C.3.8. Developmental Neurotoxicity Study (OECD TG 426)

Status: Assay validated by the OECD.

820. Modalities detected: (Anti)-eEstrogen, (anti)androgen, thyroid, steroidogenesis.

Endpoints: Gestation length, litter size, sex ratio (F1, F2), litter/pup weight, pup survival index, sexual maturation (age at vaginal opening and preputial separation).

In offspring: motor activity (including habituation), motor and sensory function, learning and memory.

Brain weight and histopathological examination. Morphometric (quantitative) evaluation of the brain.

Other (non-neurological) tissues may be taken at post-mortem on a case-by-case basis.

Background to the assay

821. OECD TG 426 determines the potential for developmental neurotoxicity of chemicals. The assay provides data, including dose-response characterisations, on the potential functional and morphological effects on the developing nervous system of the offspring that may arise from exposure *in utero* and during early life. Developmental neurotoxicity cohorts may also be added to other OECD studies, and is included in the Extended One-Generation Reproductive Toxicity Study (EOGRTS – OECD TG 443). In OECD TG 426, test substance is administered to animals during gestation and lactation (PND 21). It may extend post-weaning in young adulthood (PND 60-70). Dams are tested to assess effects in pregnant and lactating females and may also provide comparative information (dams versus offspring). Offspring are tested during postnatal development and adulthood for gross neurologic and behavioural abnormalities, physical development, behavioural ontogeny, motor activity, motor and sensory function, learning and memory, brain weights, and neuropathology. The preferred species is the rat. The recommended route of administration is oral, by gavage, via the diet or in drinking water. The study is not specifically designed to detect endocrine active substances (EASs), but has endpoints relevant for the assessment of possible endocrine disruption. Some endocrine disruptors (EDs) have been shown to cause developmental neurotoxicity and therefore this assay should detect such effects. Disturbance of the thyroid hormonal system, particularly reduction of thyroid hormones in the fetus, has been shown to cause developmental neurotoxicity (Crofton, 2008; Zoeller, 2010). The exposure of the fetus (which may be a sensitive life stage for endocrine disruption effects) and the duration of dosing makes it an assay that can be used when assessing effects relevant to endocrine disruption. In addition, it provides data on effects related to reproduction and development, in particular the endocrine-sensitive endpoint of sexual maturation.

822. OECD TG 426 was adopted in October 2007 following an expert consultation on neurotoxicity (OECD, 2003a). Additional information on the conduct and interpretation of this test guideline (TG) can be found in Guidance Document (GD) No. 43 on

"Reproductive Toxicity Testing and Assessment" (OECD, 2008b) and GD 20 on "Neurotoxicity Testing" (OECD, 2003b). As all the endpoints are apical, it is difficult to discern mechanism of action from this test alone. Information on mechanism of action needs to be obtained from *in vitro* estrogen/androgen/thyroid/steroidogenesis (E,A,T,S) assays or *in vivo* lower tier tests such as the Uterotrophic Bioassay (UT – OECD TG 440) and the Hershberger Bioassay (H – OECD TG 441).

When/why the assay may be used

823. This assay may form part of the package of studies required for registration of pesticides in some jurisdictions. It is likely to be conducted following a concern for neurotoxicity. It will have at least three dose levels and therefore may be used for hazard identification/characterisation.

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

825. Table C.3.8 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.

826. The results of OECD TG 426 are given in the second column. As this assay is not a screening test where a yes/no (qualitative) answer is obtained, criteria for positive results for the endocrine endpoints are not given in the test guideline. Results for the endpoints would be considered both individually and as a whole. It is not possible to provide guidance on all endpoints individually and for this test all endpoints are considered to be "apical".

827. For the purpose of this guidance, a positive result is defined as a biologically significant change in any of the endocrine endpoints (e.g. a significant alteration in vaginal opening in the absence of body weight changes).

828. A negative result for OECD TG 426 is taken to be the absence of biologically significant changes in all of the endocrine endpoints.

829. 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.

Existing data to be considered

830. 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.

831. 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.

832. When considering the results of OECD TG 426, 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

833. The scenarios (A to R) presented in Table C.3.8 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 426, 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 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.

834. Scenarios A to C represent positive results in OECD TG 426 in the presence of positive *in vitro* mechanistic data and positive, negative or equivocal *in vivo* effects data. A positive result in the *in vitro* assays in combination with a positive OECD TG 426 assay is evidence of adverse effects on reproduction/development (including neurodevelopment) via E,A,T,S mechanisms. 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 426 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 Larval Amphibian Growth and Development Assay (LAGDA) or the 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).

835. Scenarios D to F represent positive results in OECD TG 426 in the presence of negative *in vitro* mechanistic data and positive, negative or equivocal *in vivo* effects data. A positive result in OECD TG 426 is evidence of adverse effects on reproduction/development (including neurodevelopment). Differential effects on the different endpoints 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 416 study. If the metabolic profile of the test substance is not known, performing the *in vitro* assays with addition of a metabolising system may help to understand mechanism. Positive results in the OECD TG 426 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.

836. Scenarios G to I represent positive results in OECD TG 426 in the presence of various combinations of missing or equivocal data. Positive results in the OECD TG 426 assay may also indicate the potential for endocrine mediated effects in lower vertebrates. The next step to take in these eventualities will depend on the nature of the other available data and the jurisdiction in which it is being used. In some cases, equivocal data may be viewed as positive whilst in others it may or may not contribute to the weight of evidence. The interpretation may also depend on the mode of action (MOA) in question and why the data are considered equivocal, e.g. a study that is equivocal for thyroid effects may still be of value in evaluating (anti)androgenic effects. In all three scenarios, the recommended first step is to obtain reliable mechanistic (*in vitro*) data rather than proceed further with *in vivo* testing. Equivocal and missing data are alternative scenarios and two possibilities for the next step are given in most cases, but the nature of equivocal data means that decisions need to be taken on a case-by-case basis. In all cases, the role of metabolism, route of exposure and data from structural analogues should be considered before deciding on the next step.

837. Scenarios J to L represent negative results in OECD TG 426 in the presence of positive *in vitro* mechanistic data and positive, negative or equivocal *in vivo* effects data. In all scenarios, the small number of (non-neuro) endocrine-sensitive endpoints in OECD TG 426 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 426. 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 426 data.

838. Scenarios M to O represent negative results in OECD TG 426 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 426 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 426 data.

839. Scenarios P to R represent negative results in OECD TG 426 in the presence of various combinations of missing or equivocal data. As with the positive result scenarios above, the next step to take in these eventualities will have to be decided on a case-by-case basis. In all cases, the role of metabolism, route of exposure and data from structural analogues should be considered before deciding on the next step.

840. In all scenarios (A to R), the next step to take to strengthen weight of evidence will depend on the existing information. Table C.3.8 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.3.8. **Developmental Neurotoxicity Study (OECD TG 426): 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, Peripubertal (PP) Assays, or read-across from chemical analogues.

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

Scenarios	Result of OECD TG 426 (DNT study)	Existing results		Possible conclusions	Next step which could be taken to strengthen weight of evidence if necessary	Other considerations
		Mechanism (*in vitro* mechanistic data)*	Effects (*in vivo* effects of concern)**			
A	+ ***	+	+	Evidence for adverse effects on (neuro-) development in OECD TG 426, possibly via (anti)-E,A,T,S activity.	Further testing may not be required. May perform assay from Level 5 (e.g. EOGRTS or two-generation assay) if needed.	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 absorption, distribution, metabolism and excretion (ADME) characteristics of the chemical. Endocrine activity possible in lower vertebrates. Consider performing a Larval Amphibian Growth and Development Assay (LAGDA) or Medaka Extended One-Generation Reproduction Test (MEOGRT).
B	+	+	–	Evidence for adverse effects on (neuro-) development in OECD TG 426, possibly via (anti)-E,A,T,S activity.	Further testing may not be required. May perform assay from Level 5 (e.g. EOGRTS or two-generation assay) if needed.	Note that the EOGRTS provides the most information on endocrine disruption; however, for EDCs with a carcinogenic potential, OECD TG 451-3 may be more sensitive. Effects on apical endpoints may indicate E,A,T,S modalities or other mechanisms. Consider potency of effects for existing results and whether E,A,T,S mechanism is credible for reproductive/developmental effects or whether there may be non-endocrine mechanisms. Consider route of exposures for effects data and possible implications of ADME characteristics of the chemical. Hormonal activity possible in lower vertebrates. Consider performing a LAGDA or MEOGRT.
C	+	+	Eq/0	Evidence for adverse effects on (neuro-) development in OECD TG 426, possibly via (anti)-E,A,T,S activity.	Further testing may not be required. May perform assay from Level 5 (e.g. EOGRTS or two-generation assay) if needed.	Note that the EOGRTS provides the most information on endocrine disruption; however, for EDCs with a carcinogenic potential, OECD TG 451-3 may be more sensitive. Effects on apical endpoints may indicate E,A,T,S modalities or other mechanisms. Consider potency of effects for existing results and whether E,A,T,S mechanism is credible for reproductive/developmental effects or whether there may be non-endocrine mechanisms. Consider route of exposures for effects data and possible implications of ADME characteristics of the chemical. Hormonal activity possible in lower vertebrates. Consider performing a LAGDA or MEOGRT. Equivocal results may indicate chemical has multiple modes of action (MOA).

Scenarios	Result of OECD TG 426 (DNT study)	Existing results		Possible conclusions	Next step which could be taken to strengthen weight of evidence if necessary	Other considerations
		Mechanism (*in vitro* mechanistic data)*	Effects (*in vivo* effects of concern)**			
D	+	–	+	Evidence for adverse effects on (neuro-) development in OECD TG 426, but not via E,A,T,S mechanism or requires metabolic activation for activity.	Further testing may not be required. 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.	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. Hormonal activity possible in lower vertebrates. Consider performing a LAGDA or MEOGRT.
E	+	–	–	Evidence for adverse effects on (neuro-) development in OECD TG 426 via non-E,A,T,S/non-endocrine disruption mechanism or requires metabolic activation for activity.	Further testing may not be required. To further discern mechanism, could perform *in vitro* ER, AR, TR, S assays with added metabolising system.	Sufficient information to conclude evidence of concern for reproductive toxicity via unknown mechanism. Note that the EOGRTS provides the most information on endocrine disruption; however, for EDCs with a carcinogenic potential, OECD TG 451-3 may be more sensitive. Effects on apical endpoints may indicate E,A,T,S modalities or other mechanisms. Consider potency of effects for existing results and whether endocrine disruption mechanism is credible for reproductive/developmental effects or whether there may be non-endocrine mechanisms. Consider route of exposures for effects data and possible implications of ADME characteristics of the chemical. Hormonal activity possible in lower vertebrates. Consider performing a LAGDA or MEOGRT.
F	+	–	Eq/0	Evidence for adverse effects on (neuro-) development in OECD TG 426 via non-E,A,T,S/non-endocrine disruption mechanism or requires metabolic activation for activity.	Further testing may not be required. 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. Equivocal results may indicate chemical has multiple MOA. Consider route of exposures for effects data and possible implications of ADME characteristics of the chemical. Hormonal activity possible in lower vertebrates. Consider performing a LAGDA or MEOGRT.

Scenarios	Result of OECD TG 426 (DNT study)	Existing results		Possible conclusions	Next step which could be taken to strengthen weight of evidence if necessary	Other considerations
		Mechanism (*in vitro* mechanistic data)*	Effects (*in vivo* effects of concern)**			
G	+	Eq/0	+	Evidence for adverse effects on (neuro-) development in OECD TG 426, may act via E,A,T,S mechanism and may require metabolic activation for activity.	Further testing may not be required. 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. Hormonal activity possible in lower vertebrates. Consider performing a LAGDA or MEOGRT.
H	+	Eq/0	–	Evidence for adverse effects on (neuro-) development in OECD TG 426 via non-E,A,T,S/non-endocrine disruption mechanism or requires metabolic activation for activity.	Further testing may not be required. To further discern mechanism, could perform *in vitro* ER, AR, TR, S assays with added metabolising system.	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. Hormonal activity possible in lower vertebrates. Consider performing a LAGDA or MEOGRT.
I	+	Eq/0	Eq/0	Evidence for adverse effects on (neuro-) development in OECD TG 426 via unknown mechanism.	Further testing may not be required. 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. Hormonal activity possible in lower vertebrates. Consider performing a LAGDA or MEOGRT.
J	–	+	+	No evidence of adverse effects on (neuro-) development in OECD TG 426. 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.

Scenarios	Result of OECD TG 426 (DNT study)	Existing results		Possible conclusions	Next step which could be taken to strengthen weight of evidence if necessary	Other considerations
		Mechanism (*in vitro* mechanistic data)*	Effects (*in vivo* effects of concern)**			
K	–	+	–	No evidence of adverse effects in OECD TG 426. 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 426. 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 426. Effects seen in existing (lower level) studies do not lead to adverse outcome.	Consider supplemental testing, depending on existing data.	If existing data are from adequate *in vivo* studies such as 28-day, 90-day, chronic/carcinogenicity studies, than 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 426.	Consider supplemental testing, depending on existing data.	If existing data are from an adequate Level 5 assay, there may be sufficient information to conclude absence of concern for endocrine disruption. Note that the EOGRTS provides the most information on endocrine disruption; however, for EDCs with a carcinogenic potential, OECD TG 451-3 may be more sensitive.
O	–	–	Eq/0	No evidence of adverse effects in OECD TG 426. No evidence for (anti)-E,A,T,S activity *in vitro*.	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. 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 OECD TG 426 (DNT study)	Existing results		Possible conclusions	Next step which could be taken to strengthen weight of evidence if necessary	Other considerations
		Mechanism (*in vitro* mechanistic data)*	Effects (*in vivo* effects of concern)**			
P	–	Eq/0	+	No evidence of adverse effects in OECD TG 426. Effects seen in existing (lower level) studies do not lead to adverse. 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, than 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 426.	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 in OECD TG 426	Consider supplemental testing, depending on existing data.	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. Equivocal results may indicate chemical has multiple MOA. Check data on chemical analogues.