C.3.14. Two-Generation Reproduction Toxicity Study (OECD TG 416)

Status: Assay validated by the OECD.

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

Endpoints:

Time to mating, male fertility, female fertility, gestation length, number of implantations and corpora lutea, number of live births and post-implantation loss, litter size, sex ratio (F1, F2), litter/pup weight, pup survival index.

Estrus cyclicity (P, F1), sexual maturation (age at vaginal opening [VO] and preputial separation [PPS] (F1)), anogenital distance (F2, if triggered by changes in sex ratio or sexual maturation in F1), pup development (F1, F2).

Weights of: (P, F1) uterus, ovaries, testes, epididymides, prostate, seminal vesicles (+ coagulating glands) thyroid, adrenals.

Histopathologic changes in vagina, uterus (+ cervix), ovaries, testis, epididymis, prostate, seminal vesicles and coagulating glands.

Sperm numbers (testicular homogenisation resistant spermatids and cauda epididymides sperm reserve), sperm motility, sperm morphology (P, F1).

Background to the assay

942. The OECD Two-Generation Reproduction Toxicity Study is an apical assay designed to provide general information concerning the effects of a chemical on the male and female reproductive systems including gonadal function, the estrus cycle, mating, conception, gestation, parturition, lactation, weaning, and growth and development of the offspring. The rat is the preferred species. The recommended route of administration is oral, via the diet, by gavage or in drinking water. The study is not specifically designed to detect endocrine active substances (EASs), but has many endpoints relevant for the assessment of possible endocrine disruption and provides data on adverse effects related to reproduction and development. OECD TG 416 was revised in January 2001 to include a more comprehensive range of endpoints. These endpoints include sexual maturation (VO and PPS) which are particularly sensitive to EASs. One-generation studies and two-generation studies conducted prior to the adoption of the revised OECD TG 416 are therefore unlikely to provide as much data as studies conducted to the revised OECD TG 416, particularly with respect to endocrine disruption. They do, however, provide a great deal of useful data, particularly on adverse effects on reproduction, that may be sufficient for hazard assessment purposes even if the etiology of the effect(s) is not fully characterised.

943. The Extended One-Generation Reproduction Toxicity Study (EOGRTS – OECD TG 443) is the preferred Conceptual Framework Level 5 study for investigating potential endocrine disruption and is likely to replace OECD TG 416 in time. The EOGRTS contains

more endpoints than OECD TG 416 that are sensitive to endocrine disruption and may also include cohorts for investigating developmental neurotoxicity and developmental immunotoxicity. Decisions on which assay to use may also depend on regulatory considerations.

944. All versions of the test guideline (TG) require that parental males be dosed for a period of time encompassing at least one spermatogenic cycle and that parental females be dosed for at least several estrus cycles. Dosing is continuous during mating and throughout production of subsequent generations. The exposure of the fetus (which may be a sensitive life stage for endocrine disruption effects), the duration of dosing and the diversity of endpoints means that the revised OECD TG 416 may be considered to be more predictive for endocrine disruptor (ED-) mediated adverse effects via estrogen/androgen/thyroid/steroidogenesis (E,A,T,S) modalities than previous versions. As all the endpoints are apical, it is difficult to discern mechanism of action from this test alone. Information on mechanism of action needs to be obtained from *in vitro* E,A,T,S assays or *in vivo* lower tier tests such as the Uterotrophic Bioassay (UT) and the Hershberger Bioassay (H).

945. Although formal validation of OECD TG 416 with EDs has not taken place, studies have been published showing that estrogen receptor (ER) agonists (such as ethinylestradiol [NTP, 2010]), androgen receptor (AR) antagonists (such as vinclozolin [Matsuura et al., 2005]), steroidogenesis inhibitors (such as myclobutanil [Rockett et al., 2006]) and thyroid hormone modulators (such as propylthiouracil [Axelstad et al., 2008; NTP, 2003]) can all be detected by reproductive toxicity tests. However, OECD TG 416 does not include measurement of thyroid hormones. Thyroid endpoints are limited to thyroid weight and histopathology. In addition, OECD TG 416 lacks apical endpoints of developmental neurotoxicity, such as motor activity, sensory function, learning and memory, which are included in the EOGRTS (OECD TG 443). Endocrine modalities other than E,A,T,S are also detected (e.g. chemicals acting on the hypothalamic/pituitary/gonadal [HPG] axis or other hormone systems). Some chemicals interacting weakly with endocrine disrupting modalities in lower tier tests, designed to have greater sensitivity than specificity, may not have effects in this test as functional HPG axes in parents and offspring may allow compensation for weak effects. In these cases it could be interpreted that the weak effects do not lead to adverse outcomes in more comprehensive studies. Nonylphenol, for example, is a weak ER agonist in *in vitro* ER assays and in the *in vivo* UT assay, but has no effect on reproduction or development in reproductive tests (Tyl et al., 2006) although there were some effects on the offspring (slight changes in the estrous cycle length, the timing of VO and possibly also in ovarian weight and sperm/spermatid count, although in the absence of functional changes in reproduction at the dose levels tested). The observed perturbations in offspring were concluded (ECBI/48/99 HSE, UK) to be compatible with the predicable or hypothesised effects of exogenous estrogenic activity. Octylphenol is a further example of a weak ER agonist in *in vitro* ER binding assays and in the UT assay, but did not reveal effects on reproduction or development in a good quality test conducted according to OECD TG 416 (Tyl et al., 1999).

946. The adequacy of the protocol in studies where no endocrine-related effects are reported needs to be confirmed so that the absence of effects is not due to inadequacy of methods or reporting.

947. If the adequacy of the protocol is suspect, or the test was conducted before OECD TG 416 was revised, it may be possible to conduct or to use additional studies to support the reproductive toxicity test. For example, a reproduction toxicity study not including data

on sexual maturation could be supplemented by male and female Peripubertal Assays if needed. However, existing knowledge should be considered before embarking on further testing.

When/why the assay may be used

948. This assay forms part of the package of studies required for registration of pesticides in many jurisdictions. It may be carried out for high production volume chemicals of high concern, as well as being a more comprehensive test at Level 5 of the Conceptual Framework. It is likely to have at least three dose levels and therefore may be used for hazard identification/characterisation. OECD TG 416 has been replaced in many regions by the extended one-generation study (OECD TG 443), which is preferable for detecting endocrine disruption because it provides an evaluation of a number of endocrine endpoints in the juvenile and adult F1 which are not included in the two-generation study.

949. In order to provide information relevant for assessing whether or not a chemical may fulfil the WHO/IPCS (2002) definition of an 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

950. Table C.3.12 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.

951. The results of OECD TG 416 are given in the second column. As this assay is not a screening test where a yes/no (qualitative) answer is obtained, criteria for positive results for the endocrine endpoints are not given in the test guideline. Results for the endpoints would be considered both individually and as a whole. It is not possible to provide guidance on all endpoints individually and for this test all endpoints are considered to be “apical”. Serum hormone determinations are not included in OECD TG 416, therefore (unlike with some of the other Level 4 and 5 assays) the division of the endpoints into “apical” and “indicators of hormonal activity” has not been possible.

952. For the purpose of this guidance, a positive result is defined as a biologically significant change in any of the endocrine endpoints (e.g. biologically significant reductions in reproductive organ weight). Changes in related endpoints will increase their biological significance (e.g. abnormal estrous cyclicity combined with reduced fertility).

953. A negative result for OECD TG 416 is taken to be the absence of biologically significant changes in all of the endocrine endpoints measured in this TG. Studies conducted to current standards are considered to be more predictive for absence of reproductive and developmental effects.

954. Equivocal results for the guideline are not considered in the table, 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 or supplemented by a different test. 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

955. Existing "mechanism" *in vitro* data are assumed to be available from ER, 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, if any. 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 (2008). These methods, however, have not yet been validated.

956. Existing "effects" data refer to *in vivo* effects that may come from lower level assays (e.g. UT or H Assays) (Level 3); Peripubertal (PP) Assays or OECD TG 407 assays (Level 4), or there may be longer term studies (e.g. in the case of pesticide registration packages where 90-day and carcinogenicity studies may be available). 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.

957. When considering the results of the OECD TG 416, 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

958. The scenarios (A to R) presented in Table C.3.14 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 rats are the preferred species for OECD TG 416, the well-conserved nature of the hormonal pathways across taxa should be a strong indication that results 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, exposure route and species-specific metabolism 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 Toxiticy 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. Further considerations specific to each scenario are given in the table.

959. Scenarios A to C represent positive results in OECD TG 416 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 416 assay is strong evidence of adverse effects on reproduction/development and/or endocrine organs via E,A,T,S mechanisms. Effects on the different endpoints may assist with interpretation. In all scenarios a robust OECD TG 416 study should provide sufficient information to conclude evidence of concern for reproductive toxicity via an endocrine disruption mechanism. If the study is not considered to be robust (for reasons given in the paragraph above), then supplemental testing could be considered. Positive results in the OECD TG 416 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.

960. Scenarios D to F represent positive results in OECD TG 416 in the presence of negative *in vitro* mechanistic data and positive, negative or equivocal *in vivo* effects data. A positive result in OECD TG 416 is strong evidence of adverse effects on reproduction/development and/or endocrine organs. Differential effects on the different endpoints may assist with interpretation. In all scenarios a robust OECD TG 416 study should provide sufficient information to conclude evidence of concern for reproductive toxicity via an endocrine disruption mechanism. If the study is not considered to be robust (for reasons given above), then supplemental testing could be considered. Positive results in the OECD TG 416 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 FSDT, LAGDA or MEOGRT if the evidence were strong enough. Negative results in the *in vitro* assays should be viewed with caution in case a metabolite is responsible for the positive OECD TG 416 study. If the metabolic profile of the test substance is not known, then performing the *in vitro* assays with addition of a metabolising system may help to understand mechanism.

961. Scenarios G to I represent positive results in OECD TG 416 in the presence of various combinations of missing or equivocal data. 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. Positive results in the OECD TG 416 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 FSDT, LAGDA or MEOGRT if the evidence were strong enough.

962. Scenarios J to L represent negative results in OECD TG 416 in the presence of positive *in vitro* mechanistic data and positive, negative or equivocal *in vivo* effects data. In all scenarios, a robust OECD TG 416 study may provide sufficient information to conclude absence of concern for reproductive toxicity via an endocrine disruption mechanism. If the study is not considered to be robust (for reasons given above), then supplemental testing could be considered. All three scenarios could fit a chemical that is positive in *in vitro* assays but is metabolised to a non-active metabolite, leading to negative results in OECD TG 416. This possibility may be investigated to help understand mechanism. Endocrine active potency may also explain differences between *in vitro* and *in vivo* results (e.g. a weak chemical may give a positive result *in vitro* but may be negative *in vivo*). Positive *in vivo* effects data may involve E,A,T,S or non-E,A,T,S mechanisms (e.g. involving other receptors or endocrine axes), more sensitive endpoints, greater statistical power but knowledge of absorption, distribution, metabolism and excretion (ADME) may help to explain differences from the OECD TG 416 data.

963. Scenarios M to O represent negative results in OECD TG 416 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 provide sufficient information to conclude absence of concern for reproductive toxicity via an endocrine disruption mechanism. If the study is not considered to be robust (for reasons given above), then supplemental testing could be considered. Positive *in vivo* effects data may involve E,A,T,S or non-E,A,T,S mechanisms (e.g. involving other receptors or endocrine axes), but knowledge of ADME may help to explain differences from the OECD TG 416 data.

964. Scenarios P to R represent negative results in OECD TG 416 in the presence of various combinations of missing or equivocal data. As with the positive result scenarios above (see Paragraph 920), the next step to take in these eventualities will have to be decided 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.

965. In all scenarios (A to R), the next step to take to strengthen weight of evidence will depend on the existing information. Table C.3.14 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.3.14. **Two-Generation Reproduction Toxicity Study (OECD TG 416): 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 effects have been observed in other *in vivo* screens/tests which give rise to concern that the test chemical may be an endocrine disrupter. These may be other repeated dose toxicity tests, the Uterotrophic Bioassay (UT) and Hershberger Bioassay (H), Peripubertal Assays or read-across from chemical analogues.

*** *Note:* a positive result is defined as a biologically significant change in any of the endocrine endpoints (all "apical endpoints").

Scenarios	Result of OECD TG 416 (two-generation study)	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	+ ***	+	+	Evidence for adverse effects via (anti)-E,A,T,S activity in OECD TG 416.		Sufficient information to conclude evidence of concern for reproductive toxicity via endocrine disruption mechanism. Note that the Extended One-Generation Reproduction Toxiticy Study (EOGRTS) provides the most information on endocrine disruption; however, for endocrine disrupting chemicals (EDCs) with a carcinogenic potential, OECD TG 451-3 may be more sensitive. Effects on apical endpoints may indicate estrogen/androgen/thyroid/steroidogenesis (E,A,T,S) modalities or other mechanisms. Consider potency of effects for existing results and whether E,A,T,S mechanism is credible for reproductive/developmental effects or whether there may be non-endocrine mechanisms. 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 Fish Sexual Development Test (FSDT), Larval Amphibian Growth and Development Assay (LAGDA) or Medaka Extended One-Generation Reproduction Test (MEOGRT).
B	+	+	–	Evidence for adverse effects via (anti)-E,A,T,S activity in OECD TG 416.		Sufficient information to conclude evidence of concern for reproductive toxicity via endocrine disruption mechanism. Note that the EOGRTS provides the most information on endocrine disruption; however, for EDCs with a carcinogenic potential, OECD TG 451-3 may be more sensitive. Effects on apical endpoints may indicate E,A,T,S modalities or other mechanisms. Consider potency of effects for existing results and whether E,A,T,S mechanism is credible for reproductive/developmental effects or whether there may be non-endocrine mechanisms. 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.
C	+	+	Eq/0	Evidence for adverse effects via (anti)-E,A,T,S activity in OECD TG 416.		Sufficient information to conclude evidence of concern for reproductive toxicity via endocrine disruption mechanism. Note that the EOGRTS provides the most information on endocrine disruption; however, for EDCs with a carcinogenic potential, OECD TG 451-3 may be more sensitive. Effects on apical endpoints may indicate E,A,T,S modalities or other mechanisms. Consider potency of effects for existing results and whether E,A,T,S mechanism is credible for reproductive/developmental effects or whether there may be non-endocrine mechanisms. 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 modes of action (MOA).

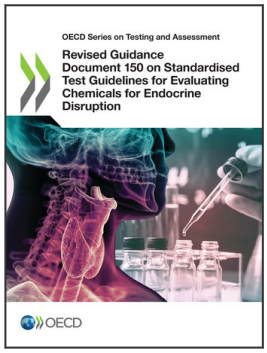
Scenarios	Result of OECD TG 416 (two-generation study)	Existing results		Possible conclusions	Next step which could be taken to strengthen weight of evidence if necessary	Other considerations
		Mechanism (*in vitro* mechanistic data)*	Effects (*in vivo* effects of concern)**			
D	+	–	+	Evidence for adverse effects in OECD TG 416 but not via E,A,T,S mechanism or may require metabolic activation for activity.	To further discern mechanism, could perform *in vitro* estrogen receptor (ER), androgen receptor (AR), thyroid hormone receptor (TR), steroidogenesis (S) assays with added metabolising system.	Sufficient information to conclude evidence of concern for reproductive toxicity via possible endocrine disruption mechanism. Note that the EOGRTS provides the most information on endocrine disruption; however, for EDCs with a carcinogenic potential, OECD TG 451-3 may be more sensitive. Effects on apical endpoints may indicate E,A,T,S modalities or other mechanisms. Consider potency of effects for existing results and whether endocrine disruption mechanism is credible for reproductive/developmental effects or whether there may be non-endocrine mechanisms. 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.
E	+	–	–	Evidence for adverse effects in OECD TG 416 via non-E,A,T,S/non-endocrine disruption mechanism or may require metabolic activation for activity.	To further discern mechanism, could perform *in vitro* ER, AR, TR, S assays with added metabolising system.	Sufficient information to conclude evidence of concern for reproductive toxicity via unknown mechanism. Note that the EOGRTS provides the most information on endocrine disruption; however, for EDCs with a carcinogenic potential, OECD TG 451-3 may be more sensitive. Effects on apical endpoints may indicate E,A,T,S modalities or other mechanisms. Consider potency of effects for existing results and whether endocrine disruption mechanism is credible for reproductive/developmental effects or whether there may be non-endocrine mechanisms. 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.
F	+	–	Eq/0	Evidence for adverse effects in OECD TG 416 via non-E,A,T,S/non-endocrine disruption mechanism or may require metabolic activation for activity.	To further discern mechanism, could perform *in vitro* ER, AR, TR, S assays with added metabolising system.	Sufficient information to conclude evidence of concern for reproductive toxicity via unknown mechanism. Note that the EOGRTS provides the most information on endocrine disruption; however, for EDCs with a carcinogenic potential, OECD TG 451-3 may be more sensitive. Effects on apical endpoints may indicate E,A,T,S modalities or other mechanisms. Consider potency of effects for existing results and whether endocrine disruption mechanism is credible for reproductive/developmental effects or whether there may be non-endocrine mechanisms. 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.

Scenarios	Result of OECD TG 416 (two-generation study)	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)**			
G	+	Eq/0	+	Evidence for adverse effects in OECD TG 416, may act via E,A,T,S mechanism and may require metabolic activation for activity.	To further discern mechanism, could perform *in vitro* ER, AR, TR, S assays with added metabolising system.	Sufficient information to conclude evidence of concern for reproductive toxicity via possible endocrine disruption mechanism. Note that the EOGRTS provides the most information on endocrine disruption; however, for EDCs with a carcinogenic potential, OECD TG 451-3 may be more sensitive. Effects on apical endpoints may indicate E,A,T,S modalities or other mechanisms. Consider potency of effects for existing results and whether endocrine disruption mechanism is credible for reproductive/developmental effects or whether there may be non-endocrine mechanisms. 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	–	Evidence for adverse effects in OECD TG 416 via non-E,A,T,S/non-endocrine disruption mechanism or may require metabolic activation for activity.	To further discern mechanism, could perform *in vitro* ER, AR, TR, S assays with added metabolising system.	Sufficient information to conclude evidence of concern for reproductive toxicity via unknown mechanism. Note that the EOGRTS provides the most information on endocrine disruption; however, for EDCs with a carcinogenic potential, OECD TG 451-3 may be more sensitive. Effects on apical endpoints may indicate E,A,T,S modalities or other mechanisms. Consider potency of effects for existing results and whether endocrine disruption mechanism is credible for reproductive/developmental effects or whether there may be non-endocrine mechanisms. 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.
I	+	Eq/0	Eq/0	Evidence for adverse effects in OECD TG 416 via unknown mechanism.	To further discern mechanism, could perform *in vitro* ER, AR, TR, S assays with added metabolising system.	Sufficient information to conclude evidence of concern for reproductive toxicity via unknown mechanism. Consider existing results and whether endocrine disruption mechanism is credible for reproductive/developmental effects or whether there may be non-endocrine mechanisms. 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.

Scenarios	Result of OECD TG 416 (two-generation study)	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 of adverse effects on reproduction/ development/endocrine organs. Effects seen in existing (lower level) studies do not lead to adverse outcome in Level 5 assay. Metabolism or potency may explain the difference from existing *in vitro* and *in vivo* data.	If test is to current OECD TG 416 standards, maybe no further testing needed. If not, consider supplemental testing, depending on existing data. To further discern mechanism, could perform *in vitro* ER, AR, TR, S assays with added metabolising system.	If existing data are from other, adequate, apical studies, question why there are differences. Note that the EOGRTS provides the most information on endocrine disruption; however, for EDCs with a carcinogenic potential, OECD TG 451-3 may be more sensitive. Consider route of exposures and possible implications for ADME characteristics of the chemical with existing studies. Further mechanistic studies with metabolism may help determine MOA.
K	–	+	–	No evidence of adverse effects on reproduction/ development/endocrine organs. Metabolism or potency may explain *in vitro*/*in vivo* differences.	If test is to current OECD TG 416 standards, maybe no further testing needed. If not, consider supplemental testing, depending on existing data. To further discern mechanism, could 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. Further mechanistic studies with metabolism may help determine MOA.
L	–	+	Eq/0	No evidence of adverse effects on reproduction/ development/endocrine organs. Metabolism or potency may explain *in vitro*/*in vivo* differences. Effects seen in existing (lower level) studies do not lead to adverse outcome in Level 5 assay.	If test is to current OECD TG 416 standards, maybe no further testing needed. If not, consider supplemental testing, depending on existing data. To further discern mechanism, could perform *in vitro* ER, AR, TR, S assays with added metabolising system.	There may be sufficient information to conclude absence of concern for endocrine disruption. Note that the EOGRTS provides the most information on endocrine disruption; however, for EDCs with a carcinogenic potential, OECD TG 451-3 may be more sensitive. Consider route of exposures and possible implications for ADME characteristics of the chemical with existing studies. Further mechanistic studies with metabolism may help determine MOA. Equivocal results may indicate chemical has multiple MOA.

Scenarios	Result of OECD TG 416 (two-generation study)	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)**			
M	–	–	+	No evidence of adverse effects on reproduction/ development/endocrine organs. Effects seen in existing (lower level) studies do not lead to adverse outcome in Level 5 assay.	If test is to current OECD TG 416 standards, maybe no further testing needed. If not, consider supplemental testing, depending on existing data.	If existing data are from adequate *in vivo* studies such as 28-day, 90-day, chronic/carcinogenicity studies, question why there are differences. Note that the EOGRTS provides the most information on endocrine disruption; however, for EDCs with a carcinogenic potential, OECD TG 451-3 may be more sensitive. Consider route of exposures and possible implications for ADME characteristics of the chemical with existing studies. Further mechanistic studies with metabolism may help determine MOA.
N	–	–	–	No evidence of adverse effects on reproduction/ development/endocrine organs.	If test is to current OECD TG 416 standards, maybe no further testing needed. If not, consider supplemental testing, depending on existing data.	If existing data are from an adequate Level 5 assay, there may be sufficient information to conclude absence of concern for endocrine disruption. Note that the EOGRTS provides the most information on endocrine disruption; however, for EDCs with a carcinogenic potential, OECD TG 451-3 may be more sensitive.
O	–	–	Eq/0	No evidence of adverse effects on reproduction/ development/endocrine organs. No evidence for (anti)-E,A,T,S activity *in vitro*.	If test is to current OECD TG 416 standards, maybe no further testing needed. If not, consider supplemental testing, depending on existing data. To further discern mechanism, could perform *in vitro* ER, AR, TR, S assays with added metabolising system.	There may be sufficient information to conclude absence of concern for endocrine disruption. Note that the EOGRTS provides the most information on endocrine disruption; however, for EDCs with a carcinogenic potential, OECD TG 451-3 may be more sensitive. Further mechanistic studies with metabolism may help determine MOA. Consider route of exposures and possible implications for ADME characteristics of the chemical with existing studies. Check data on chemical analogues.
P	–	Eq/0	+	No evidence of adverse effects on reproduction/ development/endocrine organs. Effects seen in existing (lower level) studies do not lead to adverse outcome in Level 5 assay. Effects seen in existing studies are via unknown mechanism.	If test is to current OECD TG 416 standards, maybe no further testing needed. If not, consider supplemental testing, depending on existing data. To further discern mechanism, could perform *in vitro* ER, AR, TR, S assays with added metabolising system.	If existing data are from adequate *in vivo* studies such as 28-day, 90-day, chronic/carcinogenicity studies, question why there are differences. Note that the EOGRTS provides the most information on endocrine disruption; however, for EDCs with a carcinogenic potential, OECD TG 451-3 may be more sensitive. Consider route of exposures and possible implications for ADME characteristics of the chemical with existing studies. Check data on chemical analogues. Equivocal results may indicate chemical has multiple MOA.

Scenarios	Result of OECD TG 416 (two-generation study)	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)**			
Q	–	Eq/0	–	No evidence of adverse effects on reproduction/ development/endocrine organs.	If test is to current OECD TG 416 standards, maybe no further testing needed. If not, consider supplemental testing, depending on existing data.	There may be sufficient information to conclude absence of concern for endocrine disruption. Check data on chemical analogues.
R	–	Eq/0	Eq/0	No evidence of adverse effects on reproduction/ development/endocrine organs.	If test is to current OECD TG 416 standards, maybe no further testing needed. If not, consider supplemental testing, depending on existing data.	There may be sufficient information to conclude absence of concern for endocrine disruption. Note that the EOGRTS provides the most information on endocrine disruption; however, for EDCs with a carcinogenic potential, OECD TG 451-3 may be more sensitive. Further mechanistic studies may strengthen weight of evidence. Check data on chemical analogues. Equivocal results may indicate chemical has multiple MOA.



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