OECD *in vitro* screens (Conceptual Framework Level 2)

C.1.1. Performance-based Test Guideline for Human Recombinant Estrogen Receptor (hrER) *In Vitro* Assays to Detect Chemicals with ER Binding Affinity (OECD TG 493)

Status: Assay validated by the OECD.

Modality detected/endpoints: Binding to estrogen receptor isoforms.

Background to the assay

132. The estrogen receptor (ER) binding assay is an *in vitro* screening assay to detect substances that bind to ERs. The assay has been in use for many years and there are different variations of the protocol. Older versions of the protocol utilise rat uterine cytosol as a source of ER without further purifications of ER isoforms (e.g. US EPA OPPTS 890.1250). Binding therefore occurs to a mixture of ERα and ERβ, although the primary isoform in rat uterine cytosol is ERα. The ER binding assay was chosen to be one of the suite of assays comprising the United States Environmental Protection Agency's (US EPA) "Tier 1" and the rat uterine cytosolic assay (US EPA OPPTS 890.1250) was validated in that context (US EPA, 2009). More recent protocols do not use animals as a source of ER but use human ERα recombinant protein (hrERα). OECD TG 493 (published in July 2015) is a performance-based test guideline (PBTG) that describes two methods using human ERα:

- the Freyberger-Wilson (FW) *In Vitro* Estrogen Receptor (ER) Binding Assay Using a Full Length Human Recombinant ERα
- the Chemical Evaluation and Research Institute (CERI) *In Vitro* Estrogen Receptor Binding Assay Using a Human Recombinant Ligand Binding Domain Protein.

133. The FW *In Vitro* ERα Binding Assay uses a full-length recombinant hERα produced in and isolated from baculovirus-infected insect cells. The CERI *In Vitro* ERα Binding Assay uses a truncated ER that contains only the ligand binding domain of the hERα. Both methods were validated according to OECD principles (OECD, 2005) and are the first two in this PBTG. Performance standards to enable the development and validation of similar test methods have also been published (OECD, 2015). During validation, both methods gave similar results. There was almost 100% agreement between the two test methods, based on the classifications of all the substances up to 10^{-4} M. Each substance was also correctly classified as an ER binder or non-binder. In addition, a comparison with the rat uterine cytosol assay (US EPA OPPTS 890.1250) also showed a high degree of correlation (Laws and Wilson, 2014).

134. Binding assays provide information on the ability of a compound to interact with ERs *in vitro*? but results should not be directly extrapolated to the complex signaling and regulation of the intact endocrine system *in vivo*. Binding assays determine saturation binding and competitive binding. The saturation binding assay is used to confirm the specificity and activity of the receptor preparations, while the competitive binding experiment is used to evaluate the ability of a test chemical to bind to ER and determine IC50 (the half maximal effective concentration of an inhibitory test chemical) if possible. The assay determines the ability of a chemical to displace a radiolabeled ligand

(17β-estradiol) from ER and generally provides a positive, negative or equivocal result for the ability to bind to ER.

135. Chemicals that bind to the ER may induce hormone-dependent transcriptional activity (agonist) or block normal hormone function by preventing the endogenous hormone from binding to the receptor (antagonist). The binding assay does not distinguish between these. The hormone-binding domain of the ER is highly conserved across vertebrate species and therefore represents a simple evaluation of estrogenic potential that is relevant to many taxa. A positive result in this assay requires demonstration of a concentration response curve for the ability of the test chemical to displace radiolabelled 17β-estradiol. The concentration response curve allows the determination of potency (e.g. IC50 and relative binding affinity by comparing the log [IC50] of 17β-estradiol with that of the test chemical). OECD TG 493 provides guidance on data interpretation and criteria for assigning classification based on the competitive binding curve for a test chemical. Final classification of a test chemical is as a binder, non-binder or equivocal.

136. Occasionally, there are test chemicals where additional attention is needed to appropriately analyse and interpret the binding data. Previous studies have shown cases where the analysis and interpretation of competitive receptor binding data can be complicated by an upturn of the per cent specific binding at the highest concentrations of the test chemical. Chemicals showing this characteristic often have limited solubility. The maximum concentration of chemical to be used in the assay is 1mM. The guideline provides detailed guidance on data analysis in these circumstances.

137. The ER binding assay may suffer from variability in response if not performed exactly as stated in the protocols (e.g. if the receptor concentration in the cytosol is too low or too high, or the microtiter plates are not kept cold at all times during the experiment). Performance criteria are therefore specified in order to demonstrate that the assay is functioning correctly. Reference substances are also used on each run to demonstrate the sensitivity of the experiment (reference standard: 17β-estradiol; weak positive control: norethynodrel or norethindrone; and negative control: octyltriethoxysilane). Compliance with the performance criteria should be checked before evaluating results from an assay run to ensure that most have been met. Small deviations are unlikely to have compromised the assay? but judgement should be made on a case-by-case basis. It is recommended that laboratory proficiency be demonstrated by the periodic use of proficiency substances. These are a subset of the substances provided in the performance standards for the ER binding assays (OECD, 2015). They represent the classes of chemicals commonly associated with ER binding activity, exhibiting a suitable range of potency expected for ER binding (i.e. strong to weak) and non-binders (i.e. negatives).

When/why the assay may be used

138. Although the ER binding assay may be used at any stage in the hazard assessment process, the most likely use scenario is during initial assessment of chemicals for their ability to interact with endocrine systems *in vitro* (i.e. E,A,T,S modalities). Assays for interaction with other modalities (e.g. androgen receptor [AR] and steroidogenesis interference), are likely to be conducted at the same time so that all results can be considered together. Thyroid hormone receptor (TR) and other assays concerning mechanisms of thyroid disruption may be conducted, but the methods for these are not in common use and are not validated (see Section A.6). The ER binding assay does not include the use of a xenobiotic metabolising system, but consideration should be given to the inclusion of this (Jacobs et al., 2008; OECD, 2008) depending on the circumstances (e.g.

if the metabolism of a chemical is unknown), although the methods for inclusion of xenobiotic metabolising systems are not yet validated (see Paragraph 50). Alternatively, for a chemical with known metabolites, these could also be tested in the ER binding assay. Another use scenario may be following effects obtained in higher tier tests, for example delayed or accelerated puberty onset in females, which could be indicative of an effect mediated by ER. Selection of the most appropriate tests has to be on a case-by-case basis, but also considering the need to minimise animal testing. A further example could be results obtained in other apical assays, e.g. in OECD TG 408 (90-Day Toxicity Test), where effects on reproductive organs could be investigated further by testing in the ER binding assay in combination with AR- and steroidogenesis-based assays.

Introduction to the table of scenarios

139. Table C.1.1 gives guidance on a further step to take in the event of a positive (+) or negative (-) result in the ER binding 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.

140. The results of the ER binding assay are given in the second column. Criteria for positive, negative and equivocal results are given in OECD TG 493. A test chemical is considered to be a binder if a binding curve can be fit and the lowest point on the response curve within the range of the data is less than 50% and a log IC50 can be obtained. A positive result should be obtained in at least two out of three independent test runs. It is also important that quality and proficiency criteria are demonstrated for both positive and negative results.

141. Equivocal results for the guideline are not included in the table because these data generally require further interrogation about the result itself. In the event of an equivocal result, the considerations mentioned above about control quality and proficiency criteria should be taken into account and further investigations made. Equivocal results at high concentrations may indicate solubility issues.

Existing data to be considered

142. Existing "mechanism" *in vitro* data are assumed to be available from AR-based assays (Level 2) and the Steroidogenesis Assay. Assays may also be available for interference with thyroid modalities. The ER binding assay is most likely to be performed before the ER Stably Transfected Transactivation Assay (STTA – OECD TG 455) and so the ability of the chemical to affect ER-mediated gene expression may not be known. In practice, it is possible that data from some or all of these assays may not be available, so judgement will need to be used to decide which assays to perform. The ER binding assay and ER STTA both provide data about the intrinsic ability of a chemical to interact with ER, but each has their own advantages and disadvantages. The ER binding assay will not distinguish between agonists and antagonists whilst some chemicals testing positive in the ER STTA assay may have affected the reporter gene activity through non-ER related mechanisms. Consistent results in both assays give more confidence in the presence or absence of an ER-related mode of action (MOA).

143. Existing "effects" data refer to *in vivo* effects "of concern" (i.e. data from Level 4 or 5 vertebrate assays). These may come from varied sources and will depend on the type of substance (e.g. new chemicals, high production volume [HPV] chemical, pesticide). Thus,

available data may range from repeated dose toxicity studies (28-day, 90-day), combined repeat dose/reproductive screening assays or fish screening assays, to chronic toxicity studies and multigeneration reproductive tests in vertebrate species. Some studies fail to identify EASs 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 endocrine disruptors, 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 modes of action, including endocrine modalities. Data may also be available on effects in non-mammalian species, although caution should be used when extrapolating between taxa. A chemical causing endocrine effects in fish or amphibians (for example, OECD TG 240 or TG 241) may also have endocrine effects in mammals, but the physiological consequences of the effects are likely to be different.

144. Data may also be available from Level 3 mammalian assays (Hershberger Bioassay [H] and Uterotrophic Bioassay [UT]) but as the UT assay primarily detects (*in vivo*) the same modality as ER binding, it is unlikely that it would be conducted before ER binding. An Amphibian Metamorphosis Assay (AMA) may also be available, but as this test primarily detects thyroid disruption in amphibians, it is unlikely to provide useful data for E-modalities.

145. When considering the results of the ER binding assay, all available data should be used in order to reach a conclusion and a WOE approach taken. This may include high throughput screening (HTS) 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

146. The scenarios (A to R) presented in Table C.1.1 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 493 uses hrER, the well-conserved nature of ER across taxa should be a strong indication that results in this assay are relevant to other vertebrate 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. Further considerations specific to each scenario are given in the table.

147. Scenarios A to C represent positive results in the ER binding assay in the presence of positive *in vitro* mechanistic data and positive, negative or equivocal *in vivo* effects data. A positive result in an ER binding assay is strong evidence for (anti)estrogenic activity that may or may not be supported by the *in vivo* effects data. In the case of positive *in vivo* effects data, there may be sufficient evidence to conclude concern for endocrine disruption and therefore no need for further screening. *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. 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. 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 tumours in other endocrine-sensitive tissues. MOA data to provide a clear interpretation may be required by some regulatory agencies.

148. Scenarios D to F represent positive results in the ER binding assay in the presence of negative *in vitro* mechanistic data and positive, negative or equivocal *in vivo* effects data. Unless the metabolic profile of the test substance is known, one option may be to conduct these *in vitro* assays with an added metabolising system. If the metabolic profile is known, then an *in vivo* test may be advisable. The choice of tests will depend on the available *in vivo* effects data. 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. 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 tumours in the absence of reproductive or developmental effects, as well as substances causing tumours in other endocrine-sensitive tissues. MOA data to provide a clear interpretation may be required by some regulatory agencies.

149. Scenarios G to I represent positive results in the ER binding assay 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, 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 directly to *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. In some cases, it may be necessary to conduct *in vivo* tests and some guidance is given in the final column. 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 tumours in the absence of reproductive or developmental effects, as well as substances causing tumours in other endocrine-sensitive tissues. MOA data to provide a clear interpretation may be required by some regulatory agencies.

150. Scenarios J to L represent negative results in the ER binding assay in the presence of positive *in vitro* mechanistic data and positive, negative or equivocal *in vivo* effects data. The limitations of the ER binding assay should be considered first (e.g. lack of metabolic activation, possible involvement of other binding proteins). The positive *in vitro* mechanistic data indicates possible alternative A,T,S mechanisms. To confirm lack of ER-

related activity in the presence of *in vivo* data, an ER STTA could be performed. Otherwise *in vivo* tests will confirm or refute E,A,T,S activity.

151. Scenarios M to O represent negative results in the ER binding 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. The limitations of the ER binding assay should also be considered (as described for Scenarios J to L). To confirm lack of ER-related activity in the presence of *in vivo* data, an ER STTA could be performed. Otherwise *in vivo* tests will confirm or refute E,A,T,S activity (Scenarios M and O).

152. Scenarios P to R represent negative results in the ER binding assay in the presence of various combinations of missing or equivocal data. The limitations of the ER binding assay should be considered first (as described for Scenarios J to L). As with the positive result scenarios above (Paragraph 145), the next step to take for Scenarios P to R 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.

153. In all scenarios (A to R), the next step to take to strengthen weight of evidence will depend on the existing information. Table C.1.1 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.

154. In general, a decision about whether or not to conduct *in vivo* vertebrate tests will depend on the weight of evidence of new and existing data. If most available data (e.g. the results of the ER binding assay, results from an ER STTA assay, predictions from QSARs, "read-across" from data on similar substances and results from mammalian *in vivo* assays) suggest that the substance has the potential to cause endocrine disruption via the estrogen receptor (i.e. the level of suspicion about endocrine disrupting action is high – corresponding to Scenario A), then consideration should be given to the conduct of a higher level test.

155. For non-mammalian wildlife species, higher level tests with fish or amphibians (i.e. OECD TG 234, TG 240, TG 241) are recommended. Choice about which of these tests is most appropriate will be driven *inter alia* by MOA considerations, and by whether multigeneration effects are to be expected. Such tests are unlikely to be needed if exposure of the natural environment is not expected. On the other hand, if available data only raise a low or moderate level of suspicion about endocrine disrupting action (e.g. the data appear to conflict with each other), then consideration should be given to the conduct of a fish or amphibian screen (e.g. juvenile medaka anti-androgen screening assay; EASZY; *Xenopus* embryonic thyroid signalling assay; OECD TG 231, TG 229 or TG 230). There are fewer

options available for invertebrates, but if ecdysteroid or juvenile hormone activity are suspected in arthropods (e.g. from a screening test with short-term juvenile hormone activity screening assay), various higher level tests are available, including OECD GD 201, the *Daphnia* Multigeneration Test and TG 233.

156. For mammals, similar considerations apply, but lower level tests (e.g. Level 3 or 4) 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 Extended One-Generation Reproductive Toxicity Study (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.

References

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.

Laws, S. and V. Wilson (2014), "Integrated summary report: Validation of two binding assays using human recombinant estrogen receptor alpha (hrER alpha)", Environmental Protection Agency, Washington, DC, https://cfpub.epa.gov/si/si_public_record_report.cfm?dirEntryId=307944.

OECD (2015), "Performance standards for the human recombinant estrogen receptor binding assay (intended for the developers of new or modified similar test methods)", OECD Series on Testing and Assessment, No. 222, OECD, Paris, www.oecd.org/officialdocuments/publicdisplaydocumentpdf/?cote=ENV/JM/MONO(2015)29&doclanguage=en.

OECD (2008), *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 (2005), "Guidance document on the validation and international acceptance of new or updated test methods for hazard assessment", OECD Series on Testing and Assessment, No. 34, OECD, Paris, www.oecd.org/officialdocuments/publicdisplaydocumentpdf/?doclanguage=en&cote=env/jm/mono(2005)14.

US EPA (2009), "Integrated summary report for validation of an estrogen receptor binding assay using rat uterine cytosol as source of receptor as a potential screen in the Endocrine Disrupter Screening Program Tier 1 battery", Environmental Protection Agency, Washington, DC.

Table C.1.1. **Performance-Based Test Guideline for Human Recombinant Estrogen Receptor (hrER) *In Vitro* Assays to Detect Chemicals with ER Binding Affinity (OECD TG 493): 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 androgen receptor-based assays and the Steroidogenesis Assay (Level 2). Thyroid hormone receptor 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. Data from the ER STTA are assumed to be unavailable, but a decision about the next step to be taken will also depend on the availability of this assay and QSAR data.

Existing results: ** "Effects (*in vivo* effects of concern)" assumes various information, such as data from repeat dose oral toxicity studies, reproduction/developmental toxicity screening tests, read-across from analogues, will be available.

Scenarios	Result of hrER binding 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	+	+	+	Interaction with estrogen receptor (ER) combined with effects on AR/T/S and potential for adverse effects via multiple mechanisms.	Perform ER STTA or assay from Levels 3-5, e.g. Uterotrophic Bioassay (UT) (Level 3) or female Peripubertal (PP) Assay (Level 4) or Extended One-Generation Reproductive Toxicity Study (EOGRTS) or two-generation assays or partial/full non-mammalian wildlife life cycle tests, e.g. TG 241 and TG 240 (Level 4/5).	Binding to hrER indicates strong probability of binding to ERs in other taxa. If existing data are from Level 5, 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). If existing data are from Level 4 vertebrate wildlife assays, then Level 5 assays should provide more comprehensive data on adverse effects on endocrine-relevant endpoints over more extensive parts of the life cycle of the organism, although some Level 4 assays (e.g. TG 234) may be sufficient for this purpose. If existing data are from UT assays, then Level 4 assays will provide data on multiple modalities. Consider route of exposures for existing effects data and possible transformation products and 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*. However, note that uptake and metabolism of chemicals can be different between vertebrate wildlife species.
B	+	+	–	Interaction with ER combined with effects on AR/T/S but effects not detected in *in vivo* studies. Interaction with ER does not result in adverse effects in the selected species under the conditions of the test. Metabolic differences may explain *in vitro*/*in vivo* differences.	Perform binding assay or ER STTA with added metabolising system or Assay from Levels 3-4, e.g. UT assay or fish screen (OECD TG 229/230) (Level 3) or female PP assay (Level 4).	Binding to hrER indicates strong probability of binding to ERs in other taxa. If existing data are from an adequate Level 5 study 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 Level 4 vertebrate wildlife assays, then Level 5 assays should provide more comprehensive data on adverse effects on endocrine-relevant endpoints over more extensive parts of the life cycle of the organism, although some Level 4 assays (e.g. TG 234) may be sufficient for this purpose. If existing data are from UT assays, then Level 4 assays will provide data on multiple modalities. Consider route of exposures for existing effects data and possible transformation products and implications of ADME characteristics of the chemical. The chemical itself may give positive *in vitro* results but may not be absorbed or may be metabolised to an inactive metabolite *in vivo*. However, note that uptake and metabolism of chemicals can be different between mammalian and non-mammalian wildlife species.
C	+	+	Eq/0	Interaction with ER combined with effects on AR/T/S but no or equivocal data from *in vivo* studies. Interaction with ER may not result in adverse effects in the selected species under the conditions of the test.	Perform ER STTA or Perform assay from Levels 3-4 e.g. UT assay or fish screen (OECD TG 229/230) (Level 3) or female PP assay (Level 4).	Binding to hrER indicates strong probability of binding to ERs in other taxa. Consider route of exposures for equivocal existing effects data and possible implications of ADME characteristics of the chemical. Equivocal results may indicate chemical has multiple modes of action (MOA). Check data on chemical analogues.

Scenarios	Result of hrER binding 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	+	–	+	Interaction with ER and potential for adverse effects.	Perform ER STTA or Perform assay from Levels 3-4, e.g. UT assay or fish screen (OECD TG 229/230) (Level 3) or female PP assay (Level 4).	Binding to hrER indicates strong probability of binding to ERs in other taxa. If existing data are from an adequate Level 5 assay, 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). If existing data are from Level 4 vertebrate wildlife assays, then Level 5 assays should provide more comprehensive data on adverse effects on endocrine-relevant endpoints over more extensive parts of the life cycle of the organism, although some Level 4 assays (e.g. TG 234) may be sufficient for this purpose. If existing data are from UT assay, then Level 4 assays will provide data on multiple modalities. 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	+	–	–	Interaction with ER but effects not detected in *in vivo* studies. Interaction with ER does not result in adverse effects in the selected species under the conditions of the test. Metabolic differences may explain *in vitro*/*in vivo* differences.	Perform binding assay or ER STTA with added metabolising system or Assay from Levels 3-4, e.g. UT assay or fish screen (OECD TG 229/230) (Level 3) or female PP assay (Level 4).	Binding to hrER indicates strong probability of binding to ERs in other taxa. 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 Level 4 vertebrate wildlife assays, then Level 5 assays should provide more comprehensive data on adverse effects on endocrine-relevant endpoints over more extensive parts of the life cycle of the organism, although some Level 4 assays (e.g. TG 234) may be sufficient for this purpose. If existing data are from UT assays, then Level 4 assays will provide data on multiple modalities. Consider route of exposures for existing effects data and possible transformation products and implications of ADME characteristics of the chemical. The chemical itself may give positive *in vitro* results but may not be absorbed or may be metabolised to an inactive metabolite *in vivo*. However, note that uptake and metabolism of chemicals can be different between mammalian and non-mammalian wildlife species. Check data on chemical analogues.
F	+	–	Eq/0	Interaction with ER but no or equivocal data from *in vivo* studies.	Perform ER STTA or assay from Levels 3-4, e.g. UT assay or fish screen (OECD TG 229/230) (Level 3) or female PP assay (Level 4).	Binding to hrER indicates strong probability of binding to ERs in other taxa. Consider route of exposures for equivocal existing effects data and possible implications of ADME characteristics of the chemical. Equivocal results may indicate chemical has multiple MOA. The chemical itself may give positive *in vitro* results but may not be absorbed or may be metabolised to an inactive metabolite *in vivo*. Check data on chemical analogues.

Scenarios	Result of hrER binding 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)**			
G	+	Eq/0	+	Interaction with ER and potential for adverse effects via ER. May act via E,A,T,S mechanism and may or may not require metabolic activation.	Perform ER STTA.	Binding to hrER indicates strong probability of binding to ERs in other taxa. If existing data are from an adequate Level 5 assay, 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). If existing data are from Level 4 vertebrate wildlife assays, then Level 5 assays should provide more comprehensive data on adverse effects on endocrine-relevant endpoints over more extensive parts of the life cycle of the organism, although some Level 4 assays (e.g. TG 234) may be sufficient for this purpose. If existing data are from UT assays, then Level 4 assays will provide data on multiple modalities. Check data on chemical analogues. Further mechanistic studies may help determine MOA. Equivocal results may indicate chemical has multiple MOA. A positive result could have arisen from other (E,A,T,S or non-E,A,T,S) mechanisms, e.g. HPG axis.
H	+	Eq/0	–	Interaction with ER but effects not detected in *in vivo* studies. Interaction with ER does not result in adverse effects in the selected species under the conditions of the test. Metabolic differences may explain *in vitro*/*in vivo* differences.	For the "0" scenario, perform ER STTA. For the "Eq" scenario, perform ER STTA.	Binding to hrER indicates strong probability of binding to ERs in other taxa. 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 Level 4 vertebrate wildlife assays, then Level 5 assays should provide more comprehensive data on adverse effects on endocrine-relevant endpoints over more extensive parts of the life cycle of the organism, although some Level 4 assays (e.g. TG 234) may be sufficient for this purpose. If existing data are from UT assays, then Level 4 assay will provide data on multiple modalities. Consider route of exposures for existing effects data and possible transformation products and implications of ADME characteristics of the chemical. The chemical itself may give positive *in vitro* results but may not be absorbed or may be metabolised to an inactive metabolite *in vivo*. However, note that uptake and metabolism of chemicals can be different between mammalian and non-mammalian wildlife species. Check data on chemical analogues. Further mechanistic studies may help determine MOA. Equivocal results may indicate chemical has multiple MOA.

Scenarios	Result of hrER binding 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)**			
I	+	Eq/0	Eq/0	Interaction with ER with unknown potential for effects in *in vivo* studies. May act via ER and may or may not require metabolic activation. Unknown potential for adverse effects.	For the "0" scenario, ER STTA with added metabolising system. For the "Eq" scenario, UT assay or fish screen (OECD TG 229/230) (Level 3) if existing data indicate this is needed.	Binding to hrER indicates strong probability of binding to ERs in other taxa. Consider route of exposures for equivocal existing effects data and possible implications of ADME characteristics of the chemical. The chemical itself may give positive *in vitro* results but may not be absorbed or may be metabolised to an inactive metabolite *in vivo*. Check data on chemical analogues. Equivocal results may indicate chemical has multiple MOA.
J	–	+	+	No evidence for interaction with ER. Effects on AR/T/S and potential for adverse effects via E,A,T,S mechanisms.	Perform ER binding assay or ER STTA with added metabolising system or Perform assay from Levels 3-4, e.g. UT assay or fish screen (OECD TG 229/230) (Level 3) or female PP assay (Level 4).	Lack of binding to hrER indicates binding to ERs in other taxa is unlikely. If existing data are from an adequate Level 5 assay, there may be sufficient information to conclude concern for endocrine disruption. If existing data are from Level 4 vertebrate wildlife assays, then Level 5 assays should provide more comprehensive data on adverse effects on endocrine-relevant endpoints over more extensive parts of the life cycle of the organism, although some Level 4 assays (e.g. OECD TG 234) may be sufficient for this purpose. If existing data are from UT assays, then Level 4 assays will provide data on multiple modalities. Consider route of exposures for existing effects data and possible transformation products and implications of ADME characteristics of the chemical. Check data on chemical analogues. Further mechanistic studies may help determine MOA.
K	–	+	–	No evidence for interaction with ER. Effects on AR/T/S but effects not detected in *in vivo* studies. Metabolic differences explain *in vitro*/*in vivo* A,T,S differences.	Perform assay from Levels 3-4, e.g. fish screen (OECD TG 229/230) (Level 3) or male or female PP assay (Level 4).	Lack of binding to hrER indicates binding to ERs in other taxa is unlikely. 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 Level 4 vertebrate wildlife assays then Level 5 assays should provide more comprehensive data on adverse effects on endocrine-relevant endpoints over more extensive parts of the life cycle of the organism, although some Level 4 assays (e.g. OECD TG 234 [FSDT]) may be sufficient for this purpose. If existing data are from UT assays, then Level 4 assays will provide data on multiple modalities. Metabolic deactivation of chemical may occur *in vivo* so that potential *in vitro* A,T,S activity is not realised. Consider possible routes of exposure, implications of metabolism.
L	–	+	Eq/0	No evidence for interaction with ER. Effects on AR/T/S but effects not detected in *in vivo* studies. Unknown potential for adverse effects. Metabolic differences explain *in vitro*/*in vivo* A,T,S differences.	Perform assay from Levels 3-4, e.g. fish screen (OECD TG 229/230) (Level 3), or male or female PP assay (Level 4).	Lack of binding to hrER indicates binding to ERs in other taxa is unlikely. Metabolic deactivation of chemical may occur *in vivo* so that potential *in vitro* A,T,S activity is not realised. Consider possible routes of exposure, implications of metabolism. Check data on chemical analogues. Equivocal results may indicate chemical has multiple MOA.

Scenarios	Result of hrER binding 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)**			
M	–	–	+	No evidence for interaction with ER. Metabolic differences may explain *in vitro*/*in vivo* differences. Effects seen in existing studies are via non-E,A,T,S or non-endocrine mechanisms.	Perform ER STTA with added metabolising system or Perform assay from Levels 3-4, e.g. fish screen (OECD TG 229/230) (Level 3) or male or female PP assay (Level 4).	Lack of binding to hrER indicates binding to ERs in other taxa is unlikely. If existing data are from an adequate Level 5 assay, there may be sufficient information to conclude concern for endocrine disruption. If existing data are from Level 4 vertebrate wildlife assays, then Level 5 assays should provide more comprehensive data on adverse effects on endocrine-relevant endpoints over more extensive parts of the life cycle of the organism, although some Level 4 assays (e.g. OECD TG 234 [FSDT]) may be sufficient for this purpose. If existing data are from UT assays, then Level 4 assays will provide data on multiple modalities. Metabolic activation of chemical may occur *in vivo*. Consider possible routes of exposure, implications of metabolism. Check data on chemical analogues. Further mechanistic studies may help determine MOA.
N	–	–	–	No evidence for interaction with ER. No evidence of adverse effects.	Possibly no need for further testing. If there is uncertainty, may perform assay from Levels 3-4, e.g. fish screen (OECD TG 229/230) (Level 3) or male or female PP assay (Level 4).	Lack of binding to hrER indicates binding to ERs in other taxa is unlikely. If existing data are from an adequate Level 5 assay, 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). If existing data are from Level 4 vertebrate wildlife assays, then Level 5 assays should provide more comprehensive data on adverse effects on endocrine-relevant endpoints over more extensive parts of the life cycle of the organism, although some Level 4 assays (e.g. OECD TG 234 [FSDT]) may be sufficient for this purpose. If existing data are from UT assays, then Level 4 assays will provide data on multiple modalities. Check data on chemical analogues.
O	–	–	Eq/0	No evidence for interaction with ER. Metabolic differences may explain *in vitro*/*in vivo* differences. Unknown potential for adverse effects via other mechanisms.	Perform ER STTA with added metabolising system or Fish screen (OECD TG 229/230) (Level 3) or male or female PP assay (Level 4) if existing data indicate this is needed.	Lack of binding to hrER indicates binding to ERs in other taxa is unlikely. Consider possible routes of exposure, implications of metabolism. Check data on chemical analogues.

Scenarios	Result of hrER binding 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)**			
P	–	Eq/0	+	No evidence for interaction with ER. Metabolic differences may explain *in vitro*/*in vivo* differences. Unknown potential for adverse effects via other mechanisms.	Perform ER STTA with added metabolising system.	Lack of binding to hrER indicates binding to ERs in other taxa is unlikely. If existing data are from an adequate Level 5 assay, there may be sufficient information to conclude concern for endocrine disruption. If existing data are from Level 4 vertebrate wildlife assays, then Level 5 assays should provide more comprehensive data on adverse effects on endocrine-relevant endpoints over more extensive parts of the life cycle of the organism, although some Level 4 assays (e.g. OECD TG 234 [FSDT]) may be sufficient for this purpose. If existing data are from UT assays, then Level 4 assays will provide data on multiple modalities. Consider possible routes of exposure, implications of metabolism. 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 interaction with ER. No evidence of adverse effects.	Perform ER STTA with added metabolising system.	Lack of binding to hrER indicates binding to ERs in other taxa is unlikely. 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 Level 4 vertebrate wildlife assays, then Level 5 assays should provide more comprehensive data on adverse effects on endocrine-relevant endpoints over more extensive parts of the life cycle of the organism, although some Level 4 assays (e.g. TG 234 [FSDT]) may be sufficient for this purpose. If existing data are from UT assays, then Level 4 assays will provide data on multiple modalities. Check data on chemical analogues. Further mechanistic studies may help determine MOA.
R	–	Eq/0	Eq/0	No evidence for interaction with ER. Unknown potential for adverse effects via other mechanisms.	For the "0" scenario, perform ER STTA with added metabolising system or Perform assay from Levels 3-4, e.g. UT assay or fish screen (OECD TG 229/230) (Level 3) or female PP assay (Level 4).	Lack of binding to hrER indicates binding to ERs in other taxa is unlikely. Consider possible routes of exposure, implications of metabolism. Check data on chemical analogues. Further mechanistic studies may help determine MOA. Equivocal results may indicate chemical has multiple MOA.

C.1.2. Performance-Based Test Guideline for Stably Transfected Transactivation *In Vitro* Assays to Detect Estrogen Receptor Agonists and Antagonists (ER STTA) (OECD TG 455)

Status: Assay validated by the OECD.

Modality detected/endpoints: Activation of reporter gene linked to ER (agonist assay). Inhibition of activation of reporter gene linked to ER (antagonist assay).

Background to the assay

157. The Stably Transfected hERα Transcriptional Activation Assay (ER STTA) is an *in vitro* screening assay to detect substances that bind to hER and activate the transcription of estrogen responsive genes. It is an *in vitro* tool that provides mechanistic data. Several ER STTA assays in common use can be found in the literature (e.g. Andersen et al. [2002]; Escande et al. [2006]; Takeyoshi et al. [2002]; Du et al. [2010]; Witters et al. [2010]). One of the first versions of this assay used was the "yeast estrogen screen" (Routledge and Sumpter, 1996; Odum et al., 1997; Sheahan et al., 2002) which is still widely used for screening of environmental samples. Some variants of the yeast-based assays (*Saccharomyces cerevisiae* and *Arxula adeninivorans*) carrying the human ERα-receptor have recently been standardised within the ISO 19040 series: Determination of the estrogenic potential of water and waste water, together with human cell line-based transactivation assays (see Paragraph 20 in Section A). The guidance in this building block can be cautiously used for these assays.

The previous version of OECD TG 455 described agonist interaction with hERα utilising the hERα-HeLa-9903 cell line (derived from a human cervical tumor) and a luciferase reporter gene. Antagonist interaction was provided in a separate guidance. Another OECD TG (457) for the ER STTA assay was adopted in October 2012 and described the agonist and antagonist assay using the BG1Luc cell line. However, OECD TG 455 was revised (September 2016) to become a performance-based test guideline (PBTG) and include both methods. OECD TG 457 became redundant in January 2018. The most recent version of OECD TG 455 is a PBTG that describes the two ER STTA methods. Agonism and antagonism assays are included in both test methods. The two methods are:

- the Stably Transfected TA assay using the (h) ERα-HeLa-9903 cell line
- the VM7Luc ER STTA assay using the VM7Luc4E2 cell line which predominately expresses hERα with some contribution from hERβ.

158. Note that the VM7Luc4E2 cell line was originally designated as the BG1Luc cell line. However, in July 2016 in-depth analysis of the cells revealed that the cell line used to develop the assay was not the BG1 human ovarian carcinoma cell line, but was instead a variant of the MCF7 human breast cancer cell line. The designation of the cell line was

then changed accordingly. This does not affect published validation studies nor the utility and application of this assay for screening of estrogenic/antiestrogenic test chemicals.

159. Both test methods use a human cell line stably transfected with ERα, the main difference being that the VM7Luc4E2 cell line also expresses a minor amount of endogenous ERβ. Both assays use a luciferase reporter gene. The two test methods are the first to be included in the PBTG, other test methods are in validation and may be included later. Performance standards to enable the development and validation of similar test methods have also been published (OECD, 2012a; 2012b; 2012c). During validation, both methods gave similar results. There was almost 100% agreement between the two test methods, based on the classifications of all the substances except for one (mifepristone) for the antagonist assay, and each substance was correctly classified as an ER agonist/antagonist or negative.

160. OECD TG 455 provides a positive or negative result for the ability of a chemical to induce hERα-mediated transactivation of luciferase gene expression (agonist assay) compared to a vehicle control. The antagonist assay determines whether a reduction in response occurs when cells are co-exposed to a chemical and a potent estrogen agonist compared to the potent estrogen agonist alone. Any reduction in response must occur in the absence of cytotoxicity. There is currently no universally agreed method for interpreting ER STTA data. However, both qualitative (e.g. positive/negative) and/or quantitative (e.g. EC50, PC50, IC50) assessments of ER-mediated activity should be based on empirical data and sound scientific judgment. Where possible, positive results should be characterised by both the magnitude of the effect as compared to the vehicle (solvent) control or reference estrogen and the concentration at which the effect occurs (e.g. an EC50, PC50, RPCMax, IC50, etc.).

161. Consistent results should be achieved in at least two out of two or three runs of the assay. To be acceptable, the results should also meet the performance standards given in the assay. Small deviations are unlikely to have compromised the assay, but judgement should be made on a case-by-case basis.

162. Both test methods showed a high degree of sensitivity and specificity for both estrogenic and antiestrogenic responses in the validation studies when compared with the ER binding, UT assays and published reports determining the ability of a chemical to elicit an equivalent response *in vivo*. OECD TG 455 requires strict control of assay conditions in order to maintain the accuracy and reliability of response. Demonstration of laboratory proficiency with proficiency chemicals is required at the outset: 14 for the agonist assay and 10 for the antagonist assay. These chemicals are a subset of the substances provided in the performance standards for the ER STTA (OECD, 2012b; 2012c), represent the classes of chemicals commonly associated with ER agonist or antagonist activity, exhibit a suitable range of potency expected for ER agonists/antagonists (i.e. strong to weak), and include negatives. Periodic testing with proficiency chemicals should also be carried out. In addition, each experiment requires reference chemicals. For example, the ERα-HeLa-9903 cell line test method requires for the agonist assay: a strong estrogen (E2), a weak estrogen (17α-estradiol), a very weak agonist (17α-methyltestosterone) and a negative substance (corticosterone); and for the antagonist assay: a positive substance (tamoxifen) and a negative substance (flutamide). In the assay, each plate requires positive and vehicle controls. Criteria for the degree of response with these chemicals are given in the TG. The assay also requires a minimum of 80% cell viability; this is critical for the antagonist assay where positive results can only be demonstrated in the absence of cytotoxicity. Compliance

with the quality control criteria and with the performance criteria should be accepted before evaluating results from this assay.

163. A limitation of OECD TG 455, related to the reporter gene luciferase, is the potential for chemicals to increase chemiluminescence via non-ERα mechanisms, thus possibly giving a false positive response. This has been reported for certain phytoestrogens such as genistein and daidzein but not for industrial chemicals (Kuiper et al., 1998; Escande et al., 2006). This may be recognised by incomplete or unusual dose response curves and can be tested by performing a specific antagonist assay (provided as an Appendix 2 to OECD TG 455). Other ER STTAs that do not use luciferase as a reporter gene may not have this drawback (Escande et al., 2006). Confirmation of the results (both positive and negative) could be obtained by using a cell system relying on a different reporter/read-out. For a review, see Thorne, Inglese and Auld (2010).

164. The ER STTA will not detect substances that act by other mechanisms (e.g. AR, TR and steroidogenesis interference). These chemicals will, however, be detected in AR-, TR- and steroidogenesis-specific assays and therefore results from a suite of *in vitro* tests should be considered together. The assay will not detect substances that act by affecting the hypothalamic/pituitary/gonadal (HPG) system as an *in vivo* intact axis is required for this.

When/why the assay may be used

165. Although the ER STTA may be used at any stage in the hazard assessment process, the most likely use scenario is during initial assessment of chemicals for their ability to interact with endocrine systems *in vitro* (i.e. E,A,T,S modalities). The ER STTA is frequently conducted following a positive result in the ER binding assay. Assays for interaction with other modalities (e.g. AR, ER and steroidogenesis), are likely to be conducted at the same time so that all results can be considered together. TR and other assays concerning mechanisms of thyroid disruption may be available, but they are not in common use. OECD TG 445 does not include the use of a xenobiotic metabolising system, but consideration should be given to the inclusion of this (Jacobs et al., 2008, 2013; OECD, 2008) depending on the circumstances, e.g. if the metabolism of a chemical is unknown, although the methods for inclusion of xenobiotic metabolising systems are not yet validated (see Paragraph 50). Alternatively, for a chemical with known metabolites, these could also be tested in the ER STTA.

166. Another use scenario may be following effects obtained in higher tier tests, for example accelerated puberty onset in females, but which are not exclusively indicative of an effect on ER. Selection of the most appropriate tests has to be on a case-by-case basis, but also considering the need to minimise animal testing. A further example could be results obtained in other apical assays, e.g. OECD TG 408 (90-day toxicity test); effects on reproductive organs may be investigated further by testing in the ER STTA in combination with AR- and steroidogenesis-based assays.

Introduction to the table of scenarios

167. Table C.1.2 gives guidance on a further step to take in the event of a positive (+) or negative (-) result in the ER STTA 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.

168. The results of the ER STTA are given in the second column. Criteria for positive and negative results in OECD TG 455 for both test methods are given in the test guideline. Reproducible results in at least two runs are required. If two runs do not give reproducible results (e.g. a test chemical is positive in one run and negative in the other run), or if a higher degree of certainty is required regarding the outcome of the assay, at least three independent runs should be conducted. In this case the classification is based on the two concordant results out of the three. It is important that quality and proficiency criteria are demonstrated for both positive and negative results. The concentrations tested should remain within the solubility range of the test chemicals and not demonstrate cytotoxicity.

169. Equivocal results for the guideline are not included in the table because these data generally require further interrogation about the result itself. This assay is a screen and therefore a clear positive or negative result should be obtained. In the event of an equivocal result, the considerations mentioned above about control quality and proficiency criteria should be taken into account and further investigations made.

Existing data to be considered

170. Existing "mechanism" *in vitro* data are assumed to be available from 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.

171. Existing "effects" data refer to *in vivo* effects "of concern" (i.e. data from Level 4 or 5 vertebrate wildlife assays). These may come from varied sources and will depend on the type of substance (e.g. new chemicals, high production volume [HPV] chemical, pesticide). Thus, available data may range from repeated dose toxicity studies (28-day, 90-day), combined repeat dose/reproductive screening assays or fish screening assays, to chronic toxicity studies and multigeneration reproductive tests in vertebrate wildlife species. 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 modes of action, including endocrine modalities. 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 fish or amphibians (for example, OECD TG 240 or TG 241), may also have endocrine effects in mammals, but the physiological consequences of the effects are likely to be different.

172. Data may also be available from Level 3 tests (H and UT assays), but as the UT assay primarily detects (*in vivo*) the same modality as the ER STTA, it is unlikely that it would be conducted prior to this. An AMA may also be available, but as this test primarily detects thyroid disruption in amphibians it is unlikely to provide useful data for E-modalities.

173. When considering the results of the ER STTA, all available data should be used in order to reach a conclusion and a weight of evidence approach taken. This may include

HTS data, read-across data from structural analogues and 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

174. The scenarios (A to R) presented in Table C.1.2 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 455 uses hrER, the well-conserved nature of ER across taxa should be a strong indication that results in this assay are relevant to other vertebrate 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. Further considerations specific to each scenario are given in the table.

175. Scenarios A to C represent positive results in the ER STTA assay in the presence of positive *in vitro* mechanistic data and positive, negative or equivocal *in vivo* effects data. A positive result in an ER STTA assay is strong evidence for (anti)estrogenic activity that may or may not be supported by the *in vivo* effects data. In the case of positive *in vivo* effects data, there may be sufficient evidence to conclude concern for endocrine disruption and therefore no need for further screening. *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. 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. 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 without causing reproductive or developmental effects, as well as substances causing tumors in other endocrine-sensitive tissues. Mode of action (MOA) data to provide a clear interpretation may be required by some regulatory agencies.

176. Scenarios D to F represent positive results in the ER STTA assay in the presence of negative *in vitro* mechanistic data and positive, negative or equivocal *in vivo* effects data. Unless the metabolic profile of the test substance is known, one option may be to conduct these *in vitro* assays with an added metabolising system. If the metabolic profile is known, then an *in vivo* test may be advisable. The choice of tests will depend on the available *in vivo* effects data. 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. 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.

177. Scenarios G to I represent positive results in the ER STTA assay 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, e.g. a study that is equivocal for thyroid effects may still be of value in evaluating (anti)estrogenic effects. In all three scenarios, the recommended first step is to obtain reliable mechanistic (*in vitro*) data rather than proceed directly to *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.

178. Scenarios J to L represent negative results in the ER STTA assay in the presence of positive *in vitro* mechanistic data and positive, negative or equivocal *in vivo* effects data. The limitations of the ER STTA assay should be considered first (e.g. lack of metabolic activation, possible involvement of other factors). The positive *in vitro* mechanistic data indicates possible alternative A,T,S mechanisms. To confirm lack of ER-related activity in the presence of *in vivo* data, an ER STTA with added metabolising capability could be performed. Otherwise, *in vivo* tests will confirm or refute E,A,T,S activity.

179. Scenarios M to O represent negative results in the ER STTA 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. The limitations of the ER STTA assay should also be considered (as described for Scenarios J to L). To confirm lack of ER-related activity in the presence of *in vivo* data, an ER STTA with added metabolising capability could be performed. Otherwise, *in vivo* tests will confirm or refute E,A,T,S activity (Scenarios M and O).

180. Scenarios P to R represent negative results in the ER STTA assay in the presence of various combinations of missing or equivocal data. The limitations of the ER STTA binding assay should be considered first (as described for Scenarios J to L). As with the positive result scenarios above, the next step to take for Scenarios P to R 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.

181. In all scenarios (A to R), the next step to take to strengthen weight of evidence will depend on the existing information. Table C.1.2 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.

182. In general, a decision about whether or not to conduct *in vivo* vertebrate wildlife tests will depend on the weight of evidence of new and existing data. If most available data (e.g. the results of the ER STTA assay, predictions from QSARs, "read-across" from data on similar substances and results from mammalian *in vivo* assays) suggest that the substance has the potential to cause endocrine disruption via the estrogen receptor (i.e. the level of suspicion about endocrine disrupting action is high – corresponding to Scenario A), then consideration should be given to the conduct of a higher level test.

183. For non-mammalian wildlife species, higher level tests with fish or amphibians (i.e. OECD TG 234, TG 240 or TG 241) are recommended. Choice about which of these tests is most appropriate will be driven *inter alia* by mode of action considerations, and by whether multigeneration effects are to be expected. If available data only raise a low or moderate level of suspicion about endocrine disrupting action (e.g. the data appear to conflict with each other), then consideration should be given to the conduct of a fish screen (i.e. EASZY, OECD TG 229 or TG 230).

184. Potency of any interaction with ER should also be considered in relation to cross-species effects. Ankley et al. (2016) showed that chemicals with moderate to high estrogenic potency in mammalian systems should be priority chemicals in non-mammalian vertebrates. However, applicablity to invertebrates was uncertain because of a lack of knowledge of the biological role(s) of possible ERα orthologs found in phyla such as annelids. For low-affinity chemicals, comparative analysis of *in vitro* data for low-affinity chemicals suggested that mammalian-based assays may not effectively capture ERα interactions for fish and reptiles.

185. For mammals, similar considerations apply but lower level tests (e.g. Level 3 or 4) 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.

References

Andersen, H.R. et al. (2002), "Effects of currently used pesticides in assays for estrogenicity, androgenicity, and aromatase activity *in vitro*", *Toxicology and Applied Pharmacology.*, Vol. 179/1, pp. 1-12, https://doi.org/10.1006/taap.2001.9347.

Ankley, G.T. et al. (2016), "Evaluation of the scientific underpinnings for identifying estrogenic chemicals in nonmammalian taxa using mammalian test systems", *Environmental Toxicology and Chemistry*, Vol. 35/11, pp. 2806-2816, https://doi.org/10.1002/etc.3456.

Du, G. et al. (2010), "Assessing hormone receptor activities of pyrethroid insecticides and their metabolites in reporter gene assays", *Toxicological Sciences*, Vol. 116/1, pp. 58-66, https://doi.org/10.1093/toxsci/kfq120.

Escande, A. et al. (2006), "Evaluation of ligand selectivity using reporter cell lines stably expressing estrogen receptor alpha or beta", *Biochemical Pharmacology*, Vol. 71/10, pp. 1459-1469, https://doi.org/10.1016/j.bcp.2006.02.002.

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.

Kuiper, G.G. et al. (1998), "Interaction of estrogenic chemicals and phytoestrogens with estrogen receptor beta", *Endocrinology*, Vol. 139/10, pp. 4252-3263, https://doi.org/10.1210/endo.139.10.6216.

Odum, J. et al. (1997), "The rodent uterotrophic assay: Critical protocol features, studies with nonyl phenols, and comparison with a yeast estrogenicity assay", *Regulatory Toxicology and Pharmacology*, Vol. 25/2, pp. 176-188, https://doi.org/10.1006/rtph.1997.1100.

OECD (2012a), *Detailed Review Paper on the State of the Science on Novel* In Vitro *and* In Vivo *Screening and Testing Methods and Endpoints for Evaluating Endocrine Disrupters*, OECD Series on Testing and Assessment, No. 178, OECD Publishing, Paris, http://dx.doi.org/10.1787/9789264221352-en.

OECD (2012b), "Performance standards for stably transfected transactivation *in vitro* assays to detect estrogen agonists for TG 455", OECD Series on Testing and Assessment, No. 173, OECD, Paris, www.oecd.org/officialdocuments/publicdisplaydocumentpdf/?cote=env/jm/mono(2012)18&doclanguage=en.

OECD (2012c), "Performance standards for stably transfected transactivation *in vitro* assays to detect estrogen receptor antagonists", OECD Series on Testing and Assessment, No. 174, OECD, Paris, www.oecd.org/env/ehs/testing/19_REV1.pdf.

OECD (2008), *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.

Routledge, E.J. and J.P. Sumpter (1996), "Estrogenic activity of surfactants and some of their degradation products assessed using a recombinant yeast screen", *Environmental Toxicology and Chemistry*, Vol. 15/3, pp. 241-248, https://doi.org/10.1002/etc.5620150303.

Sheahan, D.A. et al. (2002), "Reduction in the estrogenic activity of a treated sewage effluent discharge to an English river as a result of a decrease in the concentration of industrially derived surfactants", *Environmental Toxicology and Chemistry*, Vol. 21, pp. 515-419, https://doi.org/10.1002/etc.5620210307.

Takeyoshi, M. et al. (2002), "The efficacy of endocrine disrupter screening tests in detecting anti-estrogenic effects downstream of receptor-ligand interactions", *Toxicology Letters*, Vol. 126/2, pp. 91-98, https://doi.org/10.1016/S0378-4274(01)00446-5.

Thorne, N., J. Inglese and D.S. Auld (2010), "Illuminating insights into firefly luciferase and other bioluminescent reporters used in chemical biology", *Chemical Biology*, Vol. 17/6, pp. 646-657, http://dx.doi.org/10.1016/j.chembiol.2010.05.012.

Witters, H. et al. (2010), "The assessment of estrogenic or anti-estrogenic activity of chemicals by the human stably transfected estrogen sensitive MELN cell line: Results of test performance and transferability", *Reproductive Toxicology*, Vol. 30/1, pp. 60-72, https://doi.org/10.1016/j.reprotox.2010.02.008.

Table C.1.2. **Performance-Based Test Guideline for Stably Transfected Transactivation *In Vitro* Assays to Detect Estrogen Receptor Agonists and Antagonists (ER STTA) (OECD TG 455): 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 (S-) based assays (Level 2). The ER binding assay is likely to be performed prior to the Stably Transfected Human Estrogen Receptor-alpha Transactivation Assay for Detection of Estrogenic Agonist-Activity of Chemicals (ER STTA). 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 various information, such as data from repeat dose oral toxicity studies, reproduction/developmental toxicity screen tests, read-across from analogues, will be available.

Scenarios	Result of OECD TG 455 (ER STTA)	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	+	+	+	Estrogen receptor (ER) (ant)agonism combined with effects on AR/T/S and potential for adverse effects via multiple mechanisms.	Perform assay from Levels 3-5, e.g. Uterotrophic Bioassay (UT) assay (Level 3) or female Peripubertal (PP) Assay (Level 4) or Extended One-Generation Reproductive Toxicity Study (EOGRTS) or two-generation assays (Level 5) or partial/full non-mammalian wildlife life cycle tests, e.g. OECD TG 241 and TG 240 (Level 4/5).	A positive result indicates strong probability of interaction with ERs in other taxa. If existing data are from a Level 5 assay, 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)s with a carcinogenic potential, OECD TG 451-3 may be more sensitive). If existing data are from Level 4 vertebrate wildlife assays, then Level 5 assays should provide more comprehensive data on adverse effects on endocrine-relevant endpoints over more extensive parts of the life cycle of the organism, although some Level 4 assays (e.g. TG 234 [FSDT]) may be sufficient for this purpose. If existing data are from UT assays, then Level 4 assays will provide data on multiple modalities. Consider route of exposures for existing effects data and possible transformation products and 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*. However, note that uptake and metabolism of chemicals can be different between mammalian and non-mammalian wildlife species.
B	+	+	–	ER (ant)agonism combined with effects on AR/T/S but effects not detected in *in vivo* studies. Weak ER (ant)agonism does not result in adverse effects in the selected species under the conditions of the test. Metabolic differences may explain *in vitro*/*in vivo* differences.	Perform Stably Transfected Human Estrogen Receptor-alpha Transactivation Assay for Detection of Estrogenic Agonist-Activity of Chemicals (ER STTA) with added metabolising system or Assay from Levels 3-4, e.g. UT assay or fish screen (OECD TG 229/230) (Level 3) or female PP assay (Level 4).	A positive result indicates strong probability of interaction with ERs in other taxa. 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 Level 4 vertebrate wildlife assays, then Level 5 assays should provide more comprehensive data on adverse effects on endocrine-relevant endpoints over more extensive parts of the life cycle of the organism, although some Level 4 assays (e.g. TG 234 [FSDT]) may be sufficient for this purpose. If existing data are from UT assays, then Level 4 assays will provide data on multiple modalities. Consider route of exposures for existing effects data and possible transformation products and implications of ADME characteristics of the chemical. The chemical itself may give positive *in vitro* results but may not be absorbed or may be metabolised to an inactive metabolite *in vivo*. However, note that uptake and metabolism of chemicals can be different between mammalian and non-mammalian wildlife species.

Scenarios	Result of OECD TG 455 (ER STTA)	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)**			
C	+	+	Eq/0	ER (ant)agonism combined with effects on AR/T/S but no or equivocal data from *in vivo* studies. Weak ER (ant)agonism may not result in adverse effects in the selected species under the conditions of the test.	Perform assay from Levels 3-4, e.g. UT assay or fish screen (OECD TG 229/230) (Level 3) or female PP assay (Level 4).	A positive result indicates strong probability of interaction with ERs in other taxa. Consider route of exposures for equivocal existing effects data and possible implications of ADME characteristics of the chemical. Equivocal results may indicate chemical has multiple modes of action (MOA). Check data on chemical analogues.
D	+	–	+	ER (ant)agonism and potential for adverse effects.	Perform assay from Levels 3-4, e.g. UT assay or fish screen (OECD TG 229/230) (Level 3) or female PP assay (Level 4).	A positive result indicates strong probability of interaction with ERs in other taxa. If existing data are from an adequate Level 5 assay, 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). If existing data are from Level 4 vertebrate wildlife assays, then Level 5 assays should provide more comprehensive data on adverse effects on endocrine-relevant endpoints over more extensive parts of the life cycle of the organism, although some Level 4 assays (e.g. TG 234 [FSDT]) may be sufficient for this purpose. If existing data are from UT assays, then Level 4 assays will provide data on multiple modalities. 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	+	–	–	ER (ant)agonism but effects not detected in *in vivo* studies. Weak ER (ant)agonism does not result in adverse effects in the selected species under the conditions of the test. Metabolic differences may explain *in vitro*/*in vivo* differences.	Perform ER STTA with added metabolising system or Assay from Levels 3-4, e.g. UT assay or fish screen (OECD TG 229/230) (Level 3) or female PP assay (Level 4).	A positive result indicates strong probability of interaction with ERs in other taxa. 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 Level 4 vertebrate wildlife assays, then Level 5 assays should provide more comprehensive data on adverse effects on endocrine-relevant endpoints over more extensive parts of the life cycle of the organism, although some Level 4 assays (e.g. TG 234 [FSDT]) may be sufficient for this purpose. If existing data are from UT assays, then Level 4 assays will provide data on multiple modalities. Consider route of exposures for existing effects data and possible transformation products and implications of ADME characteristics of the chemical. The chemical itself may give positive *in vitro* results but may not be absorbed or may be metabolised to an inactive metabolite *in vivo*. However, note that uptake and metabolism of chemicals can be different between mammalian and non-mammalian wildlife species. Check data on chemical analogues.

Scenarios	Result of OECD TG 455 (ER STTA)	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)**			
F	+	–	Eq/0	ER (ant)agonism but no or equivocal data from *in vivo* studies. Weak ER (ant)agonism does not result in adverse effects in the selected species under the conditions of the test.	Perform assay from Levels 3-4, e.g. UT assay or fish screen (OECD TG 229/230) (Level 3) or female PP assay (Level 4).	A positive result indicates strong probability of interaction with ERs in other taxa. Consider route of exposures for equivocal existing effects data and possible implications of ADME characteristics of the chemical. Check data on chemical analogues. Equivocal results may indicate chemical has multiple MOA.
G	+	Eq/0	+	ER (ant)agonism and potential for adverse effects via ER (ant)agonism or other A,T,S mechanisms. May act via E,A,T,S mechanism and may or may not require metabolic activation.	Perform assay from Levels 3-4, e.g. UT assay or fish screen (OECD TG 229/230) (Level 3) or female PP assay (Level 4).	A positive result indicates strong probability of interaction with ERs in other taxa. If existing data are from an adequate Level 5 assay, 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). If existing data are from Level 4 vertebrate wildlife assays, then Level 5 assays should provide more comprehensive data on adverse effects on endocrine-relevant endpoints over more extensive parts of the life cycle of the organism, although some Level 4 assays (e.g. TG 234 [FSDT]) may be sufficient for this purpose. If existing data are from UT assays, then Level 4 assays will provide data on multiple modalities. Check data on chemical analogues. Further mechanistic studies may help determine MOA. Equivocal results may indicate chemical has multiple MOA. A positive result could have arisen from other (E,A,T,S or non-E,A,T,S) mechanisms (e.g. HPG axis).
H	+	Eq/0	–	ER (ant)agonism but effects not detected in *in vivo* studies. Weak ER (ant)agonism does not result in adverse effects in the selected species under the conditions of the test. Metabolic differences may explain *in vitro*/*in vivo* differences.	Perform ER STTA with added metabolising system.	A positive result indicates strong probability of interaction with ERs in other taxa. 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 Level 4 vertebrate wildlife assays, then Level 5 assays should provide more comprehensive data on adverse effects on endocrine-relevant endpoints over more extensive parts of the life cycle of the organism, although some Level 4 assays (e.g. TG 234 [FSDT]) may be sufficient for this purpose. If existing data are from UT assays, then Level 4 assays will provide data on multiple modalities. Consider route of exposures for existing effects data and possible transformation products and implications of ADME characteristics of the chemical. The chemical itself may give positive *in vitro* results but may not be absorbed or may be metabolised to an inactive metabolite *in vivo*. However, note that uptake and metabolism of chemicals can be different between mammalian and non-mammalian wildlife species. Check data on chemical analogues. Further mechanistic studies may help determine MOA. Equivocal results may indicate chemical has multiple MOA.

Scenarios	Result of OECD TG 455 (ER STTA)	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)**			
I	+	Eq/0	Eq/0	ER (ant)agonism with unknown potential for effects in *in vivo* studies. May act via ER mechanism and may or may not require metabolic activation. Unknown potential for adverse effects.	Perform ER STTA with added metabolising system or UT assay or fish screen (OECD TG 229/230) (Level 3), if existing data indicate this is needed.	A positive result indicates strong probability of interaction with ERs in other taxa. Consider route of exposures for equivocal existing effects data and possible implications of ADME characteristics of the chemical. Check data on chemical analogues. Equivocal results may indicate chemical has multiple MOA.
J	–	+	+	No evidence for ER (ant)agonism. Effects on AR/T/S and potential for adverse effects via E,A,T,S mechanisms.	Perform ER STTA with added metabolising system or Perform assay from Levels 3-4, e.g. UT assay or fish screen (OECD TG 229/230) (Level 3) or female PP assay (Level 4).	A negative result indicates interaction with ERs in other taxa is unlikely. If existing data are from an adequate Level 5 assay, there may be sufficient information to conclude concern for endocrine disruption. If existing data are from Level 4 vertebrate wildlife assays, then Level 5 assays should provide more comprehensive data on adverse effects on endocrine-relevant endpoints over more extensive parts of the life cycle of the organism, although some Level 4 assays (e.g. TG 234 [FSDT]) may be sufficient for this purpose. If existing data are from UT assays, then Level 4 assays will provide data on multiple modalities. Consider route of exposures for existing effects data and possible transformation products and implications of ADME characteristics of the chemical. Check data on chemical analogues. Further mechanistic studies may help determine MOA.
K	–	+	–	No evidence for ER (ant)agonism. Effects on AR/T/S but effects not detected in *in vivo* studies.	Perform assay from Levels 3-4, e.g. fish screen (OECD TG 229/230) (Level 3) or male or female PP assay (Level 4).	A negative result indicates interaction with ERs in other taxa is unlikely. 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 Level 4 vertebrate wildlife assays, then Level 5 assays should provide more comprehensive data on adverse effects on endocrine-relevant endpoints over more extensive parts of the life cycle of the organism, although some Level 4 assays (e.g. TG 234 [FSDT]) may be sufficient for this purpose. If existing data are from UT assays, then Level 4 assays will provide data on multiple modalities. Metabolic deactivation of chemical may occur *in vivo* so that potential *in vitro* E,A,T,S activity is not realised. Consider possible routes of exposure, implications of metabolism.
L	–	+	Eq/0	No evidence for ER (ant)agonism. Effects on AR/T/S but effects not detected in *in vivo* studies. Unknown potential for adverse effects. Metabolic differences explain *in vitro*/*in vivo* A,T,S differences.	Perform assay from Levels 3-4, e.g. fish screen (OECD TG 229/230) (Level 3) or male or female PP assay (Level 4).	A negative result indicates interaction with ERs in other taxa is unlikely. Metabolic deactivation of chemical may occur *in vivo* so that potential *in vitro* E,A,T,S activity is not realised. Consider possible routes of exposure, implications of metabolism. Check data on chemical analogues. Equivocal results may indicate chemical has multiple MOA.

Scenarios	Result of OECD TG 455 (ER STTA)	Existing results		Possible conclusions	Next step which could be taken to strengthen weight of evidence if necessary	Other considerations
		Mechanism (*in vitro* mechanistic data)*	Effects (*in vivo* effects of concern)**			
M	–	–	+	No evidence for ER (ant)agonism. Metabolic differences or route of exposure explain *in vitro*/*in vivo* differences. Effects seen in existing studies are via non-E,A,T,S or non-endocrine mechanisms.	Perform ER STTA with added metabolising system or Perform assay from Levels 3-4, e.g. fish screen (OECD TG 229/230) (Level 3) or male or female PP assay (Level 4).	A negative result indicates interaction with ERs in other taxa is unlikely. If existing data are from an adequate Level 5 assay, there may be sufficient information to conclude concern for endocrine disruption. If existing data are from Level 4 vertebrate wildlife assays, then Level 5 assays should provide more comprehensive data on adverse effects on endocrine-relevant endpoints over more extensive parts of the life cycle of the organism, although some Level 4 assays (e.g. TG 234 [FSDT]) may be sufficient for this purpose. If existing data are from UT assays, then Level 4 assays will provide data on multiple modalities. Metabolic activation of chemical may occur *in vivo*. Consider possible routes of exposure, implications of metabolism. Check data on chemical analogues. Further mechanistic studies may help determine MOA.
N	–	–	–	No evidence for ER (ant)agonism. No evidence of adverse effects.	Possibly no need for further testing. If there is uncertainty, may perform assay from Levels 3-4, e.g. fish screen (OECD TG 229/230) (Level 3) or male or female PP assay (Level 4).	A negative result indicates interaction with ERs in other taxa is unlikely. 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 Level 4 vertebrate wildlife assays, then Level 5 assays should provide more comprehensive data on adverse effects on endocrine-relevant endpoints over more extensive parts of the life cycle of the organism, although some Level 4 assays (e.g. TG 234 [FSDT]) may be sufficient for this purpose. If existing data are from UT assays, then Level 4 assays will provide data on multiple modalities. Check data on chemical analogues.
O	–	–	Eq/0	No evidence for ER (ant)agonism. Unknown potential for adverse effects via other mechanisms.	Perform ER STTA with added metabolising system or Fish screen (OECD TG 229/230) (Level 3) or male or female PP assay (Level 4) if existing data indicate this is needed.	A negative result indicates interaction with ERs in other taxa is unlikely. Consider possible routes of exposure, implications of metabolism. Check data on chemical analogues.

Scenarios	Result of OECD TG 455 (ER STTA)	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 for ER (ant)agonism. Unknown potential for adverse effects via other mechanisms.	Perform ER STTA with added metabolising system.	A negative result indicates interaction with ERs in other taxa is unlikely. If existing data are from an adequate Level 5 assay, there may be sufficient information to conclude concern for endocrine disruption. If existing data are from Level 4 vertebrate wildlife assays, then Level 5 assays should provide more comprehensive data on adverse effects on endocrine-relevant endpoints over more extensive parts of the life cycle of the organism, although some Level 4 assays (e.g. TG 234 [FSDT]) may be sufficient for this purpose. If existing data are from UT assays, then Level 4 assays will provide data on multiple modalities. Consider possible routes of exposure, implications of metabolism. 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 ER (ant)agonism. No evidence of adverse effects.	Perform ER STTA with added metabolising system.	A negative result indicates interaction with ERs in other taxa is unlikely. 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 Level 4 vertebrate wildlife assays, then Level 5 assays should provide more comprehensive data on adverse effects on endocrine-relevant endpoints over more extensive parts of the life cycle of the organism, although some Level 4 assays (e.g. TG 234 [FSDT]) may be sufficient for this purpose. If existing data are from UT assays, then Level 4 assays will provide data on multiple modalities. Check data on chemical analogues. Further mechanistic studies may help determine MOA.
R	–	Eq/0	Eq/0	No evidence for ER (ant)agonism. Unknown potential for adverse effects via other mechanisms.	For the "0" scenario, perform ER STTA with added metabolising system or Perform UT assay or fish screen (OECD TG 229/230) (Level 3), if existing data indicate this is needed.	A negative result indicates interaction with ERs in other taxa is unlikely. Consider possible routes of exposure, implications of metabolism. Check data on chemical analogues. Further mechanistic studies may help determine MOA. Equivocal results may indicate chemical has multiple MOA.

C.1.3. Stably Transfected Human Androgen Receptor Transcriptional Activation Assay for Detection of Androgenic Agonist and Antagonist Activity of Chemicals (AR STTA) (OECD TG 458)

Status: Assay validated by the OECD.

Modality detected/endpoints: Activation of reporter gene linked to AR (agonist assay). Inhibition of activation of reporter gene linked to AR.

Background to the assay

186. The Stably Transfected AR Transcriptional Activation Assay (AR STTA) is an *in vitro* screening assay to detect substances that bind to androgen receptors (AR) and activate the transcription of androgen responsive genes. It is an *in vitro* tool that provides mechanistic data. Several AR STTA assays in common use can be found in the literature (Hartig et al., 2002; Birkhøj et al., 2004; Araki et al., 2005a, 2005b), one of the first versions of this assay used was the "yeast androgen screen" (Sohoni and Sumpter, 1998), which is still widely used for screening of environmental samples. The guidance in this building block can be cautiously used for the yeast assays. OECD TG 458 was published in July 2016, following validation of this assay using the AR-EcoScreen™ cell line. Other AR STTA assays are being validated via OECD initiatives and a performance-based test guideline for this assay will be developed in the future.

187. The AR-EcoScreen™ cell line is derived from a Chinese hamster ovary cell line (CHO-K1) stably transfected with human (h) AR and uses a firefly luciferase reporter gene resulting in increased cellular expression of the luciferase enzyme in the presence of AR agonists. This cell line has also been constructed to stably express a non-inducible renilla luciferase reporter gene, the activity of which decreases in the presence of cytotoxic agents. The luciferase enzymes differ in their substrate and cofactor requirements and emit light at different wavelengths. Cell viability can therefore be determined in the same cells as those used for AR (ant)agonism. This enables pure antagonisms to be distinguished from a cytotoxicity-related decrease of luciferase activity. Other AR STTA assays utilise different viability assessments.

188. The AR STTA assay provides a positive or negative result for the ability of a chemical to induce AR-mediated transactivation of gene expression (agonist assay) compared to a vehicle control. The antagonist assay determines whether a reduction in response occurs when cells are co-exposed to chemicals and a potent androgen agonist compared to the potent androgen agonist alone. 5α- Dihydrotestosterone (DHT) is used as the co-administered agonist in OECD TG 458. R1881 is also commonly used. Any reduction in response must occur in the absence of cytotoxicity.

189. OECD TG 458 gives a positive or negative result for a test chemical when reporter gene activity is compared to controls. A measure of potency is also provided by the magnitude of the effect and the concentration at which it occurs. An AR agonistic effect is

based on the maximum response level induced by a test chemical. If this response equals or exceeds 10% of the response induced by DHT (the positive AR agonist control) (i.e. the log PC10), the test chemical is considered positive. An AR antagonistic effect is based on a cut-off of a 30% inhibitory response against DHT (i.e. the log IC30). If the response exceeds this 30% AR inhibition, then the chemical is considered a positive AR antagonist.

190. OECD TG 458 requires strict control of assay conditions in order to maintain the accuracy and reliability of response. Demonstration of laboratory proficiency with proficiency chemicals is required at the outset, ten for each of the agonist and antagonist assays. These chemicals were used in the validation of this assay (OECD, 2011), represent the classes of chemicals commonly associated with AR agonist or antagonist activity, exhibit a suitable range of potency expected for AR agonists/antagonists (i.e. strong to weak), and include negatives. Periodic testing with proficiency chemicals should also be carried out. In addition, each experiment requires reference chemicals: for the agonist assay, DHT (a strong agonist), mestanolone (a weak agonist) and (2-ethylhexyl)phthalate (DEHP) (negative); for the antagonist assay: hydroxyl flutamide (a strong antagonist), bisphenol A (a weak antagonist) and DEHP (negative) should be used. In the assay, each plate requires positive and vehicle controls. A positive control for cytotoxicity (cycloheximide) is also required for each plate. Criteria for the degree of response with these chemicals are given in the test guidance (TG). The assay requires a minimum of 80% cell viability, demonstrated by renilla luciferase activity. This is critical for the antagonist assay where positive results can only be demonstrated in the absence of cytotoxicity. Compliance with the quality control criteria and with the performance criteria should be accepted before evaluating results from this assay. The response with positive control chemicals (e.g. hydroxy-flutamide for antagonism and dihydrotestosterone for agonism) should be robust and cell viability should be above 80%.

191. Some cell lines used for the AR STTA also express the glucocorticoid receptor (GR), which may cause cross-talk interference with AR (Hartig et al., 2002). This is due to the fact that the receptor can act on the same responsive elements (androgen response elements). The level of GR expression in the cell line and therefore potential for interference should be known.

192. The AR STTA assay will not detect substances that act by other mechanisms (e.g. estrogen receptor [ER], thyroid hormone receptor [TR] and steroidogenesis interference). These chemicals will, however, be detected in ER-, TR- and steroidogenesis-specific assays and therefore results from a suite of *in vitro* tests should be considered together. The assay will not detect substances that act by affecting the hypothalamic/pituitary/ gonadal (HPG) as an *in vivo* intact axis is required for this.

When/why the assay may be used

193. Although the AR STTA assay may be used at any stage in the hazard assessment process, the most likely use scenario is during initial assessment of chemicals for their ability to interact with endocrine systems *in vitro*, i.e. estrogen/androgen/thyroid/steroidogenesis (E,A,T,S) modalities. The AR STTA assay is frequently conducted following a positive result in the AR Binding Assay. Assays for interaction with other modalities (e.g. AR, ER and steroidogenesis) are likely to be conducted at the same time so that all results can be considered together. TR and other assays concerning mechanisms of thyroid disruption may be available, but they are not in common use. AR STTAs do not include the use of a xenobiotic metabolising system, but consideration should be given to the inclusion of this (OECD, 2008; Jacobs et al., 2008, 2013) depending on the circumstances, e.g. if the

metabolism of a chemical is unknown, although the methods for inclusion of xenobiotic metabolising systems are not yet validated (see Paragraph 50). Alternatively, for a chemical with known metabolites, these could also be tested in the AR STTA assay.

194. Another use scenario may be following effects obtained in higher tier tests, for example accelerated puberty onset in males, but which are not exclusively indicative of an effect on AR. Selection of the most appropriate tests has to be on a case-by-case basis, but also considering the need to minimise animal testing.

Introduction to the table of scenarios

195. Table C.1.3 gives guidance on a further step to take in the event of a positive (+) or negative (-) result in the AR STTA 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.

196. The results of the AR STTA assay are given in the second column. Criteria for positive and negative results in OECD TG 458 are given in the test guideline. Reproducible results in at least two runs are required. If two runs do not give reproducible results (e.g. a test chemical is positive in one run and negative in the other run), at least three independent runs should be conducted. In this case, the classification is based on the two concordant results out of the three. It is important that quality and proficiency criteria are demonstrated for both positive and negative results. The concentrations tested should remain within the solubility range of the test chemicals and not demonstrate cytotoxicity.

197. Equivocal results for the AR STTA assay are not included in the table because these data require further interrogation about the result itself. This assay is a screen and therefore a clear positive or negative result should be obtained. In the event of an equivocal result, the considerations mentioned above about control quality and proficiency criteria should be taken into account and further investigations made. Equivocal results at high concentrations may may indicate solubility issues.

Existing data to be considered

198. Existing "mechanism" *in vitro* data are assumed to be available from AR-, ER- 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.

199. Existing "effects" data refer to *in vivo* effects "of concern" (i.e. data from Level 3, 4 or 5 vertebrate wildlife assays/tests). These may come from varied sources and will depend on the type of substance (e.g. new chemicals, high production volume [HPV] chemical, pesticide). Thus, available data may range from repeated dose toxicity studies (28-day, 90-day), or combined repeat dose/reproductive screening assays or fish screening assays, to chronic toxicity studies and multigeneration reproductive tests in vertebrate wildlife species. Some studies fail to identify endocrine disrupters (EDs) that weakly affect estrogen or androgen receptors as was demonstrated on the basis of data generated in the validation process of the 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 modes of action (MOA), including endocrine modalities. 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 vertebrates species may also have endocrine effects in mammals, but the physiological consequences of the effects are likely to be different.

200. Data may also be available from Level 3 tests (Hershberger [H] and Uterotrophic [UT] Assays) although these tests may not give rise to "concern" as they are hazard screening tests only. The H assay is, however, more likely to be conducted **after** the AR STTA assay (to test whether a chemical that is positive *in vitro* is also positive *in vivo*) rather than before. An Amphibian Metamorphosis Assay may also be available, but as this test primarily detects thyroid disruption in amphibians it is unlikely to provide useful data for A-modalities.

201. When considering the results of the AR STTA assay, all available data should be used in order to reach a conclusion and a weight of evidence approach taken. This may include read-across data from structural analogues and quantitative structure activity relationships (QSARs). 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

202. The scenarios (A to R) presented in Table C.1.3 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 458 uses hAR (human androgen receptor), the well-conserved nature of AR across taxa is assumed be a strong indication that results in this assay are relevant to other vertebrate 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. Further considerations specific to each scenario are given in the table.

203. Scenarios A to C represent positive results in the AR STTA assay in the presence of positive *in vitro* mechanistic data and positive, negative or equivocal *in vivo* effects data. A positive result in an AR STTA assay is strong evidence for (anti)androgenic activity that may or may not be supported by the *in vivo* effects data. In the case of positive *in vivo* effects data, there may be sufficient evidence to conclude concern for endocrine disruption and therefore no need for further screening. *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. 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. 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.

204. Scenarios D to F represent positive results in the AR STTA assay in the presence of negative *in vitro* mechanistic data and positive, negative or equivocal *in vivo* effects data. Unless the metabolic profile of the test substance is known, one option may be to conduct these *in vitro* assays with an added metabolising system. If the metabolic profile is known, then an *in vivo* test may be advisable. The choice of tests will depend on the available *in vivo* effects data. 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. 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.

205. Scenarios G to I represent positive results in the AR STTA assay 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, 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 directly to *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.

206. Scenarios J to L represent negative results in the AR STTA assay in the presence of positive *in vitro* mechanistic data and positive, negative or equivocal *in vivo* effects data. The limitations of the AR STTA assay should be considered first (e.g. lack of metabolic activation, possible involvement of other factors). The positive *in vitro* mechanistic data indicate possible alternative E,T,S mechanisms. To confirm lack of AR-related activity in the presence of *in vivo* data, an AR STTA with added metabolising capability could be performed. Otherwise *in vivo* tests will confirm or refute E,A,T,S activity.

207. Scenarios M to O represent negative results in the AR STTA 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. The limitations of the AR

STTA assay should also be considered (as described for Scenarios J to L). To confirm lack of AR-related activity in the presence of *in vivo* data, an AR STTA with added metabolising capability could be performed. Otherwise, *in vivo* tests will confirm or refute E,A,T,S activity (Scenarios M and O).

208. Scenarios P to R represent negative results in the AR STTA assay in the presence of various combinations of missing or equivocal data. The limitations of the AR STTA binding assay should be considered first (as described for Scenarios J to L). As with the positive result scenarios above (see Paragraph 203), the next step to take for Scenarios P to R 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.

209. In all scenarios (A to R), the next step to take to strengthen weight of evidence will depend on the existing information. Table C.1.3 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.

210. In general, a decision about whether or not to conduct *in vivo* vertebrate wildlife tests will depend on the weight of evidence of new and existing data. If most available data (e.g. the results of the AR STTA assay, predictions from QSARs, "read-across" from data on similar substances and results from mammalian *in vivo* assays) suggest that the substance has the potential to cause endocrine disruption via the AR (i.e. the level of suspicion about endocrine disrupting action is high – corresponding to Scenario A), then consideration should be given to the conduct of a higher level test.

211. For non-mammalian wildlife species, higher level tests with fish or amphibians (i.e. the TG 234 [FSDT], TG 240, TG 241) are recommended. Choice about which of these tests is most appropriate will be driven *inter alia* by MOA considerations, and by whether multigeneration effects are to be expected. Such tests are unlikely to be needed if exposure of the natural environment is not expected. On the other hand, if available data only raise a low or moderate level of suspicion about endocrine disrupting action (e.g. the data appear to conflict with each other), then consideration should be given to the conduct of a fish screen (i.e. JMASA, OECD TG 229 or TG 230).

212. For mammals, similar considerations apply but lower level tests (e.g. Level 3 or 4) 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.

References

Araki, N. et al. (2005a), "Screening for androgen receptor activities in 253 industrial chemicals by *in vitro* reporter gene assays using AR-EcoScreen cells", *Toxicology In Vitro*, Vol. 19/6, pp. 831-842, https://doi.org/10.1016/j.tiv.2005.04.009.

Araki, N. et al. (2005b), "Evaluation of a rapid *in vitro* androgen receptor transcriptional activation assay using AR-EcoScreen cells", *Toxicology In Vitro*, Vol. 19/3, pp. 335-352, https://doi.org/10.1016/j.tiv.2004.10.008.

Birkhøj, M. et al. (2004), "The combined antiandrogenic effects of five commonly used pesticides", *Toxicology and Applied Pharmacology*, Vol. 201/1, pp. 10-20, https://doi.org/10.1016/j.taap.2004.04.016.

Hartig, P.C. et al. (2002), "Development of two androgen receptor assays using adenoviral transduction of MMTV-luc reporter and/or hAR for endocrine screening", *Toxicological Sciences*, Vol. 66/1, pp. 82-90.

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.

OECD (2011), "Peer review report for the validation of the Stably Transfected Transcriptional Activation Assay for the detection androgenic and anti-androgenic activity of chemicals", OECD Series on Testing and Assessment, No. 161, OECD, Paris, www.oecd.org/officialdocuments/publicdisplaydocumentpdf/?cote=ENV/JM/MONO(2011)46&doclanguage=en.

OECD (2008), *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.

Sohoni, P. and J.P. Sumpter (1998), "Several environmental oestrogens are also anti-androgens", *Journal of Endocrinology*, Vol. 158/3, pp. 327-339.

Table C.1.3. **Stably Transfected Human Androgen Receptor Transcriptional Activation Assay for Detection of Androgenic Agonist and Antagonist Activity of Chemicals (AR STTA) (OECD TG 458): 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 (S-) based assays (Level 2). The AR Binding Assay is likely to be performed prior to the AR STTA assay. 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 various information, such as data from repeat dose oral toxicity studies, reproduction/developmental toxicity screen tests, read-across from analogues, will be available.

Scenarios	Result of AR STTA	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	+	+	+	Androgen receptor (AR) (ant)agonism combined with effects on estrogen receptor/thyroid/steroidogenesis (ER/T/S) and potential for adverse effects via multiple mechanisms.	Perform assay from Levels 3-5, e.g. Hershberger (H) Assay or fish screen (AFSS or JMASA) (Level 3) or male Peripubertal (PP) Assay (Level 4) or EOGRTS or two-generation assays or partial/full non-mammalian wildlife life cycle tests, e.g. OECD TG 241 and TG 240 (Level 4/5).	A positive result indicates strong probability of interaction with AR in other taxa. If existing data are from an adequate Level 5 assay, then 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). If existing data are from Level 4 vertebrate wildlife assays, then Level 5 assays should provide more comprehensive data on adverse effects on endocrine-relevant endpoints over more extensive parts of the life cycle of the organism. If existing data are from H assays or AFSS or JMASA, then Level 4 mammalian assays or Level 3 or 4 fish screens (OECD TG 229/230/234) will provide data on multiple modalities. Consider route of exposures for existing effects data and possible transformation products and 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*. However, note that uptake and metabolism of chemicals can be different between mammalian and non-mammalian wildlife species.
B	+	+	–	AR (ant)agonism combined with effects on ER/T/S but effects not detected in *in vivo* studies. Weak AR (ant)agonism does not result in adverse effects in the selected species under the conditions of the test. Metabolic differences may explain *in vitro*/*in vivo* differences.	Perform AR STTA with added metabolising system or Assay from Levels 3-4, e.g. H assay or fish screen (AFSS) (Level 3) or male PP assay (Level 4).	A positive result indicates strong probability of interaction with AR in other taxa. 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 Level 4 vertebrate wildlife assays, then Level 5 assays should provide more comprehensive data on adverse effects on endocrine-relevant endpoints over more extensive parts of the life cycle of the organism. If existing data are from H assays or AFSS or JMASA, then Level 4 mammalian assays or Level 3 or 4 fish screens (OECD TG 229/230/234) will provide data on multiple modalities. Consider route of exposures for existing effects data and possible transformation products and implications of ADME characteristics of the chemical. The chemical itself may give positive *in vitro* results but may not be absorbed or may be metabolised to an inactive metabolite *in vivo*. However, note that uptake and metabolism of chemicals can be different between mammalian and non-mammalian wildlife species.
C	+	+	Eq/0	AR (ant)agonism combined with effects on ER/T/S but no or equivocal data from *in vivo* studies. Weak AR (ant)agonism may not result in adverse effects in the selected species under the conditions of the test.	Perform assay from Levels 3-4, e.g. H assay or fish screen (AFSS) (Level 3) or male PP assay (Level 4).	A positive result indicates strong probability of interaction with AR in other taxa. Consider route of exposures for equivocal existing effects data and possible implications of ADME characteristics of the chemical. Equivocal results may indicate chemical has multiple modes of action (MOA). Check data on chemical analogues.

Scenarios	Result of AR STTA	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	+	–	+	AR (ant)agonism and potential for adverse effects.	Perform assay from Levels 3-4, e.g. H assay or fish screen (AFSS) (Level 3) or male PP assay (Level 4).	A positive result indicates strong probability of interaction with AR in other taxa. If existing data are from an adequate Level 5 assay, 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). If existing data are from Level 4 vertebrate wildlife assays, then Level 5 assays should provide more comprehensive data on adverse effects on endocrine-relevant endpoints over more extensive parts of the life cycle of the organism. If existing data are from H assays or AFSS or JMASA, then Level 4 mammalian assays or Level 3 or 4 fish screens (OECD TG 229/230/234) will provide data on multiple modalities. 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	+	–	–	AR (ant)agonism but effects not detected in *in vivo* studies. Weak AR (ant)agonism does not result in adverse effects in the selected species under the conditions of the test. Metabolic differences may explain *in vitro*/*in vivo* differences.	Perform AR STTA with added metabolising system or Assay from Levels 3-4, e.g. H assay or fish screen (AFSS) (Level 3) or male PP assay (Level 4).	A positive result indicates strong probability of interaction with AR in other taxa. 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 Level 4 vertebrate wildlife assays, then Level 5 assays should provide more comprehensive data on adverse effects on endocrine-relevant endpoints over more extensive parts of the life cycle of the organism. If existing data are from H assays or AFSS or JMASA, then Level 4 mammalian assays or Level 3 or 4 fish screens (OECD TG 229/230/234) will provide data on multiple modalities. Consider route of exposures for existing effects data and possible transformation products and implications of ADME characteristics of the chemical. The chemical itself may give positive *in vitro* results but may not be absorbed or may be metabolised to an inactive metabolite *in vivo*. However, note that uptake and metabolism of chemicals can be different between mammalian and non-mammalian wildlife species. Check data on chemical analogues.
F	+	–	Eq/0	AR (ant)agonism but no or equivocal data from *in vivo* studies. Weak AR (ant)agonism does not result in adverse effects in the selected species under the conditions of the test.	Perform assay from Levels 3-4, e.g. H assay or fish screen (AFSS) (Level 3), male PP assay (Level 4).	A positive result indicates strong probability of interaction with AR in other taxa. Consider route of exposures for equivocal existing effects data and possible implications of ADME characteristics of the chemical. Equivocal results may indicate chemical has multiple MOA. Check data on chemical analogues.

Scenarios	Result of AR STTA	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	+	AR (ant)agonism and potential for adverse effects via AR (ant)agonism or other E,T,S mechanisms. May act via E,A,T,S mechanism and may or may not require metabolic activation.	Perform assay from Levels 3-4, e.g. H assay or fish screen (AFSS) (Level 3) or male PP assay (Level 4).	A positive result indicates strong probability of interaction with AR in other taxa. If existing data are from an adequate Level 5 assay, then 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). If existing data are from Level 4 vertebrate wildlife assays, then Level 5 assays should provide more comprehensive data on adverse effects on endocrine-relevant endpoints over more extensive parts of the life cycle of the organism. If existing data are from H assays or AFSS or JMASA, then Level 4 mammalian assays or Level 3 or 4 fish screens (OECD TG 229/230/234) will provide data on multiple modalities. Check data on chemical analogues. Further mechanistic studies may help determine MOA. Equivocal results may indicate chemical has multiple MOA. A positive result could have arisen from other (E,A,T,S or non-E,A,T,S) mechanisms (e.g. HPG axis).
H	+	Eq/0	–	AR (ant)agonism but effects not detected in *in vivo* studies. Weak AR (ant)agonism does not result in adverse effects in the selected species under the conditions of the test. Metabolic differences may explain *in vitro*/*in vivo* differences.	Perform AR STTA with added metabolising system.	A positive result indicates strong probability of interaction with AR in other taxa. 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 Level 4 vertebrate wildlife assays, then Level 5 assays should provide more comprehensive data on adverse effects on endocrine-relevant endpoints over more extensive parts of the life cycle of the organism. If existing data are from H assays or AFSS or JMASA, then Level 4 mammalian assays or Level 3 or 4 fish screens (OECD TG 229/230/234) will provide data on multiple modalities. Consider route of exposures for existing effects data and possible transformation products and implications of ADME characteristics of the chemical. The chemical itself may give positive *in vitro* results but may not be absorbed or may be metabolised to an inactive metabolite *in vivo*. However, note that uptake and metabolism of chemicals can be different between mammalian and non-mammalian wildlife species. Check data on chemical analogues. Further mechanistic studies may help determine MOA. Equivocal results may indicate chemical has multiple MOA.
I	+	Eq/0	Eq/0	AR (ant)agonism with unknown potential for effects in *in vivo* studies. May act via AR mechanism and may or may not require metabolic activation. Unknown potential for adverse effects.	Perform AR STTA with added metabolising system or H assay or fish screen (AFSS) (Level 3) if existing data indicate this is needed.	A positive result indicates strong probability of interaction with AR in other taxa. Consider route of exposures for equivocal existing effects data and possible implications of ADME characteristics of the chemical. Check data on chemical analogues. Equivocal results may indicate chemical has multiple MOA.

Scenarios	Result of AR STTA	Existing results		Possible conclusions	Next step which could be taken to strengthen weight of evidence if necessary	Other considerations
		Mechanism (*in vitro* mechanistic data)*	Effects (*in vivo* effects of concern)**			
J	–	+	+	No evidence for AR (ant)agonism. Effects on ER/T/S and potential for adverse effects via E,A,T,S mechanisms.	Perform AR STTA with added metabolising system or Perform assay from Levels 3-4, e.g. H assay or fish screen AFSS (Level 3) or male PP assay (Level 4).	A negative result indicates interaction with AR in other taxa is unlikely. 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). If existing data are from Level 4 vertebrate wildlife assays, then Level 5 assays should provide more comprehensive data on adverse effects on endocrine-relevant endpoints over more extensive parts of the life cycle of the organism. If existing data are from H assay or AFSS or JMASA, then Level 4 mammalian assays or Level 3 or 4 fish screens (OECD TG 229/230/234) will provide data on multiple modalities. Consider route of exposures for existing effects data and possible transformation products and implications of ADME characteristics of the chemical. Check data on chemical analogues. Further mechanistic studies may help determine MOA.
K	–	+	–	No evidence for AR (ant)agonism. Effects on ER/T/S but effects not detected in *in vivo* studies.	Perform assay from Levels 3-4, e.g. fish screen (AFSS) (Level 3) or male or female PP assay (Level 4).	A negative result indicates interaction with AR in other taxa is unlikely. 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 Level 4 vertebrate wildlife assays, then Level 5 assays should provide more comprehensive data on adverse effects on endocrine-relevant endpoints over more extensive parts of the life cycle of the organism. If existing data are from H assays or AFSS or JMASA, then Level 4 mammalian assays or Level 3 or 4 fish screens (OECD TG 229/230/234) will provide data on multiple modalities. Metabolic deactivation of chemical may occur *in vivo* so that possible *in vitro* E,A,T,S activity is not realised. Consider possible routes of exposure, implications of metabolism.
L	–	+	Eq/0	No evidence for AR (ant)agonism. Effects on ER/T/S but effects not detected in *in vivo* studies. Unknown potential for adverse effects. Metabolic differences explain *in vitro*/*in vivo* E,A,T,S differences.	Perform assay from Levels 3-4, e.g. fish screen (AFSS) (Level 3) or male or female PP assay (Level 4).	A negative result indicates interaction with AR in other taxa is unlikely. Metabolic deactivation of chemical may occur *in vivo* so that possible *in vitro* E,A,T,S activity is not realised. Consider possible routes of exposure, implications of metabolism. Check data on chemical analogues. Equivocal results may indicate chemical has multiple MOA.

Scenarios	Result of AR STTA	Existing results		Possible conclusions	Next step which could be taken to strengthen weight of evidence if necessary	Other considerations
		Mechanism (*in vitro* mechanistic data)*	Effects (*in vivo* effects of concern)**			
M	–	–	+	No evidence for AR (ant)agonism. Metabolic differences or route of exposure explain *in vitro*/*in vivo* differences. Effects seen in existing studies are via non-E,A,T,S or non-endocrine mechanisms.	Perform AR STTA with added metabolising system or Perform assay from Levels 3-4, e.g. fish screen (AFSS) (Level 3) or male or female PP assay (Level 4).	A negative result indicates interaction with AR in other taxa is unlikely. 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). If existing data are from Level 4 vertebrate wildlife assays, then Level 5 assays should provide more comprehensive data on adverse effects on endocrine-relevant endpoints over more extensive parts of the life cycle of the organism. If existing data are from H assays or AFSS or JMASA, then Level 4 mammalian assays or Level 3 or 4 fish screens (OECD TG 229/230/234) will provide data on multiple modalities. Metabolic activation of chemical may occur *in vivo*. Consider possible routes of exposure, implications of metabolism. Check data on chemical analogues. Further mechanistic studies may help determine MOA.
N	–	–	–	No evidence for AR (ant)agonism. No evidence of adverse effects.	Possibly no need for further testing. If there is uncertainty, may perform assay from Levels 3-4, e.g. fish screen (AFSS) (Level 3) or male or female PP assay (Level 4).	A negative result indicates interaction with AR in other taxa is unlikely. 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 Level 4 vertebrate wildlife assays, then Level 5 assays should provide more comprehensive data on adverse effects on endocrine-relevant endpoints over more extensive parts of the life cycle of the organism. If existing data are from H assays or AFSS or JMASA, then Level 4 mammalian assays or Level 3 or 4 fish screens (OECD TG 229/230/234) will provide data on multiple modalities. Check data on chemical analogues.
O	–	–	Eq/0	No evidence for AR (ant)agonism. Unknown potential for adverse effects via other mechanisms.	Perform AR STTA with added metabolising system or Fish screen (AFSS) (Level 3) or male or female PP assay (Level 4) if existing data indicate this is needed.	A negative result indicates interaction with AR in other taxa is unlikely. Consider possible routes of exposure, implications of metabolism. Check data on chemical analogues.

Scenarios	Result of AR STTA	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 for AR (ant)agonism. Unknown potential for adverse effects via other mechanisms.	Perform AR STTA with added metabolising system.	A negative result indicates interaction with AR in other taxa is unlikely. 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). If existing data are from Level 4 vertebrate wildlife assays, then Level 5 assays should provide more comprehensive data on adverse effects on endocrine-relevant endpoints over more extensive parts of the life cycle of the organism. If existing data are from H assays or AFSS or JMASA, then Level 4 mammalian assays or Level 3 or 4 fish screens (OECD TG 229/230/234) will provide data on multiple modalities. Consider possible routes of exposure, implications of metabolism. 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 AR (ant)agonism. No evidence of adverse effects.	Perform AR STTA with added metabolising system.	A negative result indicates interaction with AR in other taxa is unlikely. 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 Level 4 vertebrate wildlife assays, then Level 5 assays should provide more comprehensive data on adverse effects on endocrine-relevant endpoints over more extensive parts of the life cycle of the organism. If existing data are from H assays or AFSS or JMASA, then Level 4 mammalian assays or Level 3 or 4 fish screens (OECD TG 229/230/234) will provide data on multiple modalities. Check data on chemical analogues. Further mechanistic studies may help determine MOA.
R	–	Eq/0	Eq/0	No evidence for AR (ant)agonism. Unknown potential for adverse effects via other mechanisms.	For the "0" scenario, perform AR STTA with added metabolising system or Perform H assay or fish screen (AFSS) (Level 3) if existing data indicate this is needed.	A negative result indicates interaction with AR in other taxa is unlikely. Consider possible routes of exposure, implications of metabolism. Check data on chemical analogues. Further mechanistic studies may help determine MOA. Equivocal results may indicate chemical has multiple MOA.

C.1.4. H295R Steroidogenesis Assay (OECD TG 456)

Status: Assay validated by the OECD.

Modality detected/endpoints: Interference with steroidogenesis/inhibition and induction of estradiol and testosterone synthesis.

Background to the assay

213. The H295R Steroidogenesis Assay is an *in vitro* screening assay to detect substances that affect production of estradiol and testosterone. OECD TG 456 was published in July 2011. It provides a positive or negative result for the ability of a chemical to induce or inhibit the production of estradiol and testosterone. The assay utilises a human adrenocarcinoma cell line (NCI-H295R cells) that have the characteristics of undifferentiated human fetal adrenal cells. This cell line expresses all the key enzymes involved in steroidogenesis, from cholesterol to estradiol and testosterone. This expression would allow for the detection of other hormones. An "enhanced" Steroidogenesis Assay using H295R cells, where many other hormones are analysed, has been published (Wang et al., 2014). However, the OECD assay validation only included estradiol and testosterone. The cells represent a unique *in vitro* system because *in vivo*, expression of these enzymes is developmental stage specific with no one tissue expressing all the enzymes at once.

214. Chemicals may induce steroidogenesis; this can be determined by increased production of estradiol and testosterone. Alternatively, chemicals may inhibit steroidogenesis; this can be determined by decreased production of estradiol and testosterone. Results are expressed as fold change compared with the negative control. In the validation of the assay, forskolin induced estradiol and testosterone production whilst prochloraz inhibited estradiol and testosterone production. The validation of the Steroidogenesis Assay demonstrated that whilst not always directly predictive of a specific type of response *in vivo*, the chemicals chosen in the validation studies would always be flagged as a disrupter of steroidogenesis or a reproductive toxicant (OECD, 2010). The assay is therefore used somewhat as a "black box" where a positive result indicates that a chemical is a possible disrupter of steroidogenesis but without defining the exact mechanism of action.

215. An adequate response with positive control chemicals (forskolin and prochloraz), and other proficiency chemicals, is required in the OECD test guidance (TG) to demonstrate laboratory proficiency. The assay also requires the assessment of the cytotoxic effect of a chemical, as measurement of cell viability is an important feature of the TG. A minimum of 80% cell viability is needed for the hormone production assessment to be considered adequate. Limitations of the assay are that xenobiotic metabolising capability is unknown, but likely to be limited and production of other hormones (e.g. gluco- and mineralocorticoids) by the cells may affect estradiol and testosterone levels. The current assay does not detect 5-alpha reductase inhibitors (e.g. finasteride) that inhibit the conversion of testosterone to dihydrotestosterone. Although 5-alpha reductase is present in H295R cells, dihydrotestosterone is not a validated endpoint and therefore these chemicals

will not be identified. 5-alpha reductase inhibitors are detected by OECD TG 441 (Hershberger [H] assay).

216. The assay will not detect substances that act by affecting the hypothalamic/ pituitary/gonadal (HPG) as an *in vivo* intact axis is required for this. The effect of androgen receptor (AR), estrogen receptor (ER) and thyroid hormone receptor (TR) ligands on this assay is also not clear, although the Steroidogenesis Assay is not designed to detect these substances, it is not known whether they affect steroidogenesis. These chemicals will, however, be detected in AR-, ER- and TR-specific assays and therefore results from a suite of *in vitro* tests should be considered together.

217. The Steroidogenesis Assay requires that strict control is made of the age at which the cells are used. The capacity of the cells to produce estradiol changes with increasing number of cell passages. In addition, chemicals and cell matrices may interfere with hormone measurements. The TG includes quality control measures to ensure the accuracy and reliability of results. It is recommended that compliance with the quality control criteria and with the performance criteria for the positive control substances forskolin and prochloraz and with the other proficiency chemicals is demonstrated before evaluating results from this assay. Small deviations are unlikely to have compromised the assay, but judgement should be made on a case-by-case basis.

When/why the assay may be used

218. Although the Steroidogenesis Assay may be used at any stage in the hazard assessment process, the most likely use scenario is during initial assessment of chemicals for their ability to interact with endocrine systems *in vitro*, i.e. estrogen/androgen/thyroid/ steroidogenesis (E,A,T,S) modalities. Assays for interaction with other modalities (e.g. AR and ER), are likely to be conducted at the same time so that all results can be considered together. TR and other assays concerning mechanisms of thyroid disruption may be available, but they are not in common use. Data from the aromatase assay may also be available, chemicals testing positive in this assay are likely to also give positive results in the Steroidogenesis Assay as aromatase is one of the key enzymes in the steroidogenesis pathway. The steroidogenesis TG does not include the use of a xenobiotic metabolising system, but consideration should be given to the inclusion of this (Jacobs et al., 2008, 2013; OECD, 2008) depending on the circumstances (e.g. if the metabolism of a chemical is unknown), although the methods for inclusion of xenobiotic metabolising systems are not yet validated (see Paragraph 50). Alternatively, for a chemical with known metabolites, these could also be tested in the Steroidogenesis Assay. Another use scenario may be following effects obtained in higher tier tests, for example delayed puberty onset in females, but which are not exclusively indicative of an effect on ER. Selection of the most appropriate tests has to be on a case-by-case basis, but also considering the need to minimise animal testing. A further example could be results obtained in other apical assays, e.g. OECD TG 408 (90-day toxicity test), where effects on reproductive organs may be investigated further by testing in the Steroidogenesis Assay in combination with AR- and ER-based assays.

Introduction to the table of scenarios

219. Table C.1.4 gives guidance on a further step to take in the event of a positive (+) or negative (-) result in the Steroidogenesis 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.

220. The results of the Steroidogenesis Assay are given in the second column. Criteria for positive results are given in the draft test guideline. A result is judged positive if the fold difference is statistically significant from the solvent control at two adjacent concentrations in at least two tests, or when a single concentration data point is significantly different from the solvent control, and this can be confirmed by being significantly different in at least one more run within a +/- 1 concentration increment of the respective experiment. The latter allows for effects that may be seen close to the maximum concentration (1mM). It is important that quality and proficiency criteria are demonstrated for both positive and negative results.

221. Equivocal results for the guideline are not included in the table because these data generally require further interrogation about the result itself. This assay is a screen and therefore a clear positive or negative result should be obtained. In the event of an equivocal result, the considerations mentioned above about control quality and proficiency criteria should be taken into account and further investigations made. Equivocal results at high concentrations may may indicate solubility issues.

Existing data to be considered

222. Existing "mechanism" *in vitro* data are assumed to be available from ER- and AR-based assays and the aromatase assay (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.

223. Existing "effects" data refer to *in vivo* effects "of concern" (i.e. data from Level 4 or 5 vertebrate wildlife assays). These may come from varied sources and will depend on the type of substance (e.g. new chemicals, high production volume [HPV] chemical, pesticide). Thus, available data may range from repeated dose toxicity studies (28-day, 90-day), combined repeat dose/reproductive screening assays or fish screening assays, to chronic toxicity studies and multigeneration reproductive tests in vertebrate wildlife species. Some studies fail to identify endocrine disruptors (EDs) that weakly affect estrogen or androgen receptors, as was demonstrated on the basis of data generated in the validation process of the 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. The aromatase inhibitor CGS 18320B was detected by the OECD TG 407 assay, but this chemical was developed as a pharmaceutical aromatase inhibitor and therefore is a strong ED. The ability to detect chemicals that weakly interfere with steroidogenesis is not known. 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 modes of action, including endocrine modalities. 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 vertebrates may also have endocrine effects in mammals, but the physiological consequences of the effects are likely to be different.

224. Data may also be available from Hershberger (H) and Uterotrophic (UT) Assays (Level 3), but as these assays do not generally detect steroidogenesis interference, they are only useful in these cases for purposes of elimination.

225. When considering the results of the Steroidogenesis 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 (HTS) 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

226. The scenarios (A to R) presented in Table C.1.4 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 456 uses a human cell line, steroidogenic pathways relevant for androgen and estrogen synthesis are well conserved across taxa and therefore results results in this assay are likely to be relevant to other vertebrate species. Differences in steroidogenesis pathways, however, exist across species/cell/stages of development (for reviews see Scott, Mason and Sharpe [2009]; and Payne and Hales [2004]) and this should also be taken in account. 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. Further considerations specific to each scenario are given in the table.

227. Scenarios A to C represent positive results in the Steroidogenesis Assay in the presence of positive *in vitro* mechanistic data and positive, negative or equivocal *in vivo* effects data. A positive result in a Steroidogenesis Assay is strong evidence for disruption of steroidogenesis that may or may not be supported by the *in vivo* effects data. Inhibition of steroidogenesis (but not induction) could be followed up by a confirmatory aromatase assay if this is not already available. In the case of positive *in vivo* effects data, there may be sufficient evidence to conclude concern for endocrine disruption and therefore no need for further screening. *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. 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. 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.

228. Scenarios D to F represent positive results in the Steroidogenesis Assay in the presence of negative *in vitro* mechanistic data and positive, negative or equivocal *in vivo* effects data. As above, inhibition of steroidogenesis could be followed up by a confirmatory aromatase assay if this is not already available. Unless the metabolic profile of the test substance is known, one option may be to conduct these *in vitro* assays with an added metabolising system. If the metabolic profile is known, then an *in vivo* test may be advisable. The choice of tests will depend on the available *in vivo* effects data. 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. 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.

229. Scenarios G to I represent positive results in the Steroidogenesis Assay in the presence of various combinations of missing or equivocal data. As above, inhibition of steroidogenesis could be followed up by a confirmatory aromatase assay if this is not already available. The next step to take for missing or equivocal data 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 directly to *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.

230. Scenarios J to L represent negative results in the Steroidogenesis Assay in the presence of positive *in vitro* mechanistic data and positive, negative or equivocal *in vivo* effects data. The limitations of the Steroidogenesis Assay should be considered first (e.g. lack of metabolic activation, possible involvement of other factors). The positive *in vitro* mechanistic data indicates possible alternative EAT mechanisms. To confirm lack of steroidogenesis activity in the presence of *in vivo* data, a steroidogenesis with added metabolising capability could be performed. Otherwise *in vivo* tests will confirm or refute E,A,T,S activity.

231. Scenarios M to O represent negative results in the Steroidogenesis 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. The limitations of the Steroidogenesis Assay should also be considered (as described for Scenarios J to L). To confirm lack of steroidogenesis-related activity in the presence of *in vivo* data, a Steroidogenesis Assay with added metabolising capability could be performed. Otherwise, *in vivo* tests will confirm or refute E,A,T,S activity (Scenarios M to O).

232. Scenarios P to R represent negative results in the Steroidogenesis Assay in the presence of various combinations of missing or equivocal data. The limitations of the Steroidogenesis Assay should be considered first (as described for Scenarios J to L). As with the positive result scenarios above (see Paragraph 229), the next step to take for Scenarios P to R 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.

233. In all scenarios (A to R), the next step to take to strengthen weight of evidence will depend on the existing information. Table C.1.4 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 and there are many for which no TGs yet exist. 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.

234. In general, a decision about whether or not to conduct *in vivo* vertebrate wildlife tests will depend on the weight of evidence of new and existing data. If most available data (e.g. the results of the Steroidogenesis Assay, predictions from QSARs, "read-across" from data on similar substances and results from mammalian *in vivo* assays) suggest that the substance has the potential to cause endocrine disruption via interference with steroidogenesis (i.e. the level of suspicion about endocrine disrupting action is high – corresponding to Scenario A), then consideration should be given to the conduct of a higher level test.

235. For non-mammalian wildlife species, higher level tests with fish or amphibians (i.e TG 234 [FSDT], TG 240, TG 241) are recommended. Choice about which of these tests is most appropriate will be driven *inter alia* by mode of action considerations, and by whether multigeneration effects are to be expected. Such tests are unlikely to be needed if exposure of the natural environment is not expected. On the other hand, if available data only raise a low or moderate level of suspicion about endocrine disrupting action (e.g. the data appear to conflict with each other), then consideration should be given to the conduct of a fish or amphibian screen (i.e. OECD TG 229 or TG 230). There are fewer options available for invertebrates, but if ecdysteroid or juvenile hormone activity are suspected in arthropods (e.g. from a screening test with SJHASA), various higher level tests are available, including OECD GD 201, the *Daphnia* Multigeneration Test, and TG 233.

236. For mammals, similar considerations apply, but lower level tests (e.g. Level 3 or 4) 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.

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.

OECD (2010), "Peer review report for the H295R Cell-Based Assay for Steroidogenesis", OECD Series on Testing and Assessment, No. 133, OECD, Paris, www.oecd.org/officialdocuments/publicdisplaydocumentpdf/?cote=env/jm/mono(2010)32&doclanguage=en.

OECD (2008), *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.

Payne, A.H. and D.B. Hales (2004), "Overview of steroidogenic enzymes in the pathway from cholesterol to active steroid hormones", *Endocrine Reviews*, Vol. 25/6, pp. 947-970, https://doi.org/10.1210/er.2003-0030.

Scott, H.M., J.I. Mason and R.M. Sharpe (2009), "Steroidogenesis in the fetal testis and its susceptibility to disruption by exogenous compounds", *Endocrine Reviews*, Vol. 30/7, pp. 883-925, https://doi.org/10.1210/er.2009-0016.

Wang, S. et al. (2014), "Extending an *in vitro* panel for estrogenicity testing: The added value of bioassays for measuring antiandrogenic activities and effects on steroidogenesis", *Toxicological Sciences*, Vol. 141/1, pp. 78-89, https://doi.org/10.1093/toxsci/kfu103.

Table C.1.4. **H295R Steroidogenesis Assay (OECD TG 456): 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-) and androgen receptor (AR-) based assays (Level 2). Data on aromatase inhibition may also be available. 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 various information, such as data from repeat dose oral toxicity studies, reproduction/developmental toxicity screen tests, read-across from analogues, will be available.

Scenarios	Result of steroid-ogenesis 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	+	+	+	Inhibition/induction of steroidogenesis combined with effects on ER/AR/T/S and potential for adverse effects via multiple mechanisms.	Perform assay from Levels 3-5, e.g. male or female pubertal assay (Level 4) or EOGRTS or two-generation assays or partial/full non-mammalian wildlife life cycle tests, e.g. OECD TG 241 and TG 240 (Level 4/5).	A positive result indicates a possibility of interference with steroidogenesis in other taxa. If existing data are from a Level 5 assay, 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). If existing data are from Level 3 or 4 vertebrate wildlife assays, then Level 5 assays should provide more comprehensive data on adverse effects on endocrine-relevant endpoints over more extensive parts of the life cycle of the organism, although some Level 4 assays (e.g. TG 234 [FSDT]) may be sufficient for this purpose. Compare Steroidogenesis Assay results with other *in vitro* results to help discern mechanism. Consider route of exposures for existing effects data and possible transformation products and implications of absorption, distribution, metabolism and excretion (ADME) characteristics of the chemical. The chemical itself may give positive *in vitro* results but may not be absorbed or may be metabolised to an inactive metabolite *in vivo*. However, note that uptake and metabolism of chemicals can be different between mammalian and non-mammalian wildlife species.
B	+	+	–	Inhibition/induction of steroidogenesis combined with effects on ER/AR/T but effects not detected in *in vivo* studies. Weak steroidogenesis inhibition/induction does not result in adverse effects in the selected species under the conditions of the test. Metabolic differences may explain *in vitro*/*in vivo* differences.	Perform Steroidogenesis Assay with added metabolising system or Assay from Levels 3-4, e.g. fish screen (OECD TG 229/230/234) (Level 3) or male or female pubertal assay (Level 4).	A positive result indicates a possibility of interference with steroidogenesis in other taxa. 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 Level 3 or 4 vertebrate wildlife assays, then Level 5 assays should provide more comprehensive data on adverse effects on endocrine-relevant endpoints over more extensive parts of the life cycle of the organism, although some Level 4 assays (e.g. TG 234 [FSDT]) may be sufficient for this purpose. Compare Steroidogenesis Assay results with other *in vitro* results to help discern mechanism. Consider route of exposures for existing effects data and possible transformation products and implications of ADME characteristics of the chemical. The chemical itself may give positive *in vitro* results but may not be absorbed or may be metabolised to an inactive metabolite *in vivo*. However, note that uptake and metabolism of chemicals can be different between mammalian and non-mammalian wildlife species.

Scenarios	Result of steroid-ogenesis 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)**			
C	+	+	Eq/0	Inhibition/induction of steroidogenesis combined with effects on ER/AR/T but no or equivocal data from *in vivo* studies. Weak steroidogenesis inhibition/induction may not result in adverse effects in the selected species under the conditions of the test.	Perform assay Levels 3-4, e.g. fish screen (OECD TG 229/230/234) (Level 3) or male or female pubertal assay (Level 4).	A positive result indicates a possibility of interference with steroidogenesis in other taxa. Compare Steroidogenesis Assay results with other *in vitro* results to help discern mechanism. Consider route of exposures for equivocal existing effects data and possible implications of ADME characteristics of the chemical. Equivocal results may indicate chemical has multiple modes of action (MOA). Check data on chemical analogues.
D	+	–	+	Inhibition/induction of steroidogenesis and potential for adverse effects.	Perform assay from Levels 3-4, e.g. fish screen (OECD TG 229/230/234) (Level 3) or male or female pubertal assay (Level 4).	A positive result indicates a possibility of interference with steroidogenesis in other taxa. If existing data are from an adequate Level 5 assay, 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). If existing data are from Level 3 or 4 vertebrate wildlife assays, then Level 5 assays should provide more comprehensive data on adverse effects on endocrine-relevant endpoints over more extensive parts of the life cycle of the organism, although some Level 4 assays (e.g. TG 234 [FSDT]) may be sufficient for this purpose. 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	+	–	–	Inhibition/induction of steroidogenesis but effects not detected in *in vivo* studies. Weak steroidogenesis inhibition/induction does not result in adverse effects in the selected species under the conditions of the test. Metabolic differences may explain *in vitro*/*in vivo* differences.	Perform Steroidogenesis Assay with added metabolising system or Assay from Levels 3-4, e.g. fish screen (OECD TG 229/230/234) (Level 3) or male or female pubertal assay (Level 4).	A positive result indicates a possibility of interference with steroidogenesis in other taxa. 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 Level 3 or 4 vertebrate wildlife assays, then Level 5 assays should provide more comprehensive data on adverse effects on endocrine-relevant endpoints over more extensive parts of the life cycle of the organism, although some Level 4 assays (e.g. TG 234 [FSDT]) may be sufficient for this purpose. Consider route of exposures for existing effects data and possible transformation products and implications of ADME characteristics of the chemical. The chemical itself may give positive *in vitro* results but may not be absorbed or may be metabolised to an inactive metabolite *in vivo*. However, note that uptake and metabolism of chemicals can be different between mammalian and non-mammalian wildlife species. Check data on chemical analogues.

Scenarios	Result of steroid-ogenesis 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)**			
F	+	–	Eq/0	Inhibition/induction of steroidogenesis but no or equivocal data from *in vivo* studies. Weak steroidogenesis inhibition/induction may not result in adverse effects in the selected species under the conditions of the test.	Perform assay Levels 3-4, e.g. fish screen (OECD TG 229/230/234) (Level 3) or male or female pubertal assay (Level 4).	A positive result indicates a possibility of interference with steroidogenesis in other taxa. Consider route of exposures for equivocal existing effects data and possible implications of ADME characteristics of the chemical. Equivocal results may indicate chemical has multiple MOA. Check data on chemical analogues.
G	+	Eq/0	+	Inhibition/induction of steroidogenesis and potential for adverse effects via steroidogenesis interference or other EAT mechanisms. May act via non-steroidogenesis interference mechanism and may or may not require metabolic activation.	Perform assay from Levels 3-4, e.g. fish screen (OECD TG 229/230/234) (Level 3) or male or female pubertal assay (Level 4).	A positive result indicates a possibility of interference with steroidogenesis in other taxa. If existing data are from an adequate Level 5 assay, 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). If existing data are from Level 3 or 4 vertebrate wildlife assays, then Level 5 assays should provide more comprehensive data on adverse effects on endocrine-relevant endpoints over more extensive parts of the life cycle of the organism, although some Level 4 assays (e.g. TG 234 [FSDT]) may be sufficient for this purpose. 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.
H	+	Eq/0	–	Inhibition/induction of steroidogenesis but effects not detected in *in vivo* studies. Weak steroidogenesis inhibition/induction does not result in adverse effects in the selected species under the conditions of the test. Metabolic differences may explain *in vitro*/*in vivo* differences.	Perform Steroidogenesis Assay with added metabolising system.	A positive result indicates a possibility of interference with steroidogenesis in other taxa. 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 Level 3 or 4 vertebrate wildlife assays, then Level 5 assays should provide more comprehensive data on adverse effects on endocrine-relevant endpoints over more extensive parts of the life cycle of the organism. Consider route of exposures for existing effects data and possible transformation products and implications of ADME characteristics of the chemical. The chemical itself may give positive *in vitro* results but may not be absorbed or may be metabolised to an inactive metabolite *in vivo*. However, note that uptake and metabolism of chemicals can be different between non-mammalian wildlife species. Check data on chemical analogues. Further mechanistic studies may help determine MOA.

Scenarios	Result of steroid-ogenesis 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)**			
I	+	Eq/0	Eq/0	Steroidogenesis inhibition/induction with unknown potential for effects in *in vivo* studies. May act via non-steroidogenesis interference mechanism and may or may not require metabolic activation. Unknown potential for adverse effects.	Perform Steroidogenesis Assay with added metabolising system or assay from Levels 3-4, e.g. fish screen (OECD TG 229/230/234) (Level 3) or male or female pubertal assay (Level 4) if existing data indicate this is needed.	A positive result indicates a possibility of interference with steroidogenesis in other taxa. Consider route of exposures for equivocal existing effects data and possible implications of ADME characteristics of the chemical. Check data on chemical analogues. Equivocal results may indicate chemical has multiple MOA.
J	–	+	+	No evidence for steroidogenesis interference. Effects on ER/AR/T and potential for adverse effects via EAT mechanisms.	Perform Steroidogenesis Assay with added metabolising system or Perform assay from Levels 3-4, e.g. fish screen (OECD TG 229/230/234) (Level 3) or male or female pubertal assay (Level 4).	A negative result indicates that interference with steroidogenesis in other taxa is unlikely. If existing data are from an adequate Level 5 assay, there may be sufficient information to conclude concern for endocrine disruption. If existing data are from Level 3 or 4 vertebrate wildlife assays, then Level 5 assays should provide more comprehensive data on adverse effects on endocrine-relevant endpoints over more extensive parts of the life cycle of the organism, although some Level 4 assays (e.g. TG 234 [FSDT]) may be sufficient for this purpose. Consider route of exposures for existing effects data and possible transformation products and implications of ADME characteristics of the chemical. Check data on chemical analogues. Further mechanistic studies may help determine MOA.
K	–	+	–	No evidence for steroidogenesis interference. Effects on ER/AR/T but effects not detected in *in vivo* studies. Metabolic differences explain *in vitro*/*in vivo* E,A,T,S differences.	Perform assay from Levels 3-4, e.g. fish screen (OECD TG 229/230/234) (Level 3) or male or female pubertal assay (Level 4).	A negative result indicates that interference with steroidogenesis in other taxa is unlikely. 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 Level 3 or 4 vertebrate wildlife assays, then Level 5 assays should provide more comprehensive data on adverse effects on endocrine-relevant endpoints over more extensive parts of the life cycle of the organism, although some Level 4 assays (e.g. TG 234 [FSDT]) may be sufficient for this purpose. Metabolic deactivation of chemical may occur *in vivo* so that potential *in vitro* EAT activity is not realised. Consider possible routes of exposure, implications of metabolism.

Scenarios	Result of steroid-ogenesis 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 steroidogenesis interference. Effects on ER/AR/T but effects not detected in *in vivo* studies. Unknown potential for adverse effects. Metabolic differences explain *in vitro*/*in vivo* EAT differences.	Perform assay from Levels 3-4, e.g. fish screen (OECD TG 229/230/234) (Level 3) or male or female pubertal assay (Level 4).	A negative result indicates that interference with steroidogenesis in other taxa is unlikely. Metabolic deactivation of chemical may occur *in vivo* so that potential *in vitro* EAT activity is not realised. Consider possible routes of exposure, implications of metabolism. Check data on chemical analogues. Equivocal results may indicate chemical has multiple MOA.
M	–	–	+	No evidence for steroidogenesis interference. Metabolic differences or route of exposure explain *in vitro*/*in vivo* differences. Effects seen in existing studies are via non-E,A,T,S or non-endocrine mechanisms.	Perform Steroidogenesis Assay with added metabolising system or Perform assay from Levels 3-4, e.g. fish screen (OECD TG 229/230/234) (Level 3) or male or female pubertal assay (Level 4).	A negative result indicates that interference with steroidogenesis in other taxa is unlikely. If existing data are from an adequate Level 5 assay, there may be sufficient information to conclude concern for endocrine disruption. If existing data are from Level 3 or 4 vertebrate wildlife assays, then Level 5 assays should provide more comprehensive data on adverse effects on endocrine-relevant endpoints over more extensive parts of the life cycle of the organism, although some Level 4 assays (e.g. TG 234 [FSDT]) may be sufficient for this purpose. Metabolic activation of chemical may occur *in vivo*. Consider possible routes of exposure, implications of metabolism. Check data on chemical analogues. Further mechanistic studies may help determine MOA.
N	–	–	–	No evidence for steroidogenesis interference. No evidence of adverse effects.	Possibly no need for further testing. If there is uncertainty, may perform assay from Levels 3-4, e.g. fish screen (OECD TG 229/230/234) (Level 3) or male or female pubertal assay (Level 4).	A negative result indicates that interference with steroidogenesis in other taxa is unlikely. If existing data are from adequate Level 4 or 5 assays, 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 Level 3 or 4 vertebrate wildlife assays, then Level 5 assays should provide more comprehensive data on adverse effects on endocrine-relevant endpoints over more extensive parts of the life cycle of the organism, although some Level 4 assays (e.g. TG 234 [FSDT]) may be sufficient for this purpose. Check data on chemical analogues.
O	–	–	Eq/0	No evidence for steroidogenesis interference. Unknown potential for adverse effects via other mechanisms.	Perform Steroidogenesis Assay with added metabolising system or assay from Levels 3-4, e.g. fish screen (OECD TG 229/230/234) (Level 3) or male or female pubertal assay (Level 4) if existing data indicate this is needed.	A negative result indicates that interference with steroidogenesis in other taxa is unlikely. Consider possible routes of exposure, implications of metabolism. Check data on chemical analogues.

Scenarios	Result of steroid-ogenesis 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)**			
P	–	Eq/0	+	No evidence for steroidogenesis interference. Unknown potential for adverse effects via other mechanisms.	Perform Steroidogenesis Assay with added metabolising system.	A negative result indicates that interference with steroidogenesis in other taxa is unlikely. If existing data are from an adequate Level 5 assay, there may be sufficient information to conclude concern for endocrine disruption, although some Level 4 assays (e.g. TG 234 [FSDT]) may be sufficient for this purpose. Consider possible routes of exposure, implications of metabolism. 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 steroidogenesis interference. No evidence of adverse effects.	Perform Steroidogenesis Assay with added metabolising system.	A negative result indicates that interference with steroidogenesis in other taxa is unlikely. 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 Level 3 or 4 vertebrate wildlife assays, then Level 5 assays should provide more comprehensive data on adverse effects on endocrine-relevant endpoints over more extensive parts of the life cycle of the organism, although some Level 4 assays (e.g. TG 234 [FSDT]) may be sufficient for this purpose. Check data on chemical analogues. Further mechanistic studies may help determine MOA.
R	–	Eq/0	Eq/0	No evidence for steroidogenesis interference. Unknown potential for adverse effects via other mechanisms.	For the "0" scenario, perform Steroidogenesis Assay with added metabolising system or Perform assay from Levels 3-4, e.g. fish screen (OECD TG 229/230/234) (Level 3) or male or female pubertal (Level 4) if existing data indicate this is needed.	A negative result indicates that interference with steroidogenesis in other taxa is unlikely. Consider possible routes of exposure, implications of metabolism. Check data on chemical analogues. Further mechanistic studies may help determine MOA. Equivocal results may indicate chemical has multiple MOA.