C.3.7. Prenatal Developmental Toxicity Study (OECD TG 414)

Status: Assay validated by the OECD.

793. Modalities detected: (anti)estrogen, (anti)androgen, thyroid.

794. Endpoints: Number of implantations and corpora lutea; post-implantation loss, litter size, sex ratio, litter/fetal weight; fetal anogenital distance; fetal external, soft tissue and skeletal changes. Observations of external fetal sex (determined by gross examination) and internal (gonadal sex) in all fetuses. Indications of incomplete testicular descent/cryptorchidism in male fetuses.

Hormones: T4, T3 and TSH in dams. Other hormones "if relevant".

Background to the assay

795. The OECD Prenatal Developmental Toxicity Study is an apical assay designed to provide general information concerning the effects of prenatal exposure to a chemical on the pregnant test animal and on the developing organism. This may include assessment of maternal effects as well as death, structural abnormalities or altered growth in the foetus. The primary purpose of this study is to provide data on adverse effects related to development. The current version of the guideline was adopted in January 2001. Previous versions of this test guideline (TG) had a less extensive exposure period and fewer endpoints. The study was not designed to detect endocrine active substances (EASs), but has some endpoints relevant for the assessment of possible endocrine disruption and is currently being updated to include more. Following a feasibility study (OECD, 2015), this assay was updated in July 2018 to include some endocrine-relevant endpoints as the exposure periods cover some of the sensitive periods during development (prenatal period). It should be noted that if an assay was conducted before 2018, it is unlikely to include these extra endpoints.

796. Test substance is administered from implantation throughout pregnancy. The rat is the preferred rodent species and the rabbit the preferred non-rodent species. Route of administration is typically via oral gavage, but other routes may be used. The exposure of the fetus (which may be a sensitive life stage for endocrine disruption effects) means that some endocrine effects on development may be detected in this assay. Anogenital distance (AGD), appearance of external genitalia and sex ratio are examples of apical endpoints that may be affected by via estrogen- or androgen-mediated activity. T4 and TSH may be affected by disturbance of the thyroid hormonal system. Most endpoints are apical and therefore it may be difficult to discern mode of action (MOA) from this test alone. Information on potential endocrine mode of action may need to be obtained from *in vitro* estrogen/androgen/thyroid/steroidogenesis (E,A,T,S) assays or *in vivo* lower tier tests such as a Uterotrophic Bioassay (UT) and a Hershberger Bioassay (H), for example. The small number of endocrine-sensitive endpoints means that an absence of effect in this assay alone cannot lead to a conclusion that a substance does not have endocrine disrupting effects. Additional data from more comprehensive assays may be required.

797. AGD is included in OECD TG 414 as a sensitive endpoint of endocrine effects; however, its utility as an apical endpoint or as a biological indicator of endocrine action may require further experience in its use. Reduced anogenital distance in male offspring is a hallmark of anti-androgenicity. Nevertheless, alteration of AGD can occur via other MOA (e.g. Miyagawa et al. [2011]; Seifert et al. [2009]). However current OECD guidance on AGD can be found in OECD GD 43 and GD 151 and it is clear that it should be considered as an apical endpoint. 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]".

Thyroid hormone analysis and interpretation in OECD TG 414

798. The revised TG 414 requires the determination of serum thyroid hormones in dams. Detection/measurement of thyroid hormones (T4, TSH) in some rodent studies can be challenging. In order to provide assurance that thyroid hormone measurements are reliable, laboratories should be able to demonstrate that they are proficient with the assay. Only such a demonstration would enable regulatory authorities to interpret and use the data. 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

799. This assay forms part of the package of studies required for registration of pesticides in many jurisdictions. It forms part of the standard information requirements in certain chemical legislations (e.g. REACH for chemicals which are manufactured or imported in quantities of 1 000 tonnes or more). It may also be carried out for high production volume chemicals of high concern. It is likely to have at least three dose levels and therefore may be used for hazard identification/characterisation.

800. 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 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

801. Table C.3.7 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.

802. The results of OECD TG 414 are given in the second column. This assay is not a screening test where a yes/no (qualitative) answer is obtained. Results for the endpoints would be considered both individually and as a whole. It is not possible to provide guidance on all endpoints individually, 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 wildlife and mammalian tests. Both groups of endpoints 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".

803. For this guideline "apical" endpoints are developmental parameters (including anogenital distance, genital abnormalities and sex ratio). "Indicators of hormonal activity" are hormones (including T4, TSH).

804. Three possible outcomes for a positive result are therefore envisaged in Table C.3.7:

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.

805. A positive result for apical endpoints could be biologically significant changes in fetal anogenital distance, accompanied changes in sex ratio. 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.

806. A negative result for OECD TG 414 is taken to be the absence of biologically significant changes in all of the endocrine endpoints measured in this study. Studies conducted to current standards are considered to be more predictive for absence of reproductive and developmental effects.

807. 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 may be required as confirmation.

808. Equivocal results for the guideline are not considered in Table C.3.7, 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.

Existing data to be considered

809. 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.

810. 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.

811. When considering the results of OECD TG 414, 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

812. The scenarios (A to R) presented in Table C.3.7 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 and rabbits are the preferred species for OECD TG 414, the well-conserved nature of the hormonal pathways across taxa should be an 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 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. Further considerations specific to each scenario are given in the table.

813. Scenarios A to C represent positive results in OECD TG 414 in the presence of positive *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 *in vitro* assays in combination with a positive OECD TG 414 assay is evidence of adverse effects on development and/or endocrine endpoints via E,A,T,S mechanisms. Effects on only apical endpoints or only indicators of hormonal activity may assist with interpretation. 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 414 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), Larval Amphibian Growth and Development Assay (LAGDA) or Medaka Extended One-Generation Reproduction Test (MEOGRT) if the evidence is 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).

814. Scenarios D to F represent positive results in OECD TG 414 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 OECD TG 414 is evidence of adverse effects on development. Effects on only apical endpoints or only indicators of hormonal activity may assist with interpretation. Negative results in the *in vitro* assays should be viewed with caution in case a metabolite is responsible for the positive OECD TG 414 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. The choice of tests will depend on the available *in vivo* effects data. Positive results in the OECD TG 414 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.

815. Scenarios G to I represent positive results in OECD TG 414 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 MOA in question and why the data are considered equivocal. In all three scenarios, the recommended first step is to obtain reliable mechanistic (*in vitro*) data rather than proceed further with *in vivo* testing. Positive results in the OECD TG 414 assay may also indicate the potential for endocrine mediated effects in lower vertebrates. 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.

816. Scenarios J to L represent negative results in OECD TG 414 in the presence of positive *in vitro* mechanistic data and positive, negative or equivocal *in vivo* effects data. A negative result is taken to be negative findings for both indicators of hormonal activity and apical endpoints (unlike the situation with positive outcomes), therefore there is only

one possible negative outcome. In all scenarios, the small number of endocrine-sensitive endpoints in OECD TG 414 means that an absence of effect in this assay alone cannot lead to a conclusion that a substance does not have endocrine disrupting effects. Additional data from more comprehensive assays are required. 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 414. 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 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 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 414 data.

817. Scenarios M to O represent negative results in OECD TG 414 in the presence of negative *in vitro* mechanistic data and positive, negative or equivocal *in vivo* effects data. In all scenarios, the small number of endocrine-sensitive endpoints in OECD TG 414 means that an absence of effect in this assay alone cannot lead to a conclusion that a substance does not have endocrine disrupting effects. 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 414 data.

818. Scenarios P to R represent negative results in OECD TG 414 in the presence of various combinations of missing or equivocal data. As with the positive result scenarios above (see Paragraph 787), 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.

819. In all scenarios (A to R), the next step to take to strengthen weight of evidence will depend on the existing information. Table C.3.6 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.

References

Jacobs, M. et al. (2013), "*In vitro* metabolism and bioavailability tests for endocrine active substances: What is needed next for regulatory purposes?", *ALTEX – Alternatives to Animal Experimentation*, Vol. 30/3, pp. 331-351.

Jacobs, M.N. et al. (2008), "The use of metabolising systems for *in vitro* testing of endocrine disrupters", *Current Drug Metabolism*, Vol. 9/8, pp. 796-826.

Kucheryavenko, O. et al. (2018), "Report from the BfR Expert Hearing on Practicability of Hormonal Measurements", *Archives of Toxicology* (in prep.).

Miyagawa, S. et al. (2011), "The role of sonic hedgehog-Gli2 pathway in the masculinization of external genitalia", *Endocrinology*, Vol. 152/7, pp. 2894-2903, https://doi.org/10.1210/en.2011-0263.

OECD (2015), "Feasibility study for minor enhancements of TG 421/422 with ED relevant endpoints", Series on Testing and Assessment, No. 217, OECD, Paris, www.oecd.org/officialdocuments/publicdisplaydocumentpdf/?cote=env/jm/mono(2015)24&doclanguage=en.

OECD (2008a), *Detailed Review Paper on the Use of Metabolising Systems for* In Vitro *Testing of Endocrine Disrupters*, OECD Series on Testing and Assessment, No. 97, OECD Publishing, Paris, https://doi.org/10.1787/9789264085497-en.

OECD (2008b), "Guidance document on mammalian reproductive toxicity testing and assessment", OECD Series on Testing and Assessment, No. 43, OECD, Paris, www.oecd.org/officialdocuments/publicdisplaydocumentpdf/?cote=ENV/JM/MONO(2008)16&doclanguage=en.

Seifert, A.W. et al. (2009), "Multiphasic and tissue-specific roles of sonic hedgehog in cloacal septation and external genitalia development", *Development*, Vol. 136/23, pp. 3949-3957, http://dx.doi.org/10.1242/dev.042291.

WHO/IPCS (2002), "Global assessment of the state-of-the-science of endocrine disrupters", Damstra, T. et al. (eds.) WHO/PCS/EDC/02.2, World Health Organization, Geneva, www.who.int/ipcs/publications/new_issues/endocrine_disruptors/en.

Table C.3.7. **Prenatal Developmental Toxicity Study (OECD TG 414): 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 (PP) 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 developmental parameters (number of implantations and corpora lutea; post implantation loss, litter size, sex ratio, litter/fetal weight; fetal anogenital distance; fetal external, soft tissue and skeletal changes).

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

Scenarios	Result of OECD TG 414 (dev tox)	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 of adverse effects on endocrine/ apical endpoints via estrogen/androgen/ thyroid/steroidogenesis (E,A,T,S) mechanism. 2) Evidence of effects on hormonal endpoints via E,A,T,S mechanism. Apical endpoints may be less sensitive or unaffected. 3) Evidence of adverse effects via E,A,T,S mechanism. Indicators of hormonal activity may be less sensitive or unaffected.	Perform assay from Level 5 (e.g. EOGRTS or two-generation assay).	May be sufficient information to conclude evidence of concern for deveopmental toxicity via endocrine disruption mechanism. Note that the EOGRTS rovides the most information on endocrine disruption. 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 absorption, distribution, metabolism and excretion (ADME) characteristics of the chemical. Endocrine 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 of adverse effects on endocrine/ apical endpoints via E,A,T,S mechanism. 2) Evidence of effects on hormonal endpoints via E,A,T,S mechanism. Apical endpoints may be less sensitive or unaffected. 3) Evidence of adverse effects via E,A,T,S mechanism. Indicators of hormonal activity may be less sensitive or unaffected.	Perform assay from Level 5 (e.g. EOGRTS or two-generation assay).	Note that the 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 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.

Scenarios	Result of OECD TG 414 (Dev tox)	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 of adverse effects on endocrine/ apical endpoints via E,A,T,S mechanism. 2) Evidence of effects on hormonal endpoints via E,A,T,S mechanism. Apical endpoints may be less sensitive or unaffected. 3) Evidence of adverse effects via E,A,T,S mechanism. Indicators of hormonal activity may be less sensitive or unaffected.	Perform assay from Level 5 (e.g. EOGRTS or two-generation assay).	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. Equivocal results may indicate chemical has multiple modes of action (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.
D	+	–	+	1) Evidence of adverse effects on endocrine/apical endpoints but not via E,A,T,S mechanism or requires metabolic activation for activity. 2) Evidence of effects on hormonal endpoints, possibly requires metabolic activation for activity. Apical endpoints may be less sensitive or unaffected. 3) Evidence of adverse effects on apical endpoints but not via E,A,T,S mechanism or requires metabolic activation for activity. Indicators of hormonal activity may be less sensitive or unaffected.	Perform assay from Level 5 (e.g. EOGRTS or two-generation assay). 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.	May be 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 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.

Scenarios	Result of OECD TG 414 (Dev tox)	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 of adverse effects on apical endpoints but not via E,A,T,S mechanism or requires metabolic activation for activity. 2) Evidence of effects on hormonal endpoints, possibly requires metabolic activation for activity. Apical endpoints may be less sensitive or unaffected. 3) Evidence of adverse effects on apical endpoints but not via E,A,T,S mechanism or requires metabolic activation for activity. Indicators of hormonal activity may be less sensitive or unaffected.	Perform assay from Level 5 (e.g. EOGRTS or two-generation assay). To further discern mechanism, could perform *in vitro* ER, AR, TR, S assays with added metabolising system.	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	1) Evidence of adverse effects on apical endpoints via non-E,A,T,S/non-endocrine mechanism or requires metabolic activation for activity. 2) Evidence of effects on hormonal endpoints, possibly requires metabolic activation for activity. Apical endpoints may be less sensitive or unaffected. 3) Evidence of adverse effects on apical endpoints, via non-E,A,T,S/non-endocrine disruption mechanism or requires metabolic activation for activity. Indicators of hormonal activity may be less sensitive or unaffected.	Perform assay from Level 5 (e.g. EOGRTS or two-generation assay). To further discern mechanism, could perform *in vitro* ER, AR, TR, S assays with added metabolising system.	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. Equivocal results may indicate chemical has multiple MOA. 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.

Scenarios	Result of OECD TG 414 (Dev tox)	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 of adverse effects on apical endpoints, may act via E,A,T,S mechanism and may require metabolic activation for activity. 2) Evidence of effects on hormonal endpoints, 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 of adverse effects on apical endpoints, 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.	Perform assay from Level 5 (e.g. EOGRTS or two-generation assay). To further discern mechanism, could perform *in vitro* ER, AR, TR, S assays with added metabolising system.	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. Equivocal results may indicate chemical has multiple MOA. 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
H	+	Eq/0	–	1) Evidence of adverse effects on apical endpoints via non-E,A,T,S/non-endocrine disruption mechanism or requires metabolic activation for activity. 2) Evidence of effects on hormonal endpoints, possibly requires metabolic activation for activity. 3) Evidence of adverse effects on apical endpoints via non-E,A,T,S/non-endocrine disruption mechanism or requires metabolic activation for activity. Indicators of hormonal activity may be less sensitive or unaffected.	Perform assay from Level 5 (e.g. EOGRTS or two-generation assay). To further discern mechanism, could perform *in vitro* ER, AR, TR, S assays with added metabolising system.	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
I	+	Eq/0	Eq/0	1) Evidence of adverse effects on apical endpoints via unknown mechanism. 2) Evidence of effects on hormonal endpoints via unknown mechanism. Apical endpoints may be less sensitive or unaffected. 3) Evidence of adverse effects on apical endpoints via unknown mechanism. Hormonal endpoints may be less sensitive or unaffected.	Perform assay from Level 5 (e.g. EOGRTS or two-generation assay). To further discern mechanism, could perform *in vitro* ER, AR, TR, S assays with added metabolising system.	Consider existing results and whether endocrine disruption mechanism is credible for reproductive/developmental effects or whether there may be non-endocrine mechanisms. Equivocal results may indicate chemical has multiple 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.

Scenarios	Result of OECD TG 414 (Dev tox)	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)**			
J	–	+	+	No evidence of adverse effects in OECD TG 414. Effects seen in existing (lower level) studies do not lead to adverse outcome. Metabolism or potency explains the difference from existing *in vitro* and *in vivo* data.	Consider supplemental testing, depending on existing data. To further discern mechanism, could perform *in vitro* ER, AR, TR, S assays with added metabolising system.	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 in OECD TG 414. Metabolism or potency explains *in vitro*/*in vivo* differences.	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 in OECD TG 414. Metabolism or potency explains *in vitro*/*in vivo* differences. Effects seen in existing (lower level) studies do not lead to adverse outcome.	Consider supplemental testing, depending on existing data. To further discern mechanism, could perform *in vitro* ER, AR, TR, S assays with added metabolising system.	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.
M	–	–	+	No evidence of adverse effects in OECD TG 414. Effects seen in existing (lower level) studies do not lead to adverse outcome.	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. Further mechanistic studies with metabolism may help determine MOA.
N	–	–	–	No evidence of adverse effects in OECD TG 414.	Consider existing data, there may be no need for further testing.	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.

Scenarios	Result of OECD TG 414 (Dev tox)	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)**			
O	–	–	Eq/0	No evidence of adverse effects in OECD TG 414. No evidence for (anti)-E,A,T,S activity *in vitro*.	Consider existing data, there may be no need for further testing. To further discern mechanism, could perform *in vitro* ER, AR, TR, S assays with added metabolising system.	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 in OECD TG 414. Effects seen in existing (lower level) studies do not lead to adverse outcome. Effects seen in existing studies are via unknown mechanism.	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.
Q	–	Eq/0	–	No evidence of adverse effects in OECD TG 414.	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. Check data on chemical analogues.
R	–	Eq/0	Eq/0	No evidence of adverse effects in OECD TG 414.	To further discern mechanism, could perform *in vitro* ER, AR, TR, S assays with added metabolising system.	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.