
- Analytical method for quantification of the test chemical, including quantification limit.
- Available data or results from any preliminary studies on the stability or solubility of the test chemical.
- Results regarding lack of fluorescence emission at wavelength of 450 and 500 nm (λ_{EM} = 500–550 nm); as well as on lack of is fluorescence within the eleutheroembryos for substances shown to be fluorescent at these wavelengths.

9.2. Test species

- Scientific name, transgenic line, supplier or source, and culture conditions.
- The percentage of dead and malformed eleutheroembryos removed from the batch prior to performing the assay.

9.3. Test conditions

- Test procedure used (e.g., concentrations tested, temperature, duration, semi-static, volume, number of organisms per mL).
- Details of test medium characteristics (reference of mineral water or spring water, description of tap water treatment (e.g., charcoal filtration…) or artificial test medium used and any measurements made.
- Method of preparation of stock solutions and frequency of renewal (the solvent and its concentration should be given, when used).
- Brand and references of 6-well plates used for exposure and any plates used for fluorescence quantification.
- References and settings of the fluorescence microscope used for quantification. The method used for image analysis should also be provided.

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9.4. Results

- Results of the range-finding test(s) that allow the determination of the MTC and/or the selection of the test concentrations for the definitive test.
- The nominal test concentrations and, where possible, results of all chemical analyses to determine the concentration of the test chemical in the test vessels; the measured exposure concentration as an appropriate statistical average (e.g., arithmetic mean, time-weighted mean etc.) where appropriate; the recovery efficiency of the analytical method and the limit of quantification should also be reported.
- The numbers of dead and malformed organisms in each run and the group(s) and days on which they occurred.
- Fluorescence quantification raw data (e.g., individual fluorescence raw data). Ideally, data should be collected in tab or csv format with the following metadata present in the file: date; chemical name; concentration used; solvent; machine name; signal collection parameters for the machine, laboratory name, eleutheroembryo batch number and fluorescence values.
- Approach for the statistical analysis and treatment of data including statistical test used and whether and why any data censuring was conducted.
- Demonstration that all validity criteria of the guideline were met.
- The means of fluorescence of each experimental group including all control and test chemical concentrations and their SEM (standard error of the mean) should be presented both by a graphical representation and also in a table together with the sample size.
- The percentage increase or decrease of fluorescence for each concentration compared to its respective control in spiked and unspiked modes.
- Optionally and where appropriate, results of the evaluation of the potential effects of the solvent: a statistical comparison of the solvent control group and the test medium control group if included in the present study or a result from a previous study.
- Other observed biological effects or measurements: report any other biological effects which were observed or measured (e.g., abnormal behaviour, malformations or abnormal pigmentation).
- An explanation for any deviation from the test guideline or deviation from the validity criteria, and considerations of potential consequences on the outcome of the test.
- Where appropriate, a discussion presenting the concentrations found active in spiked and/or unspiked mode.
- A conclusion presenting whether the test chemical is found to be active or inactive on the androgen axis in the RADAR assay.

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Annex 1: ABBREVIATIONS AND DEFINITIONS

11KT: 11-Ketotestosterone.

17MT: 17α-Methyltestosterone.

AR: Androgen receptors.

BaP: Benzo(a)pyrene.

CYP: Cytochrome P450.

DMSO: Dimethyl sulfoxide.

DPF: Day post fertilisation.

DPH: Day post hatch.

EC_X: The concentration giving an effect at the X% of maximum level, for example EC50 is the concentration giving 50% of the maximum effect.

Eleutheroembryo: The eleutheroembryonic life stage is post-hatch, but before the embryo is capable of independently feeding on exogenous food supplies and is a stage of on-going embryonic development. Applying this definition to *O. latipes* positions this period of development from stage 39 (hatching stage) to stage 42 (formation of structures required for prey capture including the teeth of the upper jaw, the otolith, and the shape of all fins) (Iwamatsu, 2004).

ER: Estrogen receptors.

GD: OECD guidance document.

GFP: Green fluorescent protein.

LOEC: The lowest observed effect concentration is the lowest tested concentration at which the test chemical is observed to have a statistically significant effect (at $p < 0.05$).

mDHT: 17α-Methyl-5α-dihydrotestosterone; mestanolone.

Medaka medium: A specific artificial medium that can be used as the test medium (see Annex 5).

MS222: Tricaine methanesulfonate.

MTC: Maximum tolerated concentration. MTC is defined as the highest test concentration of the chemical which results no more than 10% mortality.

NOEC: The no observed effect concentration is the tested concentration immediately below the LOEC.

SEM: Standard error of the mean.

SMILES: Simplified molecular input line entry specification.

RADAR assay: Rapid androgen disruption activity reporter assay.

Run: A run is defined here as an experiment performed using independent solutions.

Spiked mode: Part of a RADAR assay performmed in the presence of 3 µg/L of 17MT.

***spg1-gfp*:** Transgenic medaka line harbouring a genetic construction consisting of a 4159 base pairs of the three-spined stickleback *spiggin 1* gene promoter upstream of GFP coding sequence.

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Test medium: The medium used for the assay, this could be any water permitting normal growth and development of *O. latipes* including medaka medium (see Annex 5), glass bottled still mineral water, spring water, well water and charcoal-filtered tap water.

Unspiked mode: Part of a RADAR assay performed in the absence of 17MT.

UVCB: Substances of unknown or variable composition, complex reaction products or biological
materials.

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Annex 2: OVERVIEW OF TEST CONDITIONS OF THE RADAR ASSAY

Table.2. Overview of the test conditions for the RADAR assay

Test organism	**spg1-gfp O. latipes eleutheroembryo**	
Endpoint	Fluorescence of individual eleutheroembryos	
Exposure period	DPH0 (beginning of the test) to DPH3 (end of the test)	
Exposure duration	72 h ± 2 h	
Exposure regime	Renewal after 24 h and 48 h. No feeding.	
pH	6.5 to 8	
Incubation conditions during exposure	26 ± 1oC, dark	
Organisms per concentration	5 organisms per well (6-well plate) x 4 wells (total of 20 organisms per concentration and run)	
Volume of test medium	8 mL per well	
Test medium	Water permitting normal growth and development of O. latipes (refer to §27).	
Number of experiments	Experiments are run 3 times for each test chemical with freshly prepared solutions.	
Criteria for selecting test individuals	Developmental stage (DPH0), health of organisms (alive and no malformations).	
Validity criteria	For each run: Combined mortality and/ or malformations of ≤ 10% in all control groups. Fluorescence induction >300% in the 17MT 10 µg/L control c.f. the solvent control. For the pool of the three runs: Fluorescence induction >10% in the 17MT 10 µg/L control c.f. the 17MT 3 µg/L control and a concentration-response relationship should be observed (see §20).	
At least five uncompromised test concentrations. A test concentration is considered uncompromised for the purpose of the test when this test concentration is considered uncompromised in each of the three runs of the test. A test concentration (20 individuals) is considered uncompromised in a run when combined mortality and malformations in the group is ≤ 10%.		
Controls	Test medium and/ or solvent control	Test medium and/or test medium plus solvent
	17α-methyltestosterone (17MT)	17MT (3, 10 µg/L)
	17MT+ Flutamide	17MT (3 µg/L) + Flutamide (500 µg/L)

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Annex 3: CALIBRATION: DETERMINATION OF THE OPTIMAL IMAGING SETTINGS

The goal of the calibration step is to ensure that all laboratories attain a similar amplitude of response and sensitivity to the reference compounds 17MT and flutamide despite differences in imaging equipment used to read the experiment. The calibration will require two steps:

1. Determining the optimal imaging settings to allow a satisfactory amplitude of GFP induction to be obtained with a concentration of 50 µg/L 17MT.
2. Applying these settings for the quantitation of three runs of a concentration-response experiment with 17MT and flutamide to check the amplitude of induction using increasing concentrations of 17MT and flutamide as well as the lowest concentration of 17MT and flutamide that elicits a detectable GFP response.

The example protocol, described in two steps below, involves the use of 0.2% DMSO in all exposure solutions. This is an example; the same procedure can be performed with an alternative solvent or alternative concentration of solvent. The calibration procedure does not need to be repeated if the solvent is changed when performing a RADAR assay or if the assay is performed for the first time without a solvent.

10.1. 1 - Selecting image capture settings

The first step is to determine the correct image capture settings for the calibration experiment. Once determined, these settings will be used for future experiments. In order to select the image capture settings, 50 eleutheroembryos should be exposed to 50 µg/L of 17MT and the settings should be adjusted as indicated in the following protocol. A single run is required for this step.

- Setting up the exposure media
  - The test group consists of 10 wells, with each well containing 5 eleutheroembryos of the *spg1-gfp* line.
  - The final concentration of DMSO is 0.2% in all wells.
  - Prepare a solution of 50 mg/L 17MT in DMSO.
    - Aliquot the solution of 50 mg/L 17MT with 200 µL per aliquot.
    - Conserve the aliquots at -20°C for a maximum of 2 months.
  - Prepare the following exposure solution of 50 µg/L 17MT containing 0.2% DMSO.

Test Medium	100 mL
17MT 50 mg/L in DMSO	100 µL
DMSO	100 µL

- Starting the exposure
  - Add 5 *spg1-gfp* transgenic eleutheroembryos to each well.
  - Remove the maximum amount of liquid without drying the eleutheroembryos (maximum remaining volume 800 µL).

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- Fill each well with 8 mL of the exposure solution.
- Incubate the plates at 26 °C in the dark. Do not feed the eleutheroembryos during the experiment.

- Renewal of the media at 24h and 48h
  - Note the mortality and evacuate any dead eleutheroembryos.
  - Prepare exposure solutions according to the table above.
  - Remove the maximum amount of liquid.
  - Fill the wells with 8 mL of their respective medium.
  - Incubate the plates at 26 °C in the dark. Do not feed the eleutheroembryos during the experiment.
- Rinsing eleutheroembryos at 72 h
  - Prepare 6-well rinsing plates containing 8 mL of water permitting normal growth and development of *O. latipes* (refer to §27) in each well.
  - Transfer all eleutheroembryos from an exposure group from their treatment plate to the rinsing plate.
- Reading eleutheroembryos at 72 h
  - If necessary, anesthetise the eleutheroembryos exposed to 50 µg/L of 17MT by placing 2 mL of MS222 at 1 g/L in each well of the 6-well plates. Be careful to anesthetise only 1 plate at a time.
  - Place the eleutheroembryos so that the dorsal side can be imaged by the imaging system.
  - Adjust the zoom and focus on the fluorescence microscope to determine the maximal zoom that allows imaging of both kidneys.
  - Check the other eleutheroembryos on the plate to ensure that the selected zoom allows both kidneys to be visualised in the same image. If this is not the case readjust the zoom and begin the process again.
  - If possible, reset the white balance of the camera.
  - Set the gain on the camera settings to zero and adjust the exposure time to the point where the kidneys are as bright as possible without appearing white.
  - If the exposure needs to be set above 100 ms to result in saturation of the GFP signal (white areas in the GFP signal), increase the gain and restart.
  - Check the other eleutheroembryos on the plate to ensure that the selected exposure time does not result in a significant portion of the kidneys to be white. If this is not the case adjust the exposure time and begin the process again.
  - Save and note the selected settings for the camera and conserve the settings file to be recalled at each future imaging session.
  - Capture an image of each eleutheroembryo.
  - After all images are taken, euthanise the eleutheroembryos.
  - Analyse the images by following the instructions in §48 to §53.

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- Example images of eleutheroembryos after exposure to an androgen (dorsal view) are shown below (Annex 7).

10.2. 2 - Determining linearity and sensitivity to 17MT and flutamide

The second step is to determine the linearity and sensitivity to 17MT and flutamide. In order to perform this step, groups of 20 eleutheroembryos are exposed to a concentration range of 17MT and to a concentration range of flutamide in the presence of 3 µg/L of 17MT. Three independent runs are required for this step.

- Setting up the exposure media
  - Each test group consists of 4 wells, with each well containing 5 eleutheroembryos of the *spg1-gfp* line.
  - The final concentration of DMSO is 0.2% in all wells.
  - Prepare a solution of 10 mg/L 17MT in DMSO.
    - Aliquot the solution of 10 mg/L 17MT with 200 µL per aliquot.
    - Conserve the aliquots at -20°C for a maximum of 2 months.
  - Prepare a solution of 500 mg/L flutamide in DMSO.
    - Aliquot the solution of 500 mg/L flutamide with 200 µL per aliquot.
    - Conserve the aliquots at -20°C for a maximum of 2 months.

Prepare the test solutions according to

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Table 4. Preparation of test solutions and intermediate solutions (grey background).

Solution Name	Final Concentration (µg/L)	Intermediary volume to prepare (mL)	Solutions to mix	Final Volume (mL)
Medaka medium 0.1% DMSO		300	300 mL of medaka medium + 300 µL of DMSO	20
Solvent control		70	70 mL of medaka medium 0.1% DMSO + 70 µL of DMSO	45
17MT 10 0.1% DMSO		175	175 mL of medaka medium + 175 µL of 17MT 10 mg/L	35
17MT 3 0.1% DMSO		300	90 mL of 17MT 10 0.1% DMSO + 210 mL of medaka medium 0.1% DMSO	10
17MT 10	10	50	50 mL of 17MT 10 0.1% DMSO + 50 µL of DMSO	50
17MT 3	3	220	220 mL of 17MT 3 0.1% DMSO + 220 µL of DMSO	45
17MT 1.5	1.5	50	25 mL of 17MT 3 + 25 mL of solvent control	50
Flutamide 500	500	70	70 mL of 17MT 3 0.1% DMSO + 70 µL of flutamide 500 mg/L	45
Flutamide 167	167	75	25 mL of flutamide 500 + 50 mL of 17MT 3	50
Flutamide 55.6	55.6	75	25 mL of flutamide 167 + 50 mL of 17MT 3	50
Flutamide 18.5	18.5	75	25 mL of flutamide 55.6 + 50 mL of 17MT 3	75

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The test groups are:

Solvent control: Test medium 0.2% DMSO

17MT 1.5 µg/L 0.2% DMSO

17MT 3 µg/L 0.2% DMSO

17MT 10 µg/L 0.2% DMSO

Flutamide 18.5 µg/L + 17MT 3 µg/L 0.2% DMSO

Flutamide 55.6 µg/L + 17MT 3 µg/L 0.2% DMSO

Flutamide 167 µg/L + 17MT 3 µg/L 0.2% DMSO

Flutamide 500 µg/L + 17MT 3 µg/L 0.2% DMSO

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Table 4. Preparation of test solutions and intermediate solutions (grey background).

Solution Name	Final Concentration (µg/L)	Intermediary volume to prepare (mL)	Solutions to mix	Final Volume (mL)
Medaka medium 0.1% DMSO		300	300 mL of medaka medium + 300 µL of DMSO	20
Solvent control		70	70 mL of medaka medium 0.1% DMSO + 70 µL of DMSO	45
17MT 10 0.1% DMSO		175	175 mL of medaka medium + 175 µL of 17MT 10 mg/L	35
17MT 3 0.1% DMSO		300	90 mL of 17MT 10 0.1% DMSO + 210 mL of medaka medium 0.1% DMSO	10
17MT 10	10	50	50 mL of 17MT 10 0.1% DMSO + 50 µL of DMSO	50
17MT 3	3	220	220 mL of 17MT 3 0.1% DMSO + 220 µL of DMSO	45
17MT 1.5	1.5	50	25 mL of 17MT 3 + 25 mL of solvent control	50
Flutamide 500	500	70	70 mL of 17MT 3 0.1% DMSO + 70 µL of flutamide 500 mg/L	45
Flutamide 167	167	75	25 mL of flutamide 500 + 50 mL of 17MT 3	50
Flutamide 55.6	55.6	75	25 mL of flutamide 167 + 50 mL of 17MT 3	50
Flutamide 18.5	18.5	75	25 mL of flutamide 55.6 + 50 mL of 17MT 3	75

- Starting the exposure
  - Add 5 *spg1-gfp* transgenic eleutheroembryos to each well.
  - Remove the maximum amount of liquid without drying the eleutheroembryos (maximum remaining volume 800 µL).
  - Proceed with the treatment of the solvent control, then the 17MT groups and then the 17MT + flutamide groups.
  - Fill each well with 8 mL of each preparation.
  - Incubate the plates at 26 °C in the dark. Do not feed the eleutheroembryos during the experiment.
- Renewal of the media at 24h and 48h
  - Note the mortality and evacuate any dead eleutheroembryos.

Prepare exposure solutions according to

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Table 4. Preparation of test solutions and intermediate solutions (grey background).

Solution Name	Final Concentration (µg/L)	Intermediary volume to prepare (mL)	Solutions to mix	Final Volume (mL)
Medaka medium 0.1% DMSO		300	300 mL of medaka medium + 300 µL of DMSO	20
Solvent control		70	70 mL of medaka medium 0.1% DMSO + 70 µL of DMSO	45
17MT 10 0.1% DMSO		175	175 mL of medaka medium + 175 µL of 17MT 10 mg/L	35
17MT 3 0.1% DMSO		300	90 mL of 17MT 10 0.1% DMSO + 210 mL of medaka medium 0.1% DMSO	10
17MT 10	10	50	50 mL of 17MT 10 0.1% DMSO + 50 µL of DMSO	50
17MT 3	3	220	220 mL of 17MT 3 0.1% DMSO + 220 µL of DMSO	45
17MT 1.5	1.5	50	25 mL of 17MT 3 + 25 mL of solvent control	50
Flutamide 500	500	70	70 mL of 17MT 3 0.1% DMSO + 70 µL of flutamide 500 mg/L	45
Flutamide 167	167	75	25 mL of flutamide 500 + 50 mL of 17MT 3	50
Flutamide 55.6	55.6	75	25 mL of flutamide 167 + 50 mL of 17MT 3	50
Flutamide 18.5	18.5	75	25 mL of flutamide 55.6 + 50 mL of 17MT 3	75

  - .
  - Remove the maximum amount of liquid using a soft transfer pipette.
  - Fill the wells with 8 mL of their respective medium.
  - Incubate the plates at 26 °C in the dark. Do not feed the eleutheroembryos during the experiment.
- Rinsing eleutheroembryos at 72 h
  - Prepare 6-well rinsing plates containing 8 mL of dechlorinated water or mineral water in in each well.
  - Transfer all eleutheroembryos from an exposure group from their treatment plate to the rinsing plate.
  - Transport the rinsing plates to the room where the reading will take place.
- Reading eleutheroembryos at 72 h
  - Load the image capture parameters that were saved at the end of the first step of the calibration experiment.
  - If necessary, anesthetise the eleutheroembryos exposed to the solvent control solution by placing 2 mL of MS222 at 1 g/L in each well of the 6-well plates. Be careful to anesthetise only 1 plate at a time.
  - After the onset of anaesthesia (1 to 5 min) if required, the eleutheroembryos are transferred to the support to be used for imaging such as a black plastic surface or clear-bottomed black 96-well plates.

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- Place the eleutheroembryos so that the dorsal side can be imaged by the imaging system.
- Capture an image of each eleutheroembryo.
- After all images are taken for an exposure group, euthanise the eleutheroembryos.
- Continue until all groups are read.
- Analyse the images by following the instructions in sections §48 to §53.

- Interpreting the results
  - Once the pooled data has been statistically analysed and graphed, the lowest LOEC should be noted for both 17MT and flutamide.
  - The LOEC should be at least 3 µg/L for 17MT and at least 500 µg/L for flutamide.
  - Both 17MT and flutamide should exhibit a concentration-response relationship over the range of concentrations tested.
  - If a concentration-response relationship is not apparent due to either poor sensitivity at lower concentrations or signal saturation at higher concentrations, then efforts should be made to adjust the image capture parameters to improve the concentration-response relationship.

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Annex 4: RECEIVING EMBRYOS: ACCLIMATION AND BATCH ACCEPTANCE

- Embryos should be received no later than 3 days before the test begins to allow a proper recovery and acclimation.
- Batches should be accepted only if dead or abnormal embryos represent less than 20% of the total number between the reception of the batch and the start of the exposure.

Guidance for embryos received three days before the start of the RADAR assay:

- Do not mix embryos fertilised on different days.
- Sort embryos to remove dead and abnormal embryos, these embryos should represent less than 20% otherwise the batch should not be used to perform the RADAR assay.
- Transfer only the living and normal embryos to a 1.4 L crystalliser or 15 cm Petri dish containing water suitable for raising medaka embryos (see Annex 5).
- The maximum density per crystalliser is 500 embryos, the maximum density per Petri dish is 200 embryos.
- Incubate embryos with illumination at approximately 26ºC with a 14:10 hour light:dark cycle. The temperature should be adjusted as required in order for the embryos to hatch around day post fertilisation (DPF) 9 or 10 (tolerance DPF 7-12). Although the eleutheroembryos must be DPH0, they can have a different number of days post fertilisation (DPF). The difference should not be more than one DPF in a single run. All eleutheroembryos should be randomly selected for the different exposure groups.
- The medium that the embryos are raised in should be changed at least once during the period of embryonic development leading to hatching.

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Annex 5: SOME CHEMICAL CHARACTERISTICS OF AN ACCEPTABLE WATER FOR RAISING MEDAKA EMBRYOS

Table 6: Characteristics of water suitable for raising medaka embryos to hatch.

Characteristic	Recommended range	Tolerance
Dechlorinated	-	Essential
Particle filtered	25 µm	Recommended
Activated charcoal filtered	-	Recommended
Conductivity	230-290 micro Siemens	
Temperature	26°C	26-30°C
Methylene blue	1 mL of 1 g/L stock per L	Recommended
pH	7.2-8.2	Essential

Alternatively, if an artificial medium is to be used, one option which has been extensively tested including within the OECD interlaboratory validation exercise is detailed here:

A stock solution of 10x Medaka Medium has the following composition:

- NaCl 5 g/L
- $CaCl_2$ 0.151 g/L
- $MgSO_4$ 0.098 g/L
- KCl 0.15 g/L
- NaOH 1N 1.25 mL/L

This solution should then be diluted ten-fold with reverse osmosis water to obtain the 1x working solution. The pH should be adjusted to between 7.2-8.0 with a solution of 1N NaOH.

In addition to artificial media, medaka embryos can also be raised in glass bottled still mineral water, spring water, well water or charcoal-filtered tap water or any medium that supports the normal growth and development of *O. latipes*.

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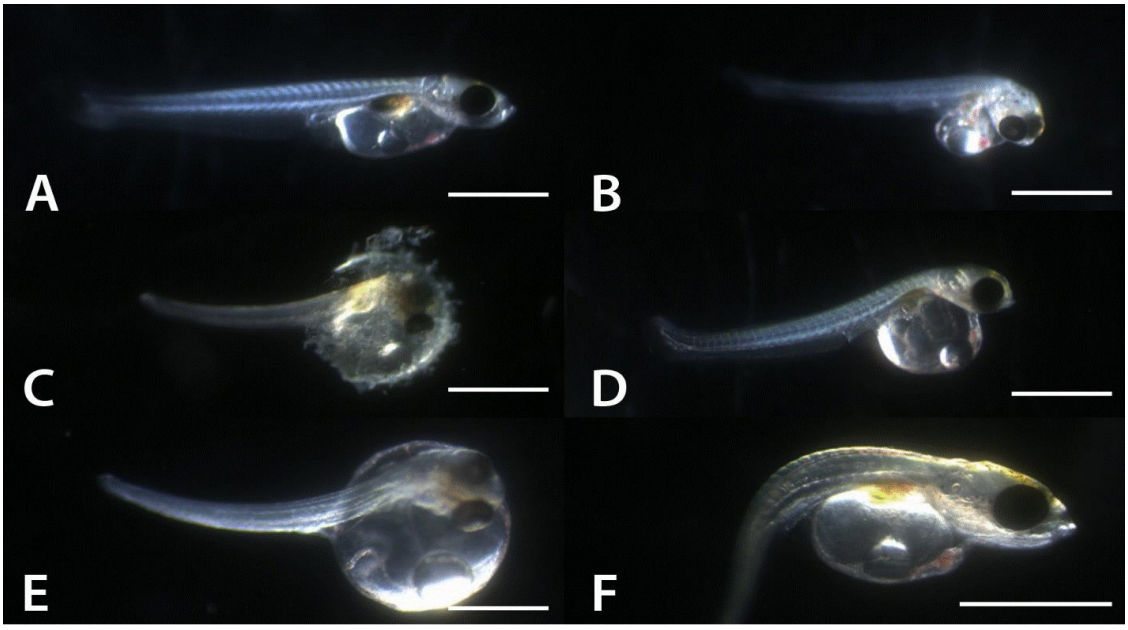
Annex 6: PHOTOGRAPHIC GUIDANCE FOR IDENTIFICATION OF NORMAL VERSUS ABNORMAL ELEUTHEROEMBRYOS

Figure 3. Photographic guidance for identification of normal versus abnormal eleutheroembryos. (A) Normal eleutheroembryo. Abnormal eleutheroembryos: (B) small, the eleutheroembryo clearly has a shorter length than other eleutheroembryos from the same batch; (C) partially hatched, the eleutheroembryo has not yet completely emerged from its egg; under developed, (D and E) both exhibit extremely large yolk sacs for a hatched medaka which still have a spherical shape; (F) malformed, the tail is curved downwards. Scale bars indicate 1 mm.



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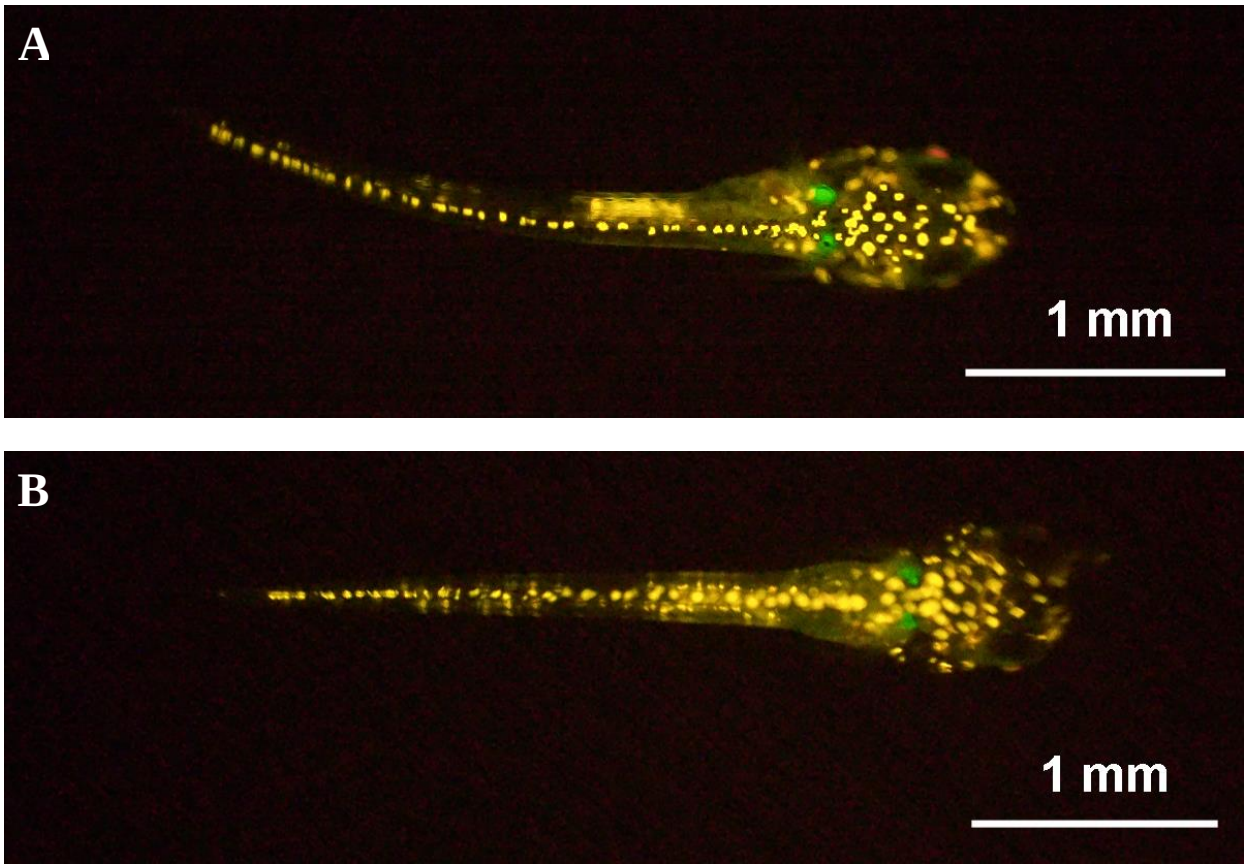
Annex 7: ELEUTHEROEMBRYO POSITIONING

Figure 3: A and B) Dorsal views of two Spg1-GFP medaka eleutheroembryos displaying green fluorescent protein (GFP) signal in the kidneys. The eleutheroembryos are facing towards the right of the image.

Figure 3 below shows the expected positioning of the eleutheroembryos for imaging. Eleutheroembryos are considered as correctly positioned if they are in a position that allows imaging of the dorsal region including the area where the kidneys are positioned.



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Annex 8: METHODS FOR THE STATISTICAL ANALYSIS OF RADAR ASSAY DATA

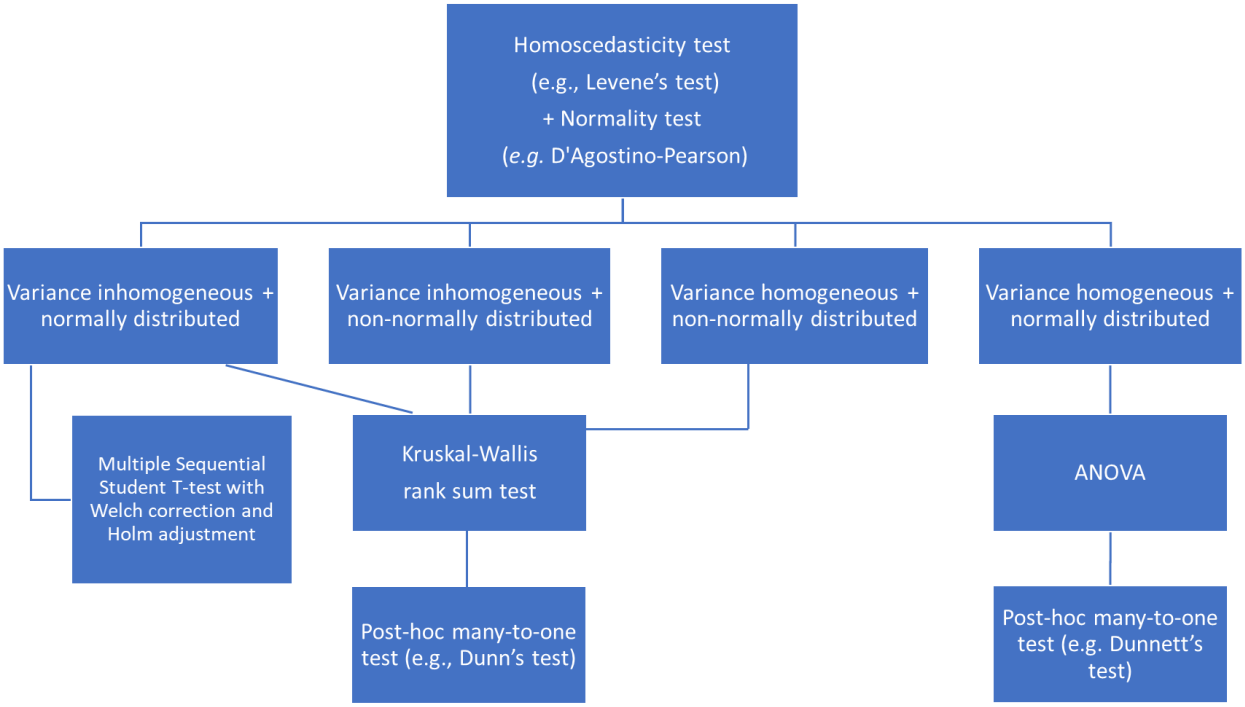
The recommended statistical approach, which was evaluated during the interlaboratory validation exercise, is to first determine whether the data for each exposure group is normally distributed by performing a D'Agostino-Pearson normality test. To determine whether variance is homogenous, a homoscedasticity test (e.g., Levene's test) should be performed.

If the data are normally distributed and homogeneous variance assumption is not violated, then an ANOVA test should be performed on the unspiked test chemical groups and the negative control (solvent control or test medium control if no solvent it used), followed by a Dunnett's post-hoc-test. Likewise, an ANOVA test should be performed on the spiked test chemical groups and the 17MT 3 µg/L control, followed by a Dunnett's post-hoc-test.

If the data follow a normal distribution but the equal variance assumption is violated, a Kruskal-Wallis test should be performed followed by a post-hoc Dunn's test or Welchs's many-to-one comparison test.

If the data do not follow a normal distribution a Kruskal-Wallis test should be performed on the unspiked test chemical groups and the negative control (solvent control or test medium control if no solvent it used), followed by a Dunn's post-hoc-test. Likewise, a Kruskal-Wallis test should be performed on the spiked test chemical groups and the 17MT 3 µg/L control, followed by a Dunn's post-hoc-test.

Figure 4: The recommended statistical workflow for comparing more than two groups when analysing the RADAR assay.



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Alternatively, a nested ANOVA test (also called mixed ANOVA) with a random effect term accounting for the variability introduced by runs or wells could be used to analyse this type of data, provided the well information is recorded during the experiment. A subsequent post-hoc test such as Dunnett's test can be applied to determine the magnitude and the significance of the difference.

If only two groups are to be compared, for example the 3 µg/L 17MT control and the 3 µg/L 17MT + 500 µg/L flutamide control, it should first be determined whether the data for each exposure group are normally distributed by performing a D'Agostino-Pearson normality test. If the data for each exposure group are normally distributed, a Welch corrected Student's T-test should be performed. If the data from one or both of the exposure groups do not follow a normal distribution, A Mann-Whitney test should be performed.

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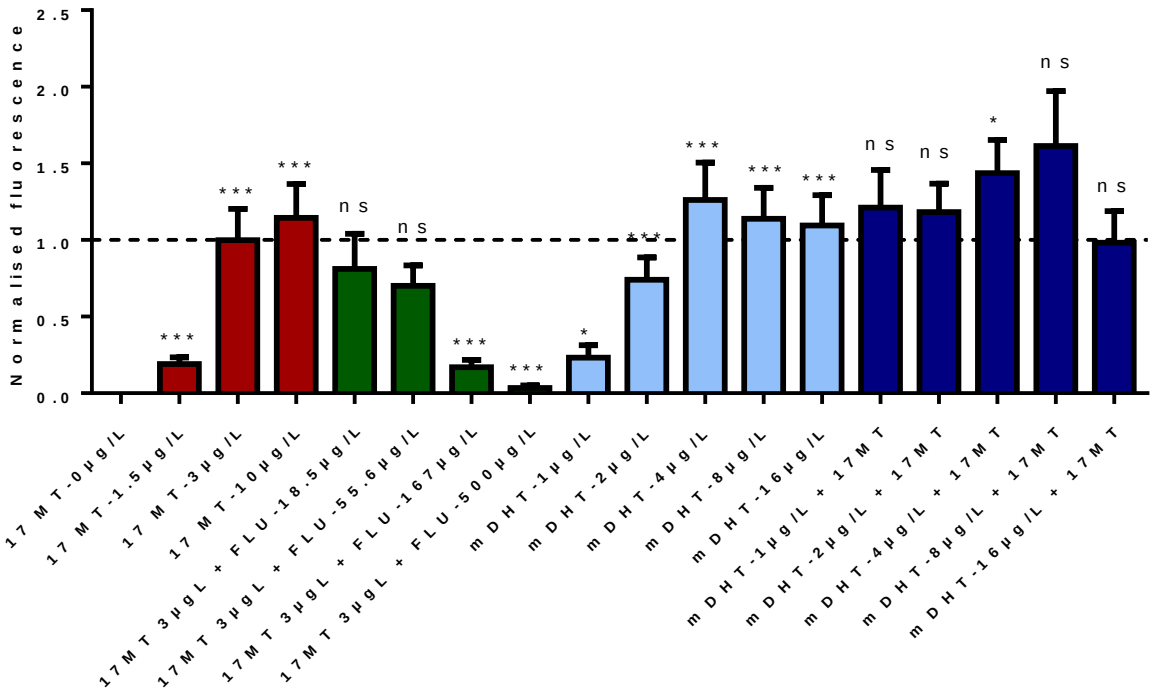
ANNEX 9: TYPICAL CONCENTRATION-RESPONSE CURVES AND THEIR INTERPRETATION

To aid with interpretation of the RADAR assay, example histograms are shown below of results obtained during the OECD validation study for the four proficiency chemicals. The interpretation of each result is discussed briefly. It should be noted that during the validation study all controls, including optional controls, were performed by all laboratories.

10.3. mDHT

Figure 5: An example result obtained with the proficiency chemical mDHT during the OECD validation study. Fluorescence was normalised to the mean fluorescence value of the 3 µg/L 17MT control. Statistical significance is shown as: * : p < 0.05 ; ** : p < 0.01 ; *** : p < 0.001 ; ns : not significant p > 0.05.



Validity criteria had already been met for the individual runs. Figure 6 shows that all validity criteria related to the performance of the controls in the pooled data set were met by the laboratory performing this RADAR assay. The mean normalised fluorescence of the 10 µg/L 17MT control group was greater than 10% higher than that of the 3 µg/L 17MT group (14% higher). The mean normalised fluorescence of the 3 µg/L 17MT + 500 µg/L flutamide group was statistically significantly different to that of the 3 µg/L 17MT group (P<0.001).

The normalised mean fluorescence of at least one concentration of mDHT in unspiked mode (light blue bars) was statistically significantly different to the 0 µg/L 17MT group

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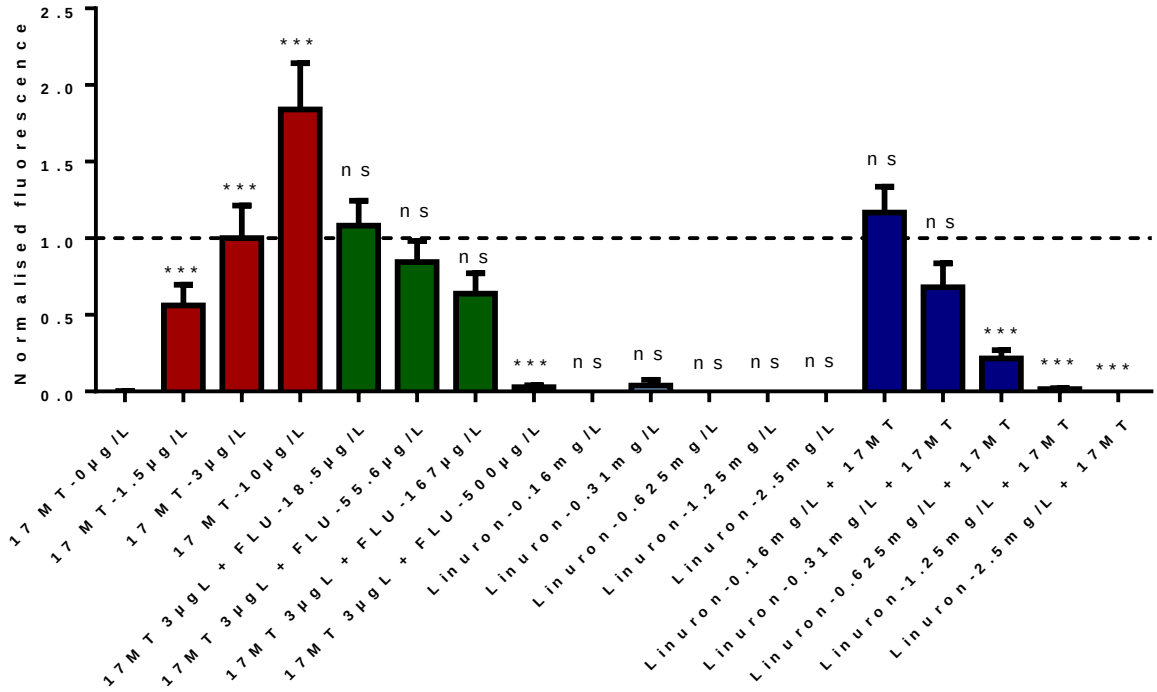
(the solvent control group) and a monotonic concentration-response profile was observed. Therefore, it was concluded that mDHT is active in the RADAR assay.

10.4. Linuron

Figure 6: An example result obtained with the proficiency chemical linuron during the OECD validation study. Fluorescence was normalised to the mean fluorescence value of the 3 µg/L 17MT control. Statistical significance is shown as: * : p < 0.05 ; ** : p < 0.01 ; *** : p < 0.001 ; ns : not significant p > 0.05.



Validity criteria had already been met for the individual runs. Figure 7 shows that all validity criteria related to the performance of the controls in the pooled data set were met by the laboratory performing this RADAR assay. The mean normalised fluorescence of the 10 µg/L 17MT control group was greater than 10% higher than that of the 3 µg/L 17MT group (84% higher). The mean normalised fluorescence of the 3 µg/L 17MT + 500 µg/L flutamide group was statistically significantly different to that of the 3 µg/L 17MT group (P<0.001).

None of the tested concentrations of linuron elicited a statistically significant difference in normalised mean fluorescence in unspiked mode (light blue bars) when compared to the 0 µg/L 17MT group (the solvent control group).

The normalised mean fluorescence of at least one concentration of linuron in spiked mode (dark blue bars) was statistically significantly different to the 3 µg/L 17MT group (the spiked mode control) and a monotonic concentration-response profile was observed. Therefore, it was concluded that linuron is active in the RADAR assay.

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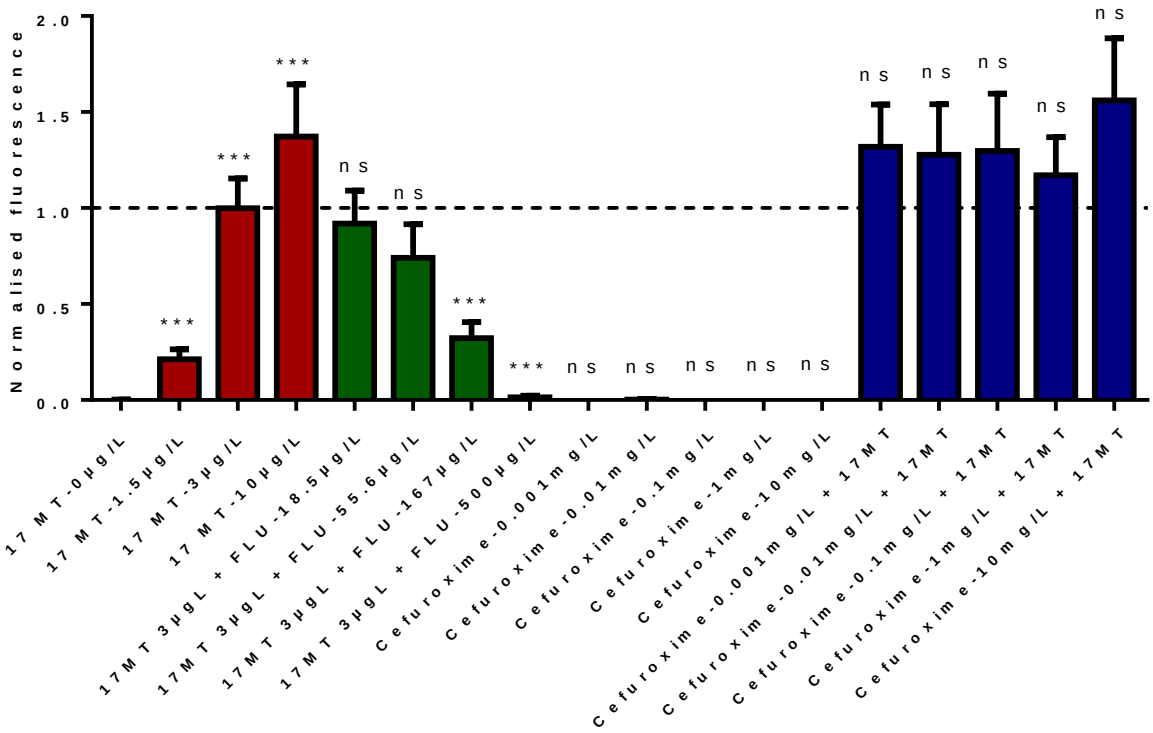
Cefuroxime

Figure 7: An example result obtained with the proficiency chemical cefuroxime during the OECD validation study. Fluorescence was normalised to the mean fluorescence value of the 3 µg/L 17MT control. Statistical significance is shown as: * : p < 0.05 ; ** : p < 0.01 ; *** : p < 0.001 ; ns : not significant p > 0.05.



Validity criteria had already been met for the individual runs. Figure 8 shows that all validity criteria related to the performance of the controls in the pooled data set were met by the laboratory performing this RADAR assay. The mean normalised fluorescence of the 10 µg/L 17MT control group was greater than 10% higher than that of the 3 µg/L 17MT group (37% higher). The mean normalised fluorescence of the 3 µg/L 17MT + 500 µg/L flutamide group was statistically significantly different to that of the 3 µg/L 17MT group (P<0.001).

None of the tested concentrations of cefuroxime elicited a statistically significant difference in normalised mean fluorescence in unspiked mode (light blue bars) when compared to the 0 µg/L 17MT group (the solvent control group).

None of the tested concentrations of cefuroxime elicited a statistically significant difference in normalised mean fluorescence in spiked mode (dark blue bars) when compared to the 3 µg/L 17MT group (the spiked mode control).

Therefore, it was concluded that cefuroxime is inactive in the RADAR assay.

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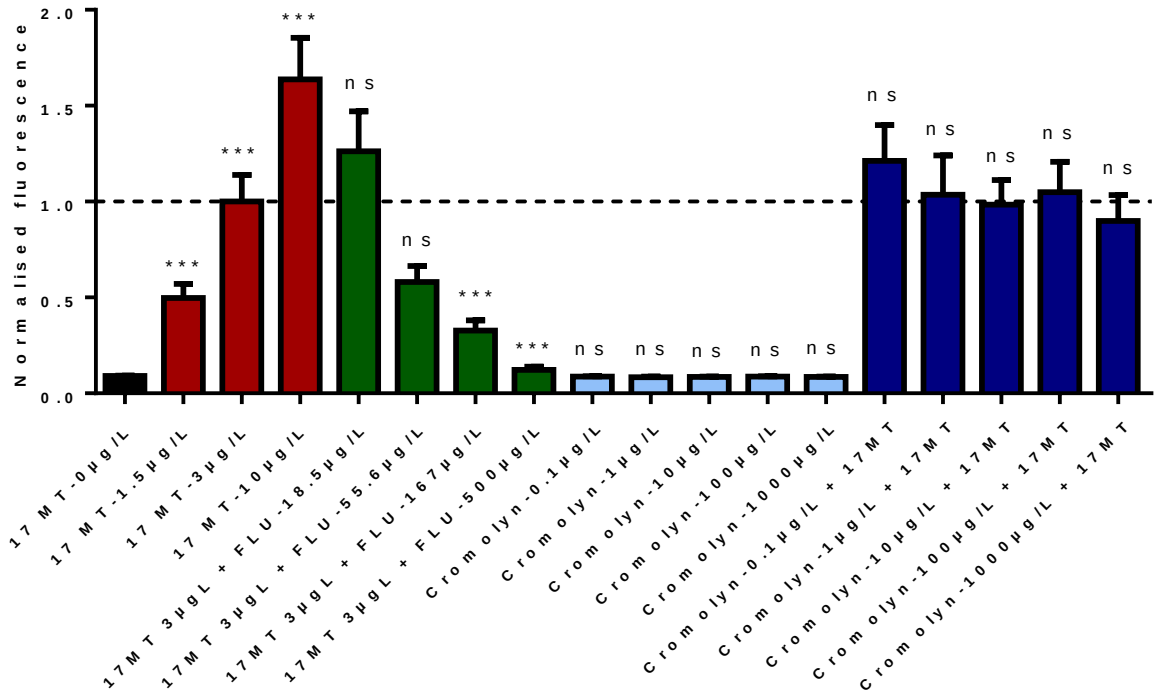
10.5. Cromolyn

Figure 8: An example result obtained with the proficiency chemical cromolyn during the OECD validation study. Fluorescence was normalised to the mean fluorescence value of the 3 µg/L 17MT control. Statistical significance is shown as: * : p < 0.05 ; ** : p < 0.01 ; *** : p < 0.001 ; ns : not significant p > 0.05.



Validity criteria had already been met for the individual runs. Figure 9 shows that all validity criteria related to the performance of the controls in the pooled data set were met by the laboratory performing this RADAR assay. The mean normalised fluorescence of the 10 µg/L 17MT control group was greater than 10% higher than that of the 3 µg/L 17MT group (64% higher). The mean normalised fluorescence of the 3 µg/L 17MT + 500 µg/L flutamide group was statistically significantly different to that of the 3 µg/L 17MT group (P<0.001).

None of the tested concentrations of cromolyn elicited a statistically significant difference in normalised mean fluorescence in unspiked mode (light blue bars) when compared to the 0 µg/L 17MT group (the solvent control group).

None of the tested concentrations of cromolyn elicited a statistically significant difference in normalised mean fluorescence in spiked mode (dark blue bars) when compared to the 3 µg/L 17MT group (the spiked mode control).

Therefore, it was concluded that cromolyn is inactive in the RADAR assay.

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Annex 10: AVAILABILITY OF THE *SPG1-GFP* LINE

Concerning access to the *spg1-gfp* medaka transgenic line, it will be accessible to laboratories from OECD member countries through WatchFrog as well as through partner laboratories. It is envisaged that these partner laboratories will form a network of distributors, possibly including the participants of the ring test as well as stock centres (TEFOR, France; The National BioResource Project, Japan; IDEA Consulting, Japan; The National Museum of Natural History, France) as with the XETA assay (TG 248). A similar network of contract research organisations to the XETA assay will also be offered the opportunity to distribute the test independently of the method developer (ibacon GmbH, Eurofins, Charles River and Scymaris).

Access to this line requires a licensing agreement. The method developer has already signed a legal document committing to applying a FRAND policy established by the OECD to the use of this method. A similar approach has already been successfully applied to the XETA assay (TG 248) and a number of *in vitro* assays.

Establishing this licensing agreement will ensure that the line is the validated line by allowing a legitimate supplier to be identified.

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