C.3.1. Uterotrophic Bioassay in Rodents (UT assay) (OECD TG 440) (including OECD GD 71 on the procedure to test for anti-estrogenicity)

Status: Assay validated by the OECD.

649. Modality detected/endpoints: estrogens (uterine wet weight and blotted weight ↑); anti-estrogens (stimulated uterine weight ↓); optional others (e.g. histopathologic changes in uterus/vagina).

Background to the assay

650. This assay is a short-term *in vivo* screening assay in female rodents for chemicals that interact with the estrogen receptor (ER). It is based on the increase in uterine weight (or uterotrophic response) that is elicited by ER agonists in animal models where endogenous estrogen levels are minimal. There are two variants of the assay; one uses immature animals, the other uses ovariectomised animals. The immature rodent assay may detect modalities acting via mechanisms other than ER, as the animals have an intact hypothalamic/pituitary/gonadal (HPG) axis, but the ability to detect these is limited. The assay may be conducted using rats or mice, but the there is more experience with the rat assay and this species was used in the OECD validation of this assay (OECD, 2006). Route of administration of test substance is via oral gavage or subcutaneous injection. This assay has been considered to be the "gold standard" bioassay screen for identifying ER agonists. A recently curated database of bioactivity with results from over 2 500 Uterotrophic Bioassays in rats and mice provides comprehensive information on this assay (Kleinstreuer et al., 2016).

651. Although this assay is a "screen", some authorities may regard an increase in uterine weight as possibly adverse. If this occurs in immature animals at a point in time when this should not occur naturally then this could represent an adverse effect in a sensitive life stage. Likewise, the ovariectomised UT assay may be regarded as a model for immature animals and therefore a uterine weight increase could be regarded as adverse. Interpretations of the results of this assay may vary according to region and regulation and should always utilise all data in a weight of evidence approach.

652. Non-aromatisable (steroidal and non-steroidal) androgens and aromatisable androgens that may be metabolised to estrogens have also been shown to increase uterine weight. In immature animals, aromatisable androgens like testosterone elicit histopathologic changes very similar to that of estradiol, suggesting that the observed changes are mediated through estrogen. For all other conditions, the observed histopathologic changes are different and are considered to be mediated via the androgen receptor (AR). In practical terms, this issue is of minor importance. Potentially aromatisable androgens can easily be identified based on their structural features, and non-steroidal androgenic chemicals are currently considered to be rare in the chemical universe. In addition, progesterone and synthetic progestins may also give a positive response (Jones and Edgren, 1973).

653. The OECD test guideline (TG 440) was adopted in October 2007 and is specific for estrogen agonists only. The validation of the assay was not considered adequate for anti-estrogens as there were insufficient pure anti-estrogens available. The test for anti-estrogens, however, is frequently used and is available as OECD GD 71 (OECD, 2007). Its use as an assay was reviewed during the validation of the UT assay (Owens and Ashby, 2002) and it continues to be used to date.

When/why the assay may be used

654. Although OECD TG 440 can be used at any stage in the assessment process, the most likely use scenario will be following a positive result in an ER transactivation assay (ER STTA) and/or an ER binding assay, in order to determine whether the positive result *in vitro* is translated into a positive result *in vivo*. It may also be used as a screen in the absence of positive *in vitro* data, when a chemical that is negative in the *in vitro* ER interaction screens is suspected of producing estrogenic metabolites *in vivo*. In this case, the first option would be to use an additional metabolising system in the *in vitro* tests, but the Uterotrophic Bioassay as an *in vivo* test will include all metabolising systems. Another possible scenario is following observation of effects in higher tier tests, for example acceleration of puberty onset in females, but which are not exclusively indicative of an effect on ER. In the European Union, chemicals included in REACH, Plant Protection Products and Biocides legislation may have been tested in OECD TG 421/422, OECD TG 416 (Two-Generation Reproductive Toxicity Study) or the Extended One-Generation Reproduction Toxicity Study (EOGRTS – OECD TG 443), the UT assay may then be used as a follow up to clarify the mode of action (MOA). The UT assay is also likely to be carried out as part of the United States Environmental Protection Agency's Endocrine Disruptor Screening Program Tier 1 screening battery. Selection of the most appropriate tests has to be on a case-by-case basis, but also considering the need to minimise animal testing.

655. It should be noted that the UT assay was designed to be sensitive and will detect weak and strong ER modulators. In the validation of the UT assay, ethinylestradiol and oestradiol were defined as "strong" estrogens whilst nonylphenol and genistein were defined as "weak" estrogens (OECD, 2006). Weakly acting chemicals may not always be detected as endocrine disruptors (EDs) when tested in higher level tests because the endocrine system in intact/adult animals has a greater ability to compensate than in the UT assay where the HPG axis is disrupted/immature. Furthermore, in case of repeat dose studies, dose levels may need adjustment to lower doses in order to cope with general toxicity.

656. The route of exposure is also an important consideration for the UT assay. OECD TG 440 states that chemicals may be administered by oral or subcutaneous routes but suggests that the route most relevant for human exposure should be used. The route will have consequences for absorption, distribution, metabolism and excretion and is an important consideration when interpreting results. Methoxychlor, for example, gave negative results when administered by subcutaneous injection but positive results when given orally (due to metabolism to estrogenic metabolites) (Laws et al., 2000).

657. In order to provide information relevant for assessing whether or not a chemical may fulfil the WHO/IPCS (2002) definition of an ED, the study design has to be sufficiently robust to demonstrate the presence or absence of effects. In the dose selection, the investigator should also consider and ensure that data generated are adequate to fulfil the regulatory requirement across OECD countries as appropriate (e.g. hazard and risk assessment and labelling, ED assessment, etc.). The top dose or concentration should be

sufficiently high to give clear systemic (i.e. non endocrine-specific) toxicity in order to ensure that a wide range of exposures (high to low) is tested. However, endocrine effects observed solely in the presence of clear systemic toxicity should be interpreted with caution and may be disregarded when sufficiently justified to be caused by secondary effects which are unlikely to be due to endocrine activity. The reason for this advice is a concern that some endocrine active substance (EAS) sensitive assays are being run at doses/concentrations of EASs that are too low to trigger direct impacts on the endocrine system. This guidance document is not the place to address this issue directly, but it should be considered when EAS-sensitive test guidelines (TGs) are revised in the future. In addition, the number and spacing of dose/concentration levels should also be adequate to fulfil the objectives of the study (e.g. to demonstrate dose response relationships if this is required).

Introduction to the table of scenarios

658. Table C.3.1 gives guidance on a further step to take in the event of a positive (+) or negative (-) result in the UT assay 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.

659. The results of OECD TG 440 are given in the second column. Criteria for positive results in OECD TG 440 are given in the test guideline itself (i.e. a statistically significant increase in uterine weight compared to the solvent control). A positive result in the assay for anti-estrogenicity would be a statistically significant decrease in uterine weight compared to the estrogen-stimulated control group. Negative results are no (statistically significant) changes in wet and blotted uterine weight. It is important that quality criteria for control uterine weights are demonstrated. It is also of note that a uterotrophic response may not always be entirely of estrogenic origin (e.g. testosterone may give a positive result, chemicals interacting with other endocrine axes may give a positive result in the immature rodent assay, diets high in phytoestrogens or energy sources may also give a positive result). Further guidance is provided in the TG. Optional endpoints may include histopathologic changes in uterus/vagina or vaginal cornification in the ovariectomised rat assay. These endpoints should supplement the uterotrophic response. Changes in these endpoints in the absence of a uterotrophic response should be considered equivocal.

660. Equivocal results for the guideline are not included in Table C.3.1 because these data require further interrogation about the result itself. In the event of an equivocal result, the considerations mentioned above about uterine weights in control animals, non ER-related changes, possible effects of phytoestrogens or high energy diets should be taken into account and further investigations made.

Existing data to be considered

661. Existing "mechanism" *in vitro* data are assumed to be available from ER (ER binding and ER STTA), AR (AR binding and AR STTA) 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. Thyroid hormone receptor (TR-) based assays are less relevant for the UT assay. Although the current *in vitro* TGs do not incorporate metabolic activation, published information on use of metabolic activation

systems is available in Jacobs et al. (2008, 2013) and OECD (2008). These methods, however, have not yet been validated.

662. Existing "effects" data refer to *in vivo* effects that may come from varied sources and will depend on the type of chemical (e.g. new chemicals, high production volume chemical, pesticide). Thus, available data may range from repeated dose toxicity studies (28-day, 90-day) or combined repeat dose/reproductive screening assays to chronic toxicity studies and multigeneration reproductive tests. Some studies fail to identify EDs that weakly affect estrogen or androgen receptors as was demonstrated on the basis of data generated in the validation process of OECD TG 407 assay with endocrine endpoints. In this validation, only moderate EDs such as nonylphenol and DDE, and strong EDs such as ethinylestradiol and flutamide (acting via ER and AR respectively), were detected. Thus OECD TG 407 cannot be regarded as a screening assay for endocrine activity. This means that when a relatively insensitive test is positive for both endocrine-specific and apical endpoints, this should be taken as an indication that the substance is a potential ED. Caution should be exercised, however, because endocrine endpoints may be impacted secondary to non-endocrine toxicity and *in vivo* apical endpoints can be affected by many MOA including endocrine modalities. The ability of a given assay to detect endocrine disruption will also vary depending on the version of the test guideline used. Older test guidelines may contain fewer endocrine-sensitive endpoints than more recent ones. If data are available from single or multigeneration studies that are adequately conducted with updated guidelines that include endpoints sensitive to EASs, then there should be no reason to conduct a UT assay as the higher tier test will provide stronger evidence for hazard identification/characterisation. Multigeneration studies conducted prior to the introduction of these endpoints will still provide valuable information on reproductive and endocrine organ toxicity, reproduction and development, but may not be sufficiently sensitive to EASs, in which case the UT assay would provide further valuable information. 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.

663. When considering the results of the UT 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

664. The scenarios (A to R) presented in Table C.3.1 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 OECD TG 440 uses rats, the well-conserved nature of ER across taxa should be a strong indication that results in this assay are relevant to other vertebrate species. Results 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. 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. It is recognised, however, that some jurisdictions may require a two-generation study. Further considerations specific to each scenario are given in the table.

665. Scenarios A to C represent positive results in the UT assay in the presence of positive *in vitro* mechanistic data and positive, negative or equivocal *in vivo* effects data. A positive result in ER-based assays in combination with a positive UT assay is strong evidence for (anti)estrogenic activity that may or may not be supported by the *in vivo* effects data. Effects on endocrine endpoints in OECD TGs 407, 408, 453 or 421/422 may provide sufficient evidence to conclude concern for endocrine disruption and therefore there is no need for further screening. Positive results in the UT assay may also indicate similar (anti)estrogenicity in lower vertebrates. These could be followed up with partial life cycle tests such as the Fish Sexual Development Test (FSDT) or the Larval Amphibian Growth and Development Assay (LAGDA). *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. Selection of the dose level and the strain of animal should also be considered. Generally, a conclusion of lack of concern for endocrine disruption in the presence of positive effects data may only be made given adequate Level 5 assays. MOA data to provide a clear interpretation may be required by some regulatory agencies. The possibility of other mechanisms should also not be overlooked (e.g. positive AR-based assays may indicate an aromatisable androgen and a positive Steroidogenesis Assay could indicate a chemical that alters endogenous estrogen levels, both situations may give a positive result in the immature rat UT assay). Other (non-E,A,T,S) mechanisms may also be considered (e.g. involving other receptors or endocrine axes).

666. Scenarios D to F represent positive results in the UT assay in the presence of negative *in vitro* mechanistic data and positive, negative or equivocal *in vivo* effects data. Negative results in the *in vitro* assays should be viewed with caution in case a metabolite is responsible for the positive UT assay. Unless the metabolic profile of the test substance is known, then one option may be to conduct these assays with an added metabolising system. If the metabolic profile is known, then a higher level *in vivo* test may be advisable. The choice of tests will depend on the available *in vivo* effects data. Positive results in the UT assay may also indicate similar (anti)estrogenicity in lower vertebrates. As in scenarios A-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. Selection of the dose level and the strain of animal should also be considered. Generally, a conclusion of lack of concern for endocrine disruption in the presence of positive effects data (Scenario E) may only be made given adequate Level 5 assays. 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. MOA data to provide a clear interpretation may be required by some regulatory agencies.

667. Scenarios G to I represent positive results in the UT assay in the presence of various combinations of missing or equivocal data. Positive results in the UT assay may also indicate similar (anti)estrogenicity 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 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. As above, generally a conclusion of lack of concern for endocrine disruption in the presence of some positive effects data (Scenario H) may only be made given adequate Level 5 assays. 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. MOA data to provide a clear interpretation may be required by some regulatory agencies.

668. Scenarios J to L represent negative results in the UT assay in the presence of positive *in vitro* mechanistic data and positive, negative or equivocal *in vivo* effects data. The *in vitro* mechanistic data given in the table could be any of the E,A,T,S tests (e.g. the AR binding or Steroidogenesis Assay). A weak aromatase inhibitor, for example, could give Scenario J from a positive result in the Steroidogenesis Assay and a positive result in the female Peripubertal Assay. All three scenarios could also arise from a chemical that binds to ER but is metabolised to a non-estrogenic metabolite leading to negative results in the UT assay and this should be considered first when investigatingthe next step. Endocrine active potency may also explain differences between *in vitro* and *in vivo* results (e.g. a chemical with weak endocrine activity may give a positive result *in vitro* but may be negative *in vivo*). Positive *in vivo* effects data may involve other E,A,T,S, non-E,A,T,S mechanisms (e.g. involving other receptors or endocrine axes), more sensitive endpoints, greater statistical power or life stages that are more sensitive to the substance than immature/ovariectomised animals in the UT assay.

669. Scenarios M to O represent negative results in the UT assay in the presence of negative *in vitro* mechanistic data and positive, negative or equivocal *in vivo* effects data. Negative results for all tests (Scenario N) may be sufficient to enable a conclusion of no concern for endocrine disruption. This will depend on the weight of evidence and may not be possible in some cases. However, in the presence of negative data from robust Level 4 and 5 assays, further animal testing is probably not justified. Where there are positive *in vivo* effects data, there could still be an estrogen-related mechanism. These effects may be related to length of exposure, route of exposure or exposure at different life stages. Other E,A,T,S or non-E,A,T,S mechanisms may also be involved.

Scenarios P to R represent negative results in the UT assay in the presence of various combinations of missing or equivocal data. As with the positive result scenarios above (see Paragraph 665), the next step to take in these eventualities will have to be decided on a case-by-case basis. However, the recommended first step is generally to obtain reliable mechanistic (*in vitro*) data rather than proceed directly to *in vivo* testing. In all cases, the role of metabolism, route of exposure and data from structural analogues should be considered before deciding on the next step.

670. In all scenarios (A-R), the next step to take to strengthen weight of evidence will depend on the existing information. Table C.3.1 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.3.1. **Uterotrophic Bioassay in Rodents (UT assay) (OECD TG 440) (including OECD GD 71 on the Procedure to Test for Anti-estrogenicity): 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 repeated dose toxicity tests (e.g. OECD TG 407, TG 408 28-day and 90-day studies), reproductive tests (e.g. reproduction screening assays or two-generation studies) or read-across from chemical analogues.

Scenarios	Result of OECD TG 440 (UT assay)	Existing results		Possible conclusions	Next step which could be taken to strengthen weight of evidence if necessary	Other considerations
		Mechanism (*in vitro* mechanistic data)*	Effects (*in vivo* effects of concern)**			
A	+	+	+	Strong evidence for estrogenic/anti-estrogenic (E/anti-E) activity with (potential for) adverse effects via estrogen receptor (ER) mechanism.	Perform assay from Level 4 (e.g. female pubertal assay) or Level 5 (e.g. Extended One-Generation Reproduction Toxicity Study [EOGRTS] or two-generation) assay.	If existing data are from Level 4 or 5 (or less sensitive) assays, there may be sufficient information to conclude evidence of concern for endocrine disruption (the EOGRTS provides the most information; however, for endocrine disrupting chemicals [EDCs] with a carcinogenic potential, OECD TG 451-3 may be more sensitive). Consider route of exposures for UT assay and existing effects data and possible implications of absorption, distribution, metabolism and excretion (ADME) characteristics of the chemical. The chemical itself may give positive *in vitro* results and *in vivo* results, but may also be metabolised to a metabolite that also has positive results *in vitro* and *in vivo*. A positive result could have arisen from other (E,A,T,S or non-E,A,T,S) mechanisms. E/anti-E activity possible in lower vertebrates. Consider performing the Fish Sexual Development Test (FSDT) or Larval Amphibian Growth and Development Assay (LAGDA).
B	+	+	–	Strong evidence for E/anti-E activity via ER but effects not detected in other *in vivo* studies in intact animals.	Perform assay from Level 4 (e.g. female pubertal assay) or Level 5 (e.g. EOGRTS or two-generation) assay.	If existing data are from an adequate Level 5 assay, there may be sufficient information to conclude absence of concern for endocrine disruption (the EOGRTS provides the most information; however, for EDCs with a carcinogenic potential, OECD TG 451-3 may be more sensitive). If existing data are from a less sensitive assay, a higher level test may be required. Consider route of exposures for UT assay and existing effects data and possible implications of ADME characteristics of the chemical. Check data on chemical analogues. A positive result could have arisen from other (E,A,T,S or non-E,A,T,S) mechanisms. E/anti-E activity possible in lower vertebrates. Consider performing an FSDT or LAGDA.
C	+	+	Eq/0	Strong evidence for E/anti-E activity via ER, but no or equivocal data from other *in vivo* studies.	Perform assay from Level 4 (e.g. female pubertal assay) or Level 5 (e.g. EOGRTS or two-generation) assay.	Check data on chemical analogues. Consider route of exposures for UT assay and existing effects data and possible implications of ADME characteristics of the chemical. Depending on route/kinetic and existing data considerations, may perform assay from Levels 4 or 5. A positive result could have arisen from other (E,A,T,S or non-E,A,T,S) mechanisms. E/anti-E activity possible in lower vertebrates. Consider performing an FSDT or LAGDA. Equivocal results may indicate chemical has multiple modes of action (MOA).

Scenarios	Result of OECD TG 440 (UT assay)	Existing results		Possible conclusions	Next step which could be taken to strengthen weight of evidence if necessary	Other considerations
		Mechanism (*in vitro* mechanistic data)*	Effects (*in vivo* effects of concern)**			
D	+	–	+	Strong evidence for E/anti-E activity. Acts via ER mechanism, but requires metabolic activation. Acts via non-ER mechanism and may or may not require metabolic activation.	Perform ER transactivation assay or binding assay with added metabolising system.	If existing data are from Level 4 or 5 (or less sensitive) assays, there may be sufficient information to conclude evidence of concern for endocrine disruption (the EOGRTS provides the most information; however, for EDCs with a carcinogenic potential, OECD TG 451-3 may be more sensitive). Further mechanistic studies may help determine MOA. A positive result could have arisen from other (E,A,T,S or non-E,A,T,S) mechanisms e.g. hypothalamic/pituitary/gonadal (HPG) axis. E/anti-E activity possible in lower vertebrates. Consider performing an FSDT or LAGDA.
E	+	–	–	Weak evidence for E/anti-E activity. Acts via non-ER mechanism. Chemical requires metabolic activation and metabolite has weak activity. Weak E/anti-E activity via ER does not result in adverse effects.	Perform ER transactivation assay or binding assay with added metabolising system OR Perform assay from Levels 4 or 5.	If existing data are from an adequate Level 5 assay, there may be sufficient information to conclude absence of concern for endocrine disruption (the EOGRTS provides the most information; however, for EDCs with a carcinogenic potential, OECD TG 451-3 may be more sensitive). If existing data are from a less sensitive assay, a higher level test may be required. Consider route of exposures for UT assay and existing effects data and possible implications of ADME characteristics of the chemical. Check data on chemical analogues A positive result could have arisen from other (E,A,T,S or non-E,A,T,S) mechanisms, e.g. HPG axis. E/anti-E activity possible in lower vertebrates. Consider performing an FSDT or LAGDA.
F	+	–	Eq/0	Weak evidence for E/anti-E activity via ER. Acts via non-ER mechanism. Requires metabolic activation and metabolite has weak/equivocal activity.	Perform ER transactivation assay or binding assay with added metabolising system OR Perform assay from Levels 4 or 5.	Check data on chemical analogues. Further mechanistic studies may help determine MOA. Level 4 or 5 studies will provide hazard data. A positive result could have arisen from other (E,A,T,S or non-E,A,T,S) mechanisms, e.g. HPG axis. E/anti-E activity possible in lower vertebrates. Consider performing an FSDT or LAGDA. Equivocal results may indicate chemical has multiple MOA.
G	+	Eq/0	+	Moderate or strong evidence for E/anti-E activity via ER. May act via ER, metabolic activation is required. Has potential for adverse effects via ER mechanism. May acts via non-ER mechanism and may or may not require metabolic activation.	For the "0" scenario, perform ER transactivation assay or binding assay. For the "Eq" scenario, perform ER transactivation assay or binding assay with added metabolising system.	If existing data are from Level 4 or 5 (or less sensitive) assays, there may be sufficient information to conclude evidence of concern for endocrine disruption (the EOGRTS provides the most information; however, for EDCs with a carcinogenic potential, OECD TG 451-3 may be more sensitive). Check data on chemical analogues. Further mechanistic studies may help determine MOA. A positive result could have arisen from other (E,A,T,S or non-E,A,T,S) mechanisms, e.g. HPG axis. E/anti-E activity possible in lower vertebrates. Consider performing an FSDT or LAGDA. Equivocal results may indicate chemical has multiple MOA.

Scenarios	Result of OECD TG 440 (UT assay)	Existing results		Possible conclusions	Next step which could be taken to strengthen weight of evidence if necessary	Other considerations
		Mechanism (*in vitro* mechanistic data)*	Effects (*in vivo* effects of concern)**			
H	+	Eq/0	–	Weak evidence for E/anti-E activity. May act via ER, metabolic activation is required. E/anti-E activity does not result in adverse effects.	For the "0" scenario, perform ER transactivation assay or binding assay. For the "Eq" scenario, perform ER transactivation assay or binding assay with added metabolising system.	If existing data are from an adequate Level 5 assay, there may be sufficient information to conclude absence of concern for endocrine disruption (the EOGRTS provides the most information; however, for EDCs with a carcinogenic potential, OECD TG 451-3 may be more sensitive). If existing data are from a less sensitive assay, a higher level test may be required. Consider route of exposures for UT assay and existing effects data and possible implications of ADME characteristics of the chemical. Check data on chemical analogues. Further mechanistic studies may help determine MOA. E/anti-E activity possible in lower vertebrates. Consider performing an FSDT or LAGDA. Equivocal results may indicate chemical has multiple MOA.
I	+	Eq/0	Eq/0	E/anti-E activity of unknown potency. May act via ER, metabolic activation is required. Unknown potential for adverse effects.	For the "0" scenario, perform ER transactivation assay or binding assay. For the "Eq" scenario, perform ER transactivation assay or binding assay with added metabolising system, or Level 4 or 5 assay if existing data indicate this is needed.	Check data on chemical analogues. Further mechanistic studies may help determine MOA. E/anti-E activity possible in lower vertebrates. Consider performing an FSDT or LAGDA. Equivocal results may indicate chemical has multiple MOA.
J	–	+	+	No evidence for E/anti-E activity *in vivo* via ER. Route of exposure, metabolic differences or potency explain differences between UT assay and existing *in vitro*/*in vivo* studies. Effects seen in existing studies are via non-ER mechanism.	Perform ER transactivation assay or binding assay with added metabolising system.	If existing data are from an adequate Level 5 assay, there may be sufficient information to conclude concern for endocrine disruption (the EOGRTS provides the most information; however, for EDCs with a carcinogenic potential, OECD TG 451-3 may be more sensitive). Effects seen in existing studies may be in a more sensitive life stage. Consider route of exposure for UT assay and possible implications of ADME characteristics of the chemical. Check data on chemical analogues. Further mechanistic studies may help determine MOA.
K	–	+	–	No evidence for E/anti-E activity *in vivo* via ER. Metabolic differences or potency explain *in vitro*/*in vivo* differences.	Perform ER transactivation assay or binding assay with added metabolising system.	If existing data are from an adequate Level 5 assay, there may be sufficient information to conclude absence of concern for endocrine disruption (the EOGRTS provides the most information; however, for EDCs with a carcinogenic potential, OECD TG 451-3 may be more sensitive). If existing data are from a less sensitive assay, a higher level test may be required. Metabolic deactivation of chemical may occur *in vivo* so that possible *in vitro* activity is not realised. Consider possible routes of exposure implications of metabolism.

Scenarios	Result of OECD TG 440 (UT assay)	Existing results		Possible conclusions	Next step which could be taken to strengthen weight of evidence if necessary	Other considerations
		Mechanism (*in vitro* mechanistic data)*	Effects (*in vivo* effects of concern)**			
L	–	+	Eq/0	No evidence for E/anti-E activity *in vivo* via ER. Metabolic differences or potency explain *in vitro*/*in vivo* difference. Unknown potential for adverse effects.	Perform ER transactivation assay or binding assay with added metabolising system OR Perform assay from Levels 4 or 5.	Metabolic deactivation of chemical may occur *in vivo* so that potential *in vitro* activity is not realised. Consider possible routes of exposure implications of metabolism. Equivocal results may indicate chemical has multiple MOA.
M	–	–	+	No evidence for E/anti-E activity *in vivo* or *in vitro* via ER. Metabolic differences or route of exposure explain *in vitro*/*in vivo* existing differences. Effects seen in existing studies are via non-ER mechanism.	Perform *in vitro* assays with added metabolising system.	Effects seen in existing studies may be in a more sensitive life stage. Check data on chemical analogues. Further mechanistic studies may help determine MOA.
N	–	–	–	No evidence for E/anti-E activity *in vivo* or *in vitro* via ER. No evidence of adverse effects.	Possibly no need for further testing. If there is uncertainty, may perform assay from Level 4.	If existing data are from an adequate Level 5 assay, there may be sufficient information to conclude absence of concern for endocrine disruption (the EOGRTS provides the most information; however, for EDCs with a carcinogenic potential, OECD TG 451-3 may be more sensitive). Check data on chemical analogues.
O	–	–	Eq/0	No evidence for E/anti-E activity *in vivo* or *in vitro* via ER. Unknown potential for adverse effects via other non-ER mechanisms.	Perform assay from Levels 4 or 5.	Consider route of exposure for UT assay and possible implications for ADME characteristics of the chemical in follow-up assay.
P	–	Eq/0	+	No evidence for E/anti-E activity *in vivo* via ER. Unknown potential for adverse effects via other mechanisms.	For the "0" scenario, perform *in vitro* E,A,T,S assays, otherwise Eq result available.	Consider route of exposure for UT assay and possible implications for differences from existing assay. Effects seen in existing studies may be in a more sensitive life stage. Check data on chemical analogues. Further mechanistic studies may help determine MOA. Equivocal results may indicate chemical has multiple MOA.
Q	–	Eq/0	–	No evidence for E/anti-E activity *in vivo* via ER. No evidence of adverse effects.	For the "0" scenario, perform *in vitro* E,A,T,S assays, otherwise Eq result available.	If existing data are from an adequate Level 5 assay, there may be sufficient information to conclude absence of concern for endocrine disruption (the EOGRTS provides the most information; however, for EDCs with a carcinogenic potential, OECD TG 451-3 may be more sensitive). If existing data are from a less sensitive assay, a higher level test may be required. Check data on chemical analogues. Further mechanistic studies may help determine MOA.
R	–	Eq/0	Eq/0	No evidence for E/anti-E activity *in vivo* via ER.	For the "0" scenario, perform *in vitro* E,A,T,S assays, otherwise Eq result available.	Consider route of exposure for UT assay and possible implications for differences from existing assay. Check data on chemical analogues. Further mechanistic studies may help determine MOA. Equivocal results may indicate chemical has multiple MOA.