C.3.4. Repeated Dose 90-Day Oral Toxicity Study in Rodents (OECD TG 408)

Status: Assay validated by the OECD.

721. Modalities detected: (anti)estrogen, (anti)androgen, thyroid, steroidogenesis.

722. Endpoints: Weight of thyroid gland, adrenals, testes, epididymides, uterus, ovaries, prostate + seminal vesicles with coagulating glands. Histopathologic changes in pituitary, thyroid gland, gonads, uterus, accessory sex organs, male and female mammary gland, testes and adrenals. Serum total T4, T3, and TSH..

723. Optional:. Estrous cyclicity. Circulating levels of, testosterone, oestradiol, follicle stimulating hormone (FSH), luteinising hormone (LH). Enumeration of cauda epididymis sperm reserves. Sperm morphology, sperm motility.

Background to the assay

724. This assay determines the general toxicity of chemicals in rodents after 90 days of oral dosing (by gavage, via the diet or in drinking water). The rat is the preferred species. It provides information on major toxic effects and target organ toxicity likely to arise from the post-weaning period until well into adulthood. OECD TG 408 was adopted in September 1998 and was updated in 2017 to add endocrine disrupter relevant endpoints intended to improve the detection of endocrine activity of test chemicals and mirrors updates to OECD TG 407 (Repeated Dose 28-Day Oral Toxicity Study in Rodents). In the updated version, an emphasis was placed on including additional thyroid parameters that could inform, alone or in combination with other information, on the potential of test chemicals to perturb the thyroid pathway. The update mirrored that of OECD TG 407 and therefore a comparison can be made with validation of the OECD TG 407 (28-Day Oral Toxicity Study) for endocrine endpoints where substances that were moderate and strong endocrine disruptors (EDs) for (anti)estrogenicity and (anti)androgenicity (e.g. ethinylestradiol and flutamide) and weak and strong modulators of thyroid hormone-related effects (e.g. propylthiouracil, T4 and methyl testosterone) were detected (OECD, 2006). Steroidogenesis inhibition was also detected, although only one (potent) chemical was used in the validation study (CGS 18320B). OECD TG 408 is likely to be more sensitive than OECD TG 407 because of the extended dosing period and the larger number of animals per group (ten male and ten female per group compared with five in OECD TG 407).

725. Experience with of serum hormone determinations in Levels 4 and 5 rodent assays has revealed that their detection/measurement in rodent studies can be challenging. A recent workshop on “Practicability of Hormonal Measurements” was organised by the BfR (Germany) and the finding from this workshop will be published (Kucheryavenko et al., 2018). The OECD Expert Group on Reproductive and Developmental Toxicity recommends that to demonstrate proficiency for thyroid hormones measurement, a laboratory should be able to show results from a separate study using a positive control substance. Laboratories may also submit their calibration curves, standard curves, as well as data on the levels of

quantification and detection. This group is also establishing a historical control database with thyroid toxicant positive controls.

When/why the assay may be used

726. This assay is likely to be used as part of a pesticide submission package and forms part of the standard information requirements in certain chemical legislations (e.g. REACH for chemicals which are manufactured or imported in quantities of 100 tonnes or more). At least three dose levels are included so that an estimate of no-adverse-effect-level can be determined and the assay used for hazard identification/characterisation. It should be noted that as this assay is not primarily designed to detect endocrine disruption, a higher degree of systemic toxicity is typically induced than is the case with the other Level 3 and 4 assays. The possibly confounding effect of systemic toxicity on endocrine endpoints therefore needs to be considered.

727. In order to provide information relevant for assessing whether or not a chemical may fulfil the WHO/IPCS (2002) definition of an endocrine disruptor (ED), the study design has to be sufficiently robust to demonstrate the presence or absence of effects. In the dose selection, the investigator should also consider and ensure that data generated are adequate to fulfil the regulatory requirement across OECD countries as appropriate (e.g. hazard and risk assessment and labelling, ED assessment, etc.). The top dose or concentration should be sufficiently high to give clear systemic (i.e. non endocrine-specific) toxicity in order to ensure that a wide range of exposures (high to low) is tested. However, endocrine effects observed solely in the presence of clear systemic toxicity should be interpreted with caution and may be disregarded when sufficiently justified to be caused by secondary effects which are unlikely to be due to endocrine activity. The reason for this advice is a concern that some endocrine active substance (EAS) sensitive assays are being run at doses/concentrations of EASs that are too low to trigger direct impacts on the endocrine system. This guidance document is not the place to address this issue directly, but it should be considered when EAS-sensitive test guidelines (TGs) are revised in the future. In addition, the number and spacing of dose/concentration levels should also be adequate to fulfil the objectives of the study (e.g. to demonstrate dose response relationships if this is required).

Introduction to the table of scenarios

728. Table C.3.4 gives guidance on a further step to take in the event of a positive (+) or negative (-) result and in the presence of positive (+), negative (-) or equivocal/absent (Eq/0) existing results. “Existing results” are subdivided into “mechanism” and “effects” data (third and fourth columns). The table is divided horizontally into a series of scenarios that represent all the combinations of these events.

729. The results of OECD TG 408 are given in the second column. As OECD TG 408 is not a screening test where a yes/no (qualitative) answer is obtained for the test as a whole, positive results would generally be assessed for individual endpoints. For the purposes of this guidance, however, a positive result is defined as a biologically significant change in any of the endocrine endpoints listed above (e.g. statistically significant reductions in reproductive organ weights). Changes in related endpoints will increase their biological significance (e.g. changes in the weights of testes and epididymides accompanied by histopathological changes). The guidance on histopathologic changes in endocrine tests

(OECD, 2009) may be helpful in interpretation. A negative result for OECD TG 408 is taken to be the absence of biologically significant changes in all endocrine endpoints.

730. In the absence of other pertinent lines of evidence, negative results in this test alone cannot be taken as evidence that the substance is not an ED. Further studies will be required as confirmation.

731. Equivocal results for the guideline are not considered in Table C.3.4, partly for brevity but also because equivocal results are by nature uncertain. A decision must eventually be reached about whether the endocrine endpoints tend to be positive or negative or whether the result must be put to one side and the test repeated (using the same or a different test guideline). Factors which may have interfered with the result (e.g. composition of the diet used, environmental influences) should be considered.

Existing data to be considered

732. Existing "mechanism" *in vitro* data are assumed to be available from estrogen receptor (ER-), androgen receptor (AR-) and steroidogenesis-based assays (Level 2). Assays may also be available for interference with thyroid modalities. In practice, it is possible that data from all of these assays may not be available, so judgement will need to be used to decide which assays to perform. Although the current *in vitro* test guidelines do not incorporate metabolic activation, published information on use of metabolic activation systems is available in Jacobs et al. (2008; 2013) and OECD (2008a). These methods, however, have not yet been validated.

733. Existing "effects" data refer to *in vivo* effects that may come from Level 3 or 4 tests in the Conceptual Framework (e.g. UT or H assays). In these cases, it should be remembered that these assays are specifically designed to be sensitive to EASs. It is unlikely that OECD TG 408 will be performed if higher tier data are already available as OECD TG 408 offers no advantage over these assays. As mentioned above, the results of the study may be interpreted as part of a battery or group of tests carried out for regulatory purposes. Data may also be available on effects in mammalian and non-mammalian wildlife species, although caution should be used when extrapolating between taxa. A chemical causing endocrine effects in non-mammalian environmental species (fish, for example) may also have endocrine effects in mammals, but the physiological consequences of the effects are likely to be different.

734. When considering the results of the OECD TG 408 assay, all available data should be used in order to reach a conclusion and a weight of evidence approach taken. This may include high throughput screening data, read-across data from structural analogues and quantitative structure activity relationship (QSAR). Several QSAR models for ER and AR binding/activation are now available (see Sections B.1.1.1 and B.1.1.2).

Scenarios: Positive and negative results combined with existing data

735. A series of scenarios (A to R) are presented in Table C.3.4 and represent all the possibilities of positive or negative results in combination with the presence or absence of existing data. The action taken will also depend on the regulatory environment, but the considerations given here are generally science based. Although the OECD TG 408 assay uses rodents, the well-conserved nature of the hormonal pathways across taxa indicate that results on endocrine endpoints in this assay may be relevant to other vertebrate species. Effects in laboratory mammal tests are also highly relevant for environmental mammalian species. Wherever possible, the recommended "next step which could be taken" avoids

unnecessary animal testing. However, sometimes conducting an animal test will be indicated and then the relevance of species, strain and exposure route should always be considered. The sensitivity and physiological function of the hormone under investigation in the test species should also be considered. In general, lower level tests should be conducted before higher level tests in order to avoid unnecessary animal usage, unless it is apparent that a Level 5 test will be required anyway or will be needed to establish the evidence to conclude on ED properties. Information on some endocrine-related tumours may be detected more comprehensively in carcinogenicity studies (OECD TG 451/453) (Level 4); for example, detection of certain types of thyroid tumors in the absence of reproductive or developmental effects, as well as substances causing tumors in other endocrine-sensitive tissues. At Level 5, the Extended One-Generation Reproduction Toxicity Study (EOGRTS – OECD TG 443) is the most sensitive reproduction assay for detecting endocrine disruption because it includes evaluation of a number of endocrine endpoints not included in the two-generation study (OECD TG 416) adopted in 2001. It is recognised, however, that some jurisdictions may require a two-generation study. Further considerations specific to each scenario are given in the table.

736. Scenarios A to C represent positive results in the OECD TG 408 assay in the presence of positive *in vitro* mechanistic data and positive, negative or equivocal *in vivo* effects data. A positive result in the *in vitro* assays in combination with a positive OECD TG 408 assay is moderate or strong evidence for estrogen/androgen/thyroid/steroidogenesis (E,A,T,S-) mediated activity that may or may not be supported by the *in vivo* effects data. In the absence of robust upper-level data, the next step may be to conduct an upper-level test. In the presence of robust *in vivo* data, there may be sufficient evidence to conclude concern for endocrine disruption and therefore no need for further testing. Positive results in the OECD TG 408 assay may also indicate the potential for endocrine mediated effects in lower vertebrates. These could be followed up with partial life cycle tests such as the Fish Sexual Development Test (FSDT), the Larval Amphibian Growth and Development Assay (LAGDA) or the Medaka Extended One-Generation Reproduction Test (MEOGRT) if the evidence were strong enough. *In vivo* assays/tests with negative results should be interpreted with caution as they may either indicate that the tests used do not have sufficient power to detect weak effects or alternatively that the effects do not present a concern for endocrine disruption. The possibility of other (non-E,A,T,S) mechanisms should also not be overlooked (e.g. involving other receptors or endocrine axes).

737. Scenarios D to F represent positive results in the OECD TG 408 assay in the presence of negative *in vitro* mechanistic data and positive, negative or equivocal *in vivo* effects data. Negative results in the *in vitro* assays should be viewed with caution in case a metabolite is responsible for the positive OECD TG 408 assay. Unless the metabolic profile of the test substance is known, one option may be to conduct these assays with an added metabolising system. If the metabolic profile is known, then a higher level *in vivo* test may be advisable. The choice of tests will depend on the available *in vivo* effects data. Positive results in the OECD TG 408 assay may also indicate the potential for endocrine mediated effects in lower vertebrates. As in Scenarios A to C, *in vivo* assays/tests with negative results should be interpreted with caution, as they may either indicate that the tests used do not have sufficient power to detect weak effects or, alternatively, that the effects do not present a concern for endocrine disruption.

738. Scenarios G to I represent positive results in the OECD TG 408 assay in the presence of various combinations of missing or equivocal data. Positive results in the OECD TG 408 assay may also indicate the potential for endocrine mediated effects in lower vertebrates. The next step to take in these eventualities will depend on the nature of the

other available data and the jurisdiction in which it is being used. In some cases, equivocal data may be viewed as positive whilst in others it may or may not contribute to the weight of evidence. The interpretation may also depend on the mode of action (MOA) in question and why the data are considered equivocal, e.g. a study that is equivocal for thyroid effects may still be of value in evaluating (anti)androgenic effects. In all three scenarios, the recommended first step is to obtain reliable mechanistic (*in vitro*) data rather than proceed further with *in vivo* testing. Equivocal and missing data are alternative scenarios and two possibilities for the next step are given in most cases, but the nature of equivocal data means that decisions need to be taken on a case-by-case basis. In all cases, the role of metabolism, route of exposure and data from structural analogues should be considered before deciding on the next step.

739. Scenarios J to L represent negative results in the OECD TG 408 assay in the presence of positive *in vitro* mechanistic data and positive, negative or equivocal *in vivo* effects data. Negative outcomes in OECD TG 408 should be viewed with caution because of the power of the assay to detect (anti)estrogens and androgens may be limited All three scenarios could also arise from a chemical that is positive in *in vitro* assays, but is metabolised to a non-active metabolite leading to negative results in the OECD TG 408 assay. This should be considered first when investigating the next step. Endocrine active potency may also explain differences between *in vitro* and *in vivo* results (e.g. a chemical with weak endocrine activity may give a positive result *in vitro* but may be negative *in vivo*). Positive *in vivo* effects data may involve other E,A,T,S, non-E,A,T,S mechanisms (e.g. involving other receptors or endocrine axes), more sensitive endpoints, greater statistical power or life stages that are more sensitive to the substance than the young adult exposed animals in OECD TG 408.

740. Scenarios M to O represent negative results in the OECD TG 408 assay in the presence of negative *in vitro* mechanistic data and positive, negative or equivocal *in vivo* effects data. Negative results for all tests (Scenario N) may be sufficient to enable a conclusion of no concern for endocrine disruption. This will depend on the weight of evidence and may not be possible. Where there are positive *in vivo* effects data, there could still be an E,A,T,S-related mechanism, the effects may be related to length of exposure, route of exposure or exposure at different life stages. Other E,A,T,S or non-E,A,T,S mechanisms may also be involved.

741. Scenarios P to R represent negative results in the OECD TG 408 assay in the presence of various combinations of missing or equivocal data. As with the positive result scenarios above, the next step to take in these eventualities will have to be decided on a case-by-case basis. However, the recommended first step is generally to obtain reliable mechanistic (*in vitro*) data rather than proceed further with *in vivo* testing. In all cases, the role of metabolism, route of exposure and data from structural analogues should be considered before deciding on the next step.

742. In all scenarios (A to R), the next step to take to strengthen weight of evidence will depend on the existing information. The table is meant to provide a succinct guide and may not cover all circumstances or possibilities. The scenarios may also suggest that chemicals have simple or single MOA, when in practice they may have multiple endocrine and non-endocrine MOA. In some cases, for example, two opposite modes of simultaneous action (e.g. estrogenic and anti-estrogenic) could, depending on dose, lead to a minimisation or abolition of effects, while in others two different MOA (e.g. estrogenic and anti-androgenic) could potentially reinforce effects. Endocrine pathways interact, mixed effects are common and there are many pathways that cannot be distinguished with currently available TGs. If multiple MOA are suspected, either from the existing results or based on QSAR/read-

across/integrated approaches, this should be investigated further if needed for regulatory decision making.

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Table C.3.4. **Repeated Dose 90-Day Oral Toxicity Study in Rodents (OECD TG 408): Guidance for scenarios of combinations of results with existing data**

This table represents possible conclusions to be drawn from assay data, and a next step which could be taken if further evidence is required about possible endocrine disrupting properties and/or effects. The guidance offered is not meant to be prescriptive, but provides science-based considerations. It encourages the use of all available data and expert judgement in a weight of evidence approach. Regional and national interpretation of results and "next steps" may vary.

The conclusions are grouped into a series of scenarios (A-R), each scenario representing a different combination of assay results, existing *in vitro* data and existing *in vivo* data. The symbol "+" indicates that the data in question represent a positive result, «-" indicates a negative result, and "Eq/0" indicates that the data are either equivocal or are not available.

Existing results: * "Mechanism (*in vitro* mechanistic data)" assumes that mechanistic data are available from estrogen receptor (ER-), androgen receptor (AR-) and steroidogenesis-based assays (Level 2). Thyroid hormone receptor (TR) and other assays concerning mechanisms of thyroid disruption may be available, but they are not in common use. In practice, data from all assays may not be available and therefore this must be taken into account when deciding on the "next step". Quantitative structure activity relationship (QSAR) predictions of estrogen and androgen binding/activation may be made for some substances.

Existing results: ** "Effects (*in vivo* effects of concern)" assumes various information, such as data from repeat dose oral toxicity studies, reproduction/developmental toxicity screen tests, read-across from analogues, will be available.

*** *Note:* a positive result is defined as a biologically significant change in any of the endocrine endpoints.

Scenarios	Result of OECD TG 408 (rodent 90-day) assay	Existing results		Possible conclusions	Next step which could be taken to strengthen weight of evidence if necessary	Other considerations
		Mechanism (*in vitro* mechanistic data)*	Effects (*in vivo* effects of concern)**			
A	+ ***	+	+	(Anti)-E,A,T,S activity. Increased evidence of (anti)-E,A,T,S activity.	Perform assay from Level 5 (e.g. EOGRTS or two-generation assay).	If existing data are from a Level 5 assay, there may be sufficient information to conclude evidence of concern for endocrine disruption (the EOGRTS provides the most information; however, for endocrine disrupting chemincals [EDCs] with a carcinogenic potential, OECD TG 451-3 may be more sensitive). Consider route of exposures for effects data and possible implications of absorption, distribution, metabolism and excretion (ADME) characteristics of the chemical. Endocrine activity possible in lower vertebrates. Consider performing a Fish Sexual Development Test (FSDT), a Larval Amphibian Growth and Development Assay (LAGDA) or a Medaka Extended One-Generation Reproduction Test (MEOGRT).
B	+	+	–	(Anti)-E,A,T,S activity. Increased evidence of (anti)-E,A,T,S activity.	Perform assay from Level 5 (e.g. EOGRTS or two-generation assay).	If existing data are from an adequate Level 5 assay, question why there are differences. If existing data are from a less sensitive assay, a higher level test may be required. Consider route of exposures and possible implications of ADME characteristics of the chemical. Endocrine activity possible in lower vertebrates. Consider performing an FSDT, LAGDA or MEOGRT.
C	+	+	Eq/0	(Anti)-E,A,T,S activity. Increased evidence of (anti)-E,A,T,S activity.	Perform assay from Level 5 (e.g. EOGRTS or two-generation assay).	Check data on chemical analogues. Consider route of exposure for OECD TG 408 and follow-up assay. Possible implications of ADME characteristics of the chemical. Endocrine activity possible in lower vertebrates. Consider performing an FSDT, LAGDA or MEOGRT. Equivocal results may indicate chemical has multiple modes of action (MOA).
D	+	–	+	(Anti)-E,A,T,S activity. Acts via non-estrogen receptor (ER),androgen receptor (AR), thyroid hormone receptor (TR), steroidogenesis (S) mechanism or requires metabolic activation for activity.	Perform *in vitro* ER, AR, TR, S assays with added metabolising system.	If existing data are from an adequate Level 5 assay, there may be sufficient information to conclude evidence of concern for endocrine disruption (the EOGRTS provides the most information; however, for EDCs with a carcinogenic potential, OECD TG 451-3 may be more sensitive). Consider route of exposures for effects data and possible implications of ADME characteristics of the chemical. Further mechanistic studies may help determine MOA. Endocrine activity possible in lower vertebrates. Consider performing an FSDT, LAGDA or MEOGRT.
E	+	–	–	(Anti)-E,A,T,S activity. Acts via non-ER, AR, TR, S mechanism or requires metabolic activation for activity. Route of exposure may account for the differences between OECD TG 408 and existing data.	Perform *in vitro* ER, AR, TR, S assays with added metabolising system OR Perform assay from Level 5 (e.g. EOGRTS or two-generation assay).	If existing data are from an adequate Level 5 assay, question why there are differences. If existing data are from a less sensitive assay, a higher level test may be required. Consider route of exposures and possible implications of ADME characteristics of the chemical. Endocrine activity possible in lower vertebrates. Consider performing an FSDT, LAGDA or MEOGRT.

Scenarios	Result of OECD TG 408 (rodent 90-day) assay	Existing results		Possible conclusions	Next step which could be taken to strengthen weight of evidence if necessary	Other considerations
		Mechanism (*in vitro* mechanistic data)*	Effects (*in vivo* effects of concern)**			
F	+	–	Eq/0	(Anti)-E,A,T,S activity. Acts via non-ER, AR, TR, S mechanism or requires metabolic activation for activity.	Perform *in vitro* ER, AR, TR, S assays with added metabolising system OR Perform assay from Level 5 (e.g. EOGRTS or two-generation assay).	Check data on chemical analogues. Further mechanistic studies may help determine MOA. Level 5 studies will provide hazard data. Endocrine activity possible in lower vertebrates. Consider performing an FSDT, LAGDA or MEOGRT. Equivocal results may indicate chemical has multiple MOA.
G	+	Eq/0	+	(Anti)-E,A,T,S activity. May act via ER, AR, TR, S mechanism (metabolic activation needed).	Perform *in vitro* ER, AR, TR, S assays for the "0" scenario, otherwise Eq result available OR Perform *in vitro* ER, AR, TR, S assays with added metabolising system.	If existing data are from a Level 5 assay, there may be sufficient information to conclude evidence of concern for endocrine disruption (the EOGRTS provides the most information; however, for EDCs with a carcinogenic potential, OECD TG 451-3 may be more sensitive). Check data on chemical analogues. Further mechanistic studies may help determine MOA. Consider route of exposures for effects data and possible implications of ADME characteristics of the chemical. Endocrine activity possible in lower vertebrates. Consider performing an FSDT, LAGDA or MEOGRT. Equivocal results may indicate chemical has multiple MOA.
H	+	Eq/0	–	(Anti)-E,A,T,S activity. Acts via unknown mechanism or may require metabolic activation for activity. Route of exposure may account for the differences between OECD TG 408 and existing data.	For the "0" scenario, perform *in vitro* ER, AR, TR, S assays with added metabolising system (otherwise Eq result available).	If existing data are from an adequate Level 5 assay, question why there are differences. Consider route of exposures and possible implications of ADME characteristics of the chemical. If existing data are from a less sensitive assay, a higher level test may be required. Check data on chemical analogues. Further mechanistic studies may help determine MOA. Endocrine activity possible in lower vertebrates. Consider performing an FSDT, LAGDA or MEOGRT. Equivocal results may indicate chemical has multiple MOA.
I	+	Eq/0	Eq/0	(Anti)-E,A,T,S activity. Acts via unknown mechanism. Unknown potential for adverse effects.	Perform *in vitro* ER, AR, TR, S assays with added metabolising system OR Perform assay from Level 5 (e.g. EOGRTS or two-generation assay).	Check data on chemical analogues. Further mechanistic studies may help determine MOA. Endocrine activity possible in lower vertebrates. Consider performing an FSDT, LAGDA or MEOGRT. Equivocal results may indicate chemical has multiple MOA.

Scenarios	Result of OECD TG 408 (rodent 90-day) assay	Existing results		Possible conclusions	Next step which could be taken to strengthen weight of evidence if necessary	Other considerations
		Mechanism (*in vitro* mechanistic data)*	Effects (*in vivo* effects of concern)**			
J	–	+	+	No evidence for (anti)-E,A,T,S activity in OECD TG 408. Weak (anti)-E,A,S activity may not be detected by this assay. Metabolism or potency explains the difference from existing *in vitro* and *in vivo* data.	Perform *in vitro* ER, AR, TR, S assays with added metabolising system OR Perform assay from Level 5 (e.g. EOGRTS or two-generation assay).	If existing data are from an adequate Level 5 assay, question why there are differences. Effects seen in existing studies may be in a more sensitive life stage. Consider route of exposures and possible implications of ADME characteristics of the chemical. Further mechanistic studies may help determine MOA.
K	–	+	–	No evidence for (anti)-E,A,T,S activity in OECD TG 408. Weak (anti)-E,A,S activity may not be detected by this assay. Metabolism or potency explains *in vitro*/*in vivo* differences.	Perform *in vitro* ER, AR, TR, S assays with added metabolising system OR Perform assay from Level 5 (e.g. EOGRTS or two-generation assay).	If existing data are from an adequate Level 5 assay, there may be sufficient information to conclude absence of concern for endocrine disruption (the EOGRTS provides the most information; however, for EDCs with a carcinogenic potential, OECD TG 451-3 may be more sensitive). If existing data are from a less sensitive assay, a higher level test may be required. Further mechanistic studies with metabolism may help determine MOA.
L	–	+	Eq/0	No evidence for (anti)-E,A,T,S activity in OECD TG 408. Weak (anti)-E,A,S activity may not be detected by this assay. Metabolism or potency explains *in vitro*/*in vivo* differences. Unknown potential for adverse effects.	Perform *in vitro* ER, AR, TR, S assays with added metabolising system OR Perform assay from Level 5 (e.g. EOGRTS or two-generation assay).	Metabolic deactivation of chemical may occur *in vivo* so that possible *in vitro* activity is not realised. Consider possible routes of exposure implications of metabolism. Equivocal results may indicate chemical has multiple MOA.
M	–	–	+	No evidence for (anti)-E,A,T,S activity in OECD TG 408. Weak (anti)-E,A,S activity may not be detected by this assay. Effects seen in existing studies are via non-E,A,T,S mechanism.	Perform assay from Level 5 (e.g. EOGRTS or two-generation assay).	If existing data are from an adequate Level 5 assay, question why there are differences. Effects seen in existing studies may be in a more sensitive life stage. Consider route of exposures and possible implications of ADME characteristics of the chemical.
N	–	–	–	No evidence for (anti)-E,A,T,S activity in OECD TG 408. Weak (anti)-E,A,S activity may not be detected by this assay. No evidence for (anti)-E,A,T,S activity *in vitro*. No evidence of adverse effects.	Perform assay from Level 5 (e.g. EOGRTS or two-generation assay).	If existing data are from an adequate Level 5 assay, there may be sufficient information to conclude absence of concern for endocrine disruption (the EOGRTS provides the most information; however, for EDCs with a carcinogenic potential, OECD TG 451-3 may be more sensitive).
O	–	–	Eq/0	No evidence for (anti)-E,A,T,S activity in OECD TG 408. Weak (anti)-E,A,S activity may not be detected by this assay. No evidence for (anti)-E,A,TS activity *in vitro*. Unknown potential for adverse effects.	Perform assay from Level 5 (e.g. EOGRTS or two-generation assay).	Consider route of exposures and possible implications for ADME characteristics of the chemical in follow-up assay.

Scenarios	Result of OECD TG 408 (rodent 90-day) assay	Existing results		Possible conclusions	Next step which could be taken to strengthen weight of evidence if necessary	Other considerations
		Mechanism (*in vitro* mechanistic data)*	Effects (*in vivo* effects of concern)**			
P	–	Eq/0	+	No evidence for (anti)-E,A,T,S activity in OECD TG 408. Weak (anti)-E,A,S activity may not be detected by this assay. Potential for adverse effects via unknown mechanism.	Perform *in vitro* ER, AR,TR, S assays with added metabolising system.	Consider route of exposure for OECD TG 408 assay and possible implications for differences from existing assay. Effects seen in existing studies may be in a more sensitive life stage. Further mechanistic studies may strengthen weight of evidence. Equivocal results may indicate chemical has multiple MOA.
Q	–	Eq/0	–	No evidence for (anti)-E,A,T,S activity in OECD TG 408. Weak (anti)-E,A,S activity may not be detected by this assay. No evidence of adverse effects.	Perform *in vitro* ER, AR, TR, S assays with added metabolising system.	If existing data are from an adequate Level 5 assay, there may be sufficient information to conclude absence of concern for endocrine disruption (the EOGRTS provides the most information; however, for EDCs with a carcinogenic potential, OECD TG 451-3 may be more sensitive). Further mechanistic studies may strengthen weight of evidence.
R	–	Eq/0	Eq/0	No evidence for (anti)-E,A,T,S activity in OECD TG 408. Weak (anti)-E,A,S activity may not be detected by this assay.	Perform *in vitro* ER, AR, TR, S assays with added metabolising system, otherwise Eq result available.	Further mechanistic studies may strengthen weight of evidence. Check data on chemical analogues. Equivocal results may indicate chemical has multiple MOA.