C.3.15. Extended One-Generation Reproductive Toxicity Study (EOGRTS) (OECD TG 443)

Status: Assay validated by the OECD.

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

Endpoints: Time to mating, male fertility, female fertility, dystocia, gestation length, number of implantations and corpora lutea, number of ovarian follicles, number of live births and post-implantation loss, viability index, litter size, sex ratio, litter/pup weight, pup survival index, placental weight, anogenital distance, presence of nipples, pup development including genitals (and presence of abnormalities), sexual maturation (age at vaginal opening and preputial separation) (F1).

Weights and/or histopathologic analysis: uterus (with oviducts and cervix), ovaries, testes, epididymides, prostate, seminal vesicles (+ coagulating glands) thyroid, adrenals, pituitary, mammary gland (P and F1).

Estrus cyclicity (P and F1).

Sperm numbers (testicular homogenization-resistant spermatids and cauda epididymal sperm reserves), sperm motility, sperm morphology (P and F1).

Hormones: T4, TSH (P and F1).

Apical endpoints from the developmental neuro- and immunotoxicity cohorts may be sensitive to endocrine modulation:

- Developmental neurotoxicity (DNT) endpoints: Auditory startle, functional observation, motor activity. Brain weight and histopathological examination.
- Developmental immunotoxicity (DIT) endpoints: Splenic lymphocyte subpopulation analysis, lymph node weight and histopathology, primary IgM antibody response to a T cell dependant antigen.

Background to the assay

967. The Extended One-Generation Reproduction Toxicity Study is an apical assay designed to evaluate specific life stages not covered by other tests and to test for effects that may occur as a result of pre- and post-natal exposure to chemicals. It is based on the proposal of Cooper et al. (2006) and includes three possible cohorts of F1 animals:

1. to assess reproductive/developmental endpoints
2. to assess effects on the developing nervous system
3. to assess effects on the developing immune system.

968. The reproductive/developmental element of the study provides a thorough evaluation of systemic, reproductive and developmental toxicity including gonadal function, the estrus cycle, epididymal sperm maturation, mating, conception, gestation, parturition, lactation, weaning, and growth and development of the offspring. The rat is the preferred

species. The assay is designed for administration of the test substance via the diet, but it can be modified for administration by other routes (drinking water, gavage, inhalation, dermal). Depending on the modules carried out in the test, effects on the developing nervous and immune systems are also assessed. These systems may also be sensitive to endocrine influences. OECD TG 443 was adopted in July 2011 (Figure 1 was corrected in October 2012 when it was found to be incorrect). The study uses fewer animals than OECD TG 416 (Two-Generation Reproduction Toxicity Study) when F2 is omitted, whilst increasing the number of pups studied in the F1 generation and the number of endpoints. Inclusion of an F2 generation may be "triggered". Triggering in other jurisdictions may depend on results obtained in the F1 generation. OECD GD 151 (2013) supports OECD TG 443, providing advice on study design including the gathering of key data on the substance to be tested, endpoints and data interpretation issues not fully covered in the test guideline (TG). Decisions on whether to assess the second generation or omit the developmental neurotoxicity or developmental immunotoxicity have to be taken on a case-by-case basis depending on existing knowledge and regulatory purpose. As endocrine activity can affect the developmental brain/immune system, inclusion of DNT and DIT cohorts in OECD TG 443 provides a more comprehensive study. The procedure and internal triggers for deciding whether a second generation should be produced are described in OECD GD 117 (2011) for those regulations under which internal triggering applies. Criteria for the study design for the EU REACH regulation is described in ECHA (2017). As the second generation is "triggered", then at present OECD TG 416 is the only OECD mammalian test that automatically covers two generations.

969. The EOGRTS was not specifically designed to detect endocrine active substances (EASs) but Cohort 1 has many endpoints relevant for the assessment of possible endocrine disruption, for example endpoints such as sexual maturation and estrous cyclicity are particularly sensitive to estrogens and androgens. Effects on the thyroid and thyroid hormones are also detected by serum T4 and TSH levels, thyroid weight and by histopathology in P and F1 generations. The assay also provides data on adverse effects related to reproduction and development which may or may not be related to endocrine disruption. Cohorts 2 and 3 also have apical endpoints that may be sensitive to endocrine modulation. The developing brain, for example, is a classical target of thyroid hormones whilst interaction of chemicals with the hypothalamic-pituitary-adrenal axis may affect both the developing immune and nervous systems (Goel et al., 2014). Motoric activity is the only sexually dimorphic behaviour included in the mandatory investigations of the DNT Cohorts 2A and 2B. Also, sexually dimorphic behaviour can be affected by exposure to compounds disrupting the hypothalamic/pituitary/gonadal (HPG) axis (Hotchkiss et al., 2002; Schantz and Widholm, 2001; Weiss, 2002).

970. Experience with of serum hormone determinations in Level 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.

971. The EOGRTS 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 (OECD TG 416) adopted in 2001. For example, anogenital distance (AGD) and nipple retention, which are measured in all offspring in this guideline, are clear and sensitive markers of endocrine disruption (anti-androgenic action), especially when both endpoints are affected.

972. Anogenital distance and nipple retention are sensitive endpoints of endocrine effects; however, their utility as apical endpoints or as biological indicators of endocrine action may require further experience in their use. Increased nipple retention and reduced AGD in male offspring are hallmarks of anti-androgenicity. Nevertheless, "retained nipples/areolae" as a qualitative endpoint may have high biological variability (e.g. Melching-Kollmuss et al. [2017]), but nipple retention is a sensitive endpoint if measured quantitatively (i.e. if the number of nipples from 0 to 12 is recorded). Alteration of AGD can occur via other modes of action (e.g. Miyagawa et al. [2011]; Seifert et al. [2009]). However, current OECD guidance on these endpoints can be found in OECD GD 43 and GD 151 and it is clear that these should be considered as apical endpoints. With regard to anogenital distance, OECD GD 43 (OECD, 2008b) states, "A statistically significant change in [anogenital distance] that cannot be explained by the size of the animal indicates effects of the exposure and should be used for setting the [no observed adverse effect level (NOAEL)]". With regard to nipple retention OECD GD 151 (OECD, 2013) states "a statistically significant change in nipple retention should be evaluated similarly to an effect on anogenital distance as both endpoints indicate an adverse effect of exposure and should be considered in setting a NOAEL".

973. Dosing is continuous, prior to and during mating, and throughout production of the subsequent generation(s). The exposure of the fetus (which is a sensitive life stage for endocrine disruption effects), the long duration of dosing and the diversity of endpoints means that the EOGRTS may be considered to be the most predictive test for endocrine disruption mediated adverse effects via estrogen/androgen/thyroid/steroidogenesis (E,A,T,S) modalities. As most of 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 a Uterotrophic Bioassay (UT) and Hershberger Bioassay (H). Beekhuijzen et al. (2016) and Moore et al. (2016) also provide recent practical guidance on this assay from specific laboratories.

When/why the assay may be used

974. The EOGRTS has replaced OECD TG 416 in many regions. As an alternative to OECD TG 416, it may form part of the package of studies required for registration of pesticides and biocides. It has now replaced OECD TG 416 as part of the standard information requirements in certain chemical legislations (e.g. REACH [ECHA, 2017]). It may also be carried out for high production volume chemicals of high concern as well as being the most 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.

975. 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

976. Table C.3.15 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.

977. The results of the EOGRTS 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 TG. 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 therefore the endpoints have been pragmatically divided into "apical" and "indicators of hormonal activity". The terminology used has been chosen to be consistent between both the non-mammalian and mammalian tests. Both groups have similar biological importance, although the "indicators of hormonal activity" in the mammalian assays are serum hormones and are generally, but not always, more variable than "apical endpoints".

978. For this guideline "apical" endpoints are reproductive, developmental and immunological parameters, including, for example, anogenital distance, presence of nipples, genital abnormalities, sexual maturation, sperm parameters, estrous cyclicity, weights and histopathologic changes in sex organs and thyroid gland. Apical endpoints indicative of DNT and DIT are included because of their possible association with thyroid and sex hormone perturbation. "Indicators of hormonal activity" are hormones (T4, TSH).

979. Three possible outcomes for a positive result are therefore envisaged in Table C.3.15:

1. indicators of hormonal activity and apical endpoints positive
2. indicators of hormonal activity positive and apical endpoints negative
3. indicators of hormonal activity negative and apical endpoints positive.

980. A positive result for apical endpoints could be biologically significant changes in pup AGD, accompanied by treatment-related histopathologic changes in parental reproductive organs or decreased fertility. A positive result for indicators of hormonal activity could be biologically significant changes in hormone profiles. A positive result for indicators of hormonal activity alone should be considered with caution, although it is

possible that these endpoints may have detected weak effects that were not detected by the apical endpoints.

981. A negative result for the EOGRTS is taken to be the absence of changes in both endocrine-relevant indicators of hormonal activity and apical endpoints. A well-conducted study is considered to be more predictive for absence of reproductive and developmental effects and for endocrine disruptive effects mediated through E,A,T,S modalities.

982. 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

983. 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.

984. 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.

985. When considering the results of the H 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

986. A series of scenarios (A to R) are presented in Table C.3.15 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 rats are the preferred species for OECD TG 443, 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. Further considerations specific to each scenario are given in the table.

987. Scenarios A to C represent positive results in the EOGRTS in the presence of positive *in vitro* mechanistic data and positive, negative or equivocal *in vivo* effects data. Each positive EOGRTS result scenario is divided into the three possible outcomes given above. A positive result in the *in vitro* assays in combination with a positive EOGRTS is strong evidence of adverse effects on reproduction/development and/or endocrine organs via E,A,T,S mechanisms. Effects on the apical endpoints or indicators of hormonal activity may assist with interpretation. In all scenarios, a robust EOGRTS 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, then supplemental testing could be considered. Positive results in the OECD TG 443 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.

988. Scenarios D to F represent positive results in the EOGRTS in the presence of negative *in vitro* mechanistic data and positive, negative or equivocal in vivo effects data. Each positive result scenario is divided into the three possible outcomes given above. A positive result in the EOGRTS is strong evidence of adverse effects on reproduction/development and/or endocrine organs. Differential effects on the different apical endpoints or indicators of hormonal activity may assist with interpretation. In all scenarios, a robust EOGRTS 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, then supplemental testing could be considered. Negative results in the *in vitro* assays should be viewed with caution in case a metabolite is responsible for the positive extended one-generation 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. Positive results in the OECD TG 443 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.

989. Scenarios G to I represent positive results in the EOGRTS in the presence of various combinations of missing or equivocal data. Each positive result scenario is divided into the three possible outcomes given above. 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 443 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.

990. Scenarios J to L represent negative results in the EOGRTS in the presence of positive *in vitro* mechanistic data and positive, negative or equivocal *in vivo* effects data. As a negative result is taken to be negative findings for both indicators of hormonal activity and apical endpoints (unlike the situation with positive outcomes), there is only one possible negative outcome. In all scenarios, a robust EOGRTS 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, 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 the extended one-generation study. 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 or greater statistical power, but knowledge of absorption, distribution, metabolism and excretion (ADME) may help to explain differences from the EOGRTS data.

991. Scenarios M to O represent negative results in the EOGRTS 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, 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 EOGRTS data.

992. Scenarios P to R represent negative results in the EOGRTS in the presence of various combinations of missing or equivocal data. As with the positive result scenarios above (see Paragraph 990), 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.

993. In all scenarios (A to R), the next step to take to strengthen weight of evidence will depend on the existing information. Table C.3.15 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.3.15. **Extended One-Generation Reproductive Toxicity Study (EOGRTS) (OECD TG 443): 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, Uterotrophic Bioassays (UT) and Hershberger Bioassays (H), Peripubertal Assays or read-across from chemical analogues.

*** *Note:* three possible outcomes for a positive result are given:

1. indicators of hormonal activity and apical endpoints positive
2. indicators of hormonal activity positive and apical endpoints negative
3. indicators of hormonal activity negative and apical endpoints positive.

"Apical endpoints" are reproductive and developmental parameters (including anogenital distance, presence of nipples, genital abnormalities), sexual maturation, sperm parameters, estrous cyclicity, weights and histopathologic changes in testes, epididymides, prostate, seminal vesicles (with coagulating glands), ovary, uterus (with oviducts and cervix), thyroid. Apical endpoints from the developmental neuro- and immunotoxicity cohorts may also be sensitive to endocrine modulation.

"Indicators of hormonal activity" are hormones (T4, TSH).

Scenarios	Result of EOGRTS	Existing results		Possible conclusions: 1) Indicators of hormonal activity and apical endpoints positive 2) Indicators of hormonal activity positive and apical endpoints negative 3) Indicators of hormonal activity negative and apical endpoints positive	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	+ ***	+	+	1) Evidence for adverse effects via (anti)-E,A,T,S activity in OECD TG 443. 2) Evidence for adverse effects via (anti)-E,A,T,S activity in OECD TG 443. Apical endpoints may be less sensitive or unaffected. 3) Evidence for adverse effects via (anti)-E,A,T,S activity in OECD TG 443. Indicators of hormonal activity may be less sensitive or unaffected.		Sufficient information to conclude evidence of concern for reproductive toxicity via endocrine disruption mechanism. Effects on indicators of hormonal activity alone may be indicative of changes not detected by apical endpoints. Effects on apical endpoints alone 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. Hormonal activity possible in lower vertebrates. Consider performing a Fish Sexual Development Test (FSDT), Larval Amphibian Growth and Development Assay (LAGDA) or Medaka Extended One-Generation Reproduction Test (MEOGRT).
B	+	+	–	1) Evidence for adverse effects via (anti)-E,A,T,S activity in OECD TG 443. 2) Evidence for adverse effects via (anti)-E,A,T,S activity in OECD TG 443. Apical endpoints may be less sensitive or unaffected. 3) Evidence for adverse effects via (anti)-E,A,T,S activity in OECD TG 443. Indicators of hormonal activity may be less sensitive or unaffected.		Sufficient information to conclude evidence of concern for reproductive toxicity via endocrine disruption mechanism. Effects on indicators of hormonal activity alone may be indicative of changes not detected by apical endpoints. Effects on apical endpoints alone 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. Hormonal activity possible in lower vertebrates. Consider performing an FSDT, LAGDA or MEOGRT.

Scenarios	Result of EOGRTS	Existing results		Possible conclusions: 1) Indicators of hormonal activity and apical endpoints positive 2) Indicators of hormonal activity positive and apical endpoints negative 3) Indicators of hormonal activity negative and apical endpoints positive	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)**			
C	+	+	Eq/0	1) Evidence for adverse effects via (anti)-E,A,T,S activity in OECD TG 443. 2) Evidence for adverse effects via (anti)-E,A,T,S activity in OECD TG 443. Apical endpoints may be less sensitive or unaffected. 3) Evidence for adverse effects via (anti)-E,A,T,S activity in OECD TG 443. Indicators of hormonal activity may be less sensitive or unaffected.		Sufficient information to conclude evidence of concern for reproductive toxicity via endocrine disruption mechanism. Effects on indicators of hormonal activity alone may be indicative of changes not detected by apical endpoints. Effects on apical endpoints alone 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. Hormonal activity possible in lower vertebrates. Consider performing an FSDT, LAGDA or MEOGRT. Equivocal results may indicate chemical has multiple modes of action (MOA).
D	+	–	+	1) Evidence for adverse effects in OECD TG 443 but not via E,A,T,S mechanism or requires metabolic activation for activity. 2) Evidence for adverse effects in OECD TG 443 but not via E,A,T,S mechanism or may require metabolic activation for activity. Apical endpoints may be less sensitive or unaffected. 3) Evidence for adverse effects in OECD TG 443 but not via E,A,T,S mechanism or requires metabolic activation for activity. Indicators of hormonal activity may be less sensitive or unaffected.	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. Effects on indicators of hormonal activity alone may be indicative of changes not detected by apical endpoints. Effects on apical endpoints alone 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. Hormonal activity possible in lower vertebrates. Consider performing an FSDT, LAGDA or MEOGRT.

Scenarios	Result of EOGRTS	Existing results		Possible conclusions: 1) Indicators of hormonal activity and apical endpoints positive 2) Indicators of hormonal activity positive and apical endpoints negative 3) Indicators of hormonal activity negative and apical endpoints positive	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)**			
E	+	–	–	1) Evidence for adverse effects in OECD TG 443 via non-E,A,T,S/ non-endocrine disruption mechanism or may require metabolic activation for activity. 2) Evidence for adverse effects in OECD TG 443 via non-E,A,T,S/ non-endocrine disruption mechanism or may require metabolic activation for activity. Apical endpoints may be less sensitive or unaffected. 3 Evidence for adverse effects in OECD TG 443 via non-E,A,T,S/ non-endocrine disruption mechanism or may require metabolic activation for activity. Indicators of hormonal activity may be less sensitive or unaffected.	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. Effects on indicators of hormonal activity alone may be indicative of changes not detected by apical endpoints. Effects on apical endpoints alone 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. Hormonal activity possible in lower vertebrates. Consider performing an FSDT, LAGDA or MEOGRT.
F	+	–	Eq/0	1) Evidence for adverse effects in OECD TG 443 via non-E,A,T,S/ non-endocrine disruption mechanism or may require metabolic activation for activity. 2) Evidence for adverse effects in OECD TG 443 via non-E,A,T,S/ non-endocrine disruption mechanism or may require metabolic activation for activity. Apical endpoints may be less sensitive or unaffected. 3) Evidence for adverse effects in OECD TG 443 via non-E,A,T,S/ non-endocrine disruption mechanism or may requirs metabolic activation for activity. Indicators of hormonal activity may be less sensitive or unaffected.	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. Effects on indicators of hormonal activity alone may be indicative of changes not detected by apical endpoints. Effects on apical endpoints alone 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. Hormonal activity possible in lower vertebrates. Consider performing an FSDT, LAGDA or MEOGRT. Equivocal results may indicate chemical has multiple MOA.

Scenarios	Result of EOGRTS	Existing results		Possible conclusions: 1) Indicators of hormonal activity and apical endpoints positive 2) Indicators of hormonal activity positive and apical endpoints negative 3) Indicators of hormonal activity negative and apical endpoints positive	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	+	1) Evidence for adverse effects in OECD TG 443, may act via E,A,T,S mechanism and may require metabolic activation for activity. 2) Evidence for adverse effects in OECD TG 443, may act via E,A,T,S mechanism and may require metabolic activation for activity. Apical endpoints may be less sensitive or unaffected. 3) Evidence for adverse effects in OECD TG 443, may act via E,A,T,S mechanism and may require metabolic activation for activity. Indicators of hormonal activity may be less sensitive or unaffected.	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. Effects on indicators of hormonal activity alone may be indicative of changes not detected by apical endpoints. Effects on apical endpoints alone 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. Hormonal activity possible in lower vertebrates. Consider performing an FSDT, LAGDA or MEOGRT. Equivocal results may indicate chemical has multiple MOA.
H	+	Eq/0	–	1) Evidence for adverse effects in OECD TG 443 via non-E,A,T,S/ non-endocrine disruption mechanism or requires metabolic activation for activity. 2) Evidence for adverse effects in OECD TG 443 via non-E,A,T,S/ non-endocrine disruption mechanism or may require metabolic activation for activity. Apical endpoints may be less sensitive or unaffected. 3 Evidence for adverse effects in OECD TG 443 via non-E,A,T,S/ non-endocrine disruption mechanism or may require metabolic activation for activity. Indicators of hormonal activity may be less sensitive or unaffected.	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. Effects on indicators of hormonal activity alone may be indicative of changes not detected by apical endpoints. Effects on apical endpoints alone 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. Hormonal activity possible in lower vertebrates. Consider performing an FSDT, LAGDA or MEOGRT. Equivocal results may indicate chemical has multiple MOA.

Scenarios	Result of EOGRTS	Existing results		Possible conclusions: 1) Indicators of hormonal activity and apical endpoints positive 2) Indicators of hormonal activity positive and apical endpoints negative 3) Indicators of hormonal activity negative and apical endpoints positive	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)**			
I	+	Eq/0	Eq/0	1) Evidence for adverse effects in OECD TG 443 via unknown mechanism. 2) Evidence for adverse effects in OECD TG 443 via unknown mechanism. Apical endpoints may be less sensitive or unaffected. 3) Evidence for adverse effects in OECD TG 443 via unknown mechanism. Indicators of hormonal activity may be less sensitive or unaffected.	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. Effects on indicators of hormonal activity alone may be indicative of changes not detected by apical endpoints. 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. Hormonal activity possible in lower vertebrates. Consider performing an FSDT, LAGDA or MEOGRT. Equivocal results may indicate chemical has multiple MOA.
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 robust, 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. If existing data are from other, adequate, apical studies, question why there are differences. 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 robust, 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.

Scenarios	Result of EOGRTS	Existing results		Possible conclusions: 1) Indicators of hormonal activity and apical endpoints positive 2) Indicators of hormonal activity positive and apical endpoints negative 3) Indicators of hormonal activity negative and apical endpoints positive	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)**			
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 robust, 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. 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.
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 robust, 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. 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 robust, 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.
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 robust, 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. 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.

Scenarios	Result of EOGRTS	Existing results		Possible conclusions: 1) Indicators of hormonal activity and apical endpoints positive 2) Indicators of hormonal activity positive and apical endpoints negative 3) Indicators of hormonal activity negative and apical endpoints positive	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 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 robust, 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. 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. Equivocal results may indicate chemical has multiple MOA.
Q	–	Eq/0	–	No evidence of adverse effects on reproduction/development/endocrine organs.	If test is robust, 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 robust, 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. Further mechanistic studies may strengthen weight of evidence. Check data on chemical analogues. Equivocal results may indicate chemical has multiple MOA.