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

C.1.5. Androgen Receptor Binding (US EPA OPPTS 890.1150)

Status: Assay validated at national level.

Modality detected/endpoints: Binding to androgen receptor.

Background to the assay

237. The AR Binding Assay is an *in vitro* screening assay to detect substances that bind to androgen receptors (AR). The assay has been in use for a number of years and there are different variations of the protocol. The most commonly used protocol utilises rat prostate cytosol as a source of AR without further purification. Human AR is also available as a recombinant proteind. The AR 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 has been validated in that context (US EPA, 2007). There is no OECD test guideline for the assay, but the US EPA (OPPTS) guideline is available (published in October 2009) (US EPA, 2009). In this context, the assay provides information on the ability of a compound to interact with AR but is not intended to be used to show that the interaction is, specifically, one-site competitive binding, or to characterise precisely the strength of the binding. The assay determines the ability of a chemical to displace a radiolabeled ligand (R1881) from AR (in a rat ventral prostate tissue homogenate) and provides a positive or negative result for the ability to bind to AR.

238. Chemicals that bind to AR 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 AR ligand binding domain among vertebrate species is well conserved, so that substances that bind to AR derived from one species are expected to bind to the AR from other vertebrate species. The results from this assay are therefore relevant to many taxa. A positive result in guideline OPPTS 890.1150 requires demonstration of a concentration response curve for the ability of the test chemical to displace radiolabelled R1881. The concentration response curve allows the determination of potency, i.e. IC50 (concentration at which 50% of radioligand is displaced by the test chemical) and relative binding affinity by comparing the log (IC50) of R1881with that of the test chemical.

239. Performance criteria are specified for the assay in order to demonstrate that the assay is functioning correctly. Proficiency chemicals are also used on each run to demonstrate the sensitivity of the experiment (reference standard: R1881 and weak positive control: dexamethasone). Compliance with the performance criteria should be checked 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

240. Although the AR 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. estrogen/androgen/thyroid/ steroidogenesis (E,A,T,S) modalities. Assays for interaction with other modalities (e.g. estrogen receptor [ER] 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 available, but they are not in common use. The AR 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 AR Binding Assay. Another use scenario may be following effects obtained in higher tier tests, for example delayed or accelerated puberty onset in males, which could be indicative of an effect mediated by 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. 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 the AR Binding Assay in combination with ER- and steroidogenesis-based assays.

Introduction to the table of scenarios

241. Table C.1.5 gives guidance on a further step to take in the event of a positive (+) or negative (-) result in the AR 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.

242. The results of the AR Binding Assay are given in the second column. Criteria for positive, negative and equivocal results are given in the OPPTS guideline. A result is judged positive if the lowest point on the fitted response curve, within the range of data, is less than 50%. This means that more than 50% of radiolabeled R1881 has been displaced from the receptor and a log IC50 can be obtained. A positive result should be obtained in at least two out of three independent test runs. Chemicals with limited solubility may be problematic in this assay if some binding is seen at high concentrations. The maximum concentration of chemical to be used in the assay is 1mM. The guideline provides detailed guidance on classification of a chemical as "binder", "equivocal", "non-binder" or "untestable" (does not reach 50% reduction in binding and is not soluble above 10^{-6} M). It is important that quality and proficiency criteria are demonstrated for both positive and negative results.

243. 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

244. Existing "mechanism" *in vitro* data are assumed to be available from ER-based assays (Level 2) and the Steroidogenesis Assay. Assays may also be available for interference with thyroid modalities. 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 AR Binding Assay and AR transactivation assays both provide data about the intrinsic ability of a chemical to interact with AR, but the binding assay will not distinguish between agonists and antagonists whilst some chemicals testing positive in the

transactivation assays may have affected the reporter gene activity through non-AR related mechanisms. Consistent results in both assays give more confidence about the presence or absence of an AR-related mode of action (MOA).

245. Existing "effects" data refer to *in vivo* effects "of concern" (i.e. data from Level 4 or 5 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 to chronic toxicity studies and multigeneration reproductive tests. 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 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 may also have endocrine effects in mammals, but the physiological consequences of the effects are likely to be different.

246. Data may also be available from Level 3 tests (Hershberger [H] and Uterotrophic [UT] Assays), but as the H assay primarily detects (*in vivo*) the same modality as AR binding, it is unlikely that it would be conducted before AR 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 A-modalities.

247. When considering the results of the AR Binding 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

248. The scenarios (A to R) presented in Table C.1.5 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 this assay uses rat AR, the well-conserved nature of AR 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.

249. Scenarios A to C represent positive results in the AR 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 AR Binding 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.

250. Scenarios D to F represent positive results in the AR 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 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.

251. Scenarios G to I represent positive results in the AR 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. 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.

252. Scenarios J to L represent negative results in the AR Binding Assay in the presence of positive *in vitro* mechanistic data and positive, negative or equivocal *in vivo* effects data. The limitations of the AR Binding Assay should be considered first (e.g. lack of metabolic activation, possible involvement of other binding proteins). 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, a Stably Transfected Human Androgen Receptor Transactivation Assay for detection of androgenic (ant)agonist-activity of chemicals (AR STTA) could be performed. Otherwise *in vivo* tests will confirm or refute E,A,T,S activity.

253. Scenarios M to O represent negative results in the AR 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 AR Binding 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 could be performed. Otherwise, *in vivo* tests will confirm or refute E,A,T,S activity (Scenarios M and O).

254. Scenarios P to R represent negative results in the AR Binding Assay in the presence of various combinations of missing or equivocal data. The limitations of the AR Binding Assay should be considered first (as described for Scenarios J to L). As with the positive result scenarios above (see Paragraph 171), 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.

255. In all scenarios (A to R), the next step to take to strengthen weight of evidence will depend on the existing information. Table C.1.5 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 test guidelines (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.

256. 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 Binding Assay, results from an AR transcription activation 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 androgen 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.

257. 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 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. JMASA, EASZY, XETA, OECD TG 231, TG 229 or TG 230 or the AFSS). 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 DMGT and TG 233.

258. 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 (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.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 (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.

US EPA (2009), "Endocrine Disrupter Screening Program Test Guidelines OPPTS 890.1150: Androgen receptor binding (rat prostate cytosol)", Environmental Protection Agency, Washington, DC, https://www.regulations.gov/document?D=EPA-HQ-OPPT-2009-0576-0003.

US EPA (2007), "Integrated summary report for the validation of an androgen receptor binding assay as a potential screen in the Endocrine Disrupter Screening Program", Environmental Protection Agency, Washington, DC.

Table C.1.5. **Androgen Receptor Binding Assay (US EPA OPPTS 890.1150): 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-) based assays and the Steroidogenesis Assay (Level 2). Thyroid hormone receptor (TR) and other assays concerning mechanisms of thyroid disruption may be available, but they are not in common use. In practice, data from all assays may not be available and therefore this must be taken into account when deciding on the "next step". Data from the Stably Transfected Human Andogen Receptor Transactivation Assay for detection of androgenic (ant)agonist-activity of chemicals (AR STTA) are assumed to be unavailable, but a decision about the next step to be taken will depend on the availability of this assay. Quantitative structure activity relationship (QSAR) predictions of androgen and estrogen 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 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 androgen receptor(AR) combined with effects on ER/T/S and potential for adverse effects via multiple mechanisms.	Perform assay AR STTA or Assay from Levels 3-4, e.g. Hershberger (H) assay (Level 3) or fish screen (AFSS or JMASA) (Level 3) or male Perpubertal (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).	Binding to mammalian AR indicates strong probability of binding to AR 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 [Fish Sexual Development Test – FSDT]) may be sufficient for this purpose. If existing data are from an H assay or AFSS, then Level 4 mammalian assays or 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 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	+	+	–	Interaction with AR combined with effects on AR/T/S but effects not detected in *in vivo* studies. Weak interaction with AR 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 AR STTA with added metabolising system or Assay from Levels 3-4, e.g. H assay or fish screen (OECD TG 229/230 or AFSS or JMASA) (Level 3) or male PP assay (Level 4).	Binding to mammalian AR indicates strong probability of binding to 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, although some Level 4 assays (e.g. TG 234 [FSDT]) may be sufficient for this purpose. If existing data are from an H assay or AFSS or JMASA, then Level 4 mammalian assays or 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.

Scenarios	Result of AR 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)**			
C	+	+	Eq/0	Interaction with AR combined with effects on ER/T/S but no or equivocal data from *in vivo* studies. Weak interaction with AR 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 (OECD TG 229/230/234 or AFSS or JMASA) (Level 3) or male PP assay (Level 4).	Binding to mammalian AR indicates strong probability of binding to 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.
D	+	–	+	Interaction with AR and potential for adverse effects.	Perform AR STTA or Perform assay from Levels 3-4, e.g. H assay or fish screen (OECD TG 229/230/234 or AFSS) (Level 3) or male PP assay (Level 4).	Binding to mammalian AR indicates strong probability of binding to 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, although some Level 4 assays (e.g. TG 234 [FSDT]) may be sufficient for this purpose. If existing data are from H assay or AFSS or JMASA, then Level 4 mammalian assays or fish screens (OECD TG 229/230) 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 AR but effects not detected in *in vivo* studies. Weak interaction with AR 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 AR STTA with added metabolising system or Assay from Levels 3-4, e.g H assay or fish screen (OECD TG 229/230/234 or AFSS or JMASA) (Level 3) or male PP assay (Level 4).	Binding to mammalian AR indicates strong probability of binding to 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, although some Level 4 assays (e.g. TG 234 [FSDT]) may be sufficient for this purpose. If existing data are from an H assay or AFSS or JMASA, then Level 4 mammalian assays or 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. Equivocal results may occur if 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*. However, note that uptake and metabolism of chemicals can be different between non-mammalian wildlife species. Check data on chemical analogues.

Scenarios	Result of AR 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)**			
F	+	–	Eq/0	Interaction with AR but no or equivocal data from *in vivo* studies. Weak interaction with AR does not result in adverse effects in the selected species under the conditions of the test.	Perform AR STTA or perform assay from Levels 3-4, e.g. H assay or fish screen (OECD TG 229/230/234 or AFSS or JMASA) (Level 3) or male PP assay (Level 4).	Binding to mammalian AR indicates strong probability of binding to AR in other taxa. AR transactivation assay results will indicate whether AR binding affects transcription. 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	+	Interaction with AR and potential for adverse effects via AR or other E,T,S mechanisms. May act via E,A,T,S mechanisms and may or may not require metabolic activation.	Perform AR STTA.	Binding to mammalian AR indicates strong probability of binding to 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, although some Level 4 assays (e.g. TG 234 [FSDT]) may be sufficient for this purpose. If existing data are from an H assay or AFSS or JMASA, then Level 4 mammalian assays or 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 occur if 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 AR but effects not detected in *in vivo* studies. Weak interaction with AR 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 AR STTA. For the "Eq" scenario perform AR STTA with added metabolising system.	Binding to mammalian AR indicates strong probability of binding to 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, although some Level 4 assays (e.g. TG 234 [FSDT]) may be sufficient for this purpose. If existing data are from an H assay or AFSS or JMASA, then Level 4 mammalian assays or 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 occur if chemical has multiple MOA.

Scenarios	Result of AR 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 AR with unknown potential for effects in *in vivo* studies. May act via AR and may or may not require metabolic activation. Unknown potential for adverse effects.	For the "0" scenario, AR STTA with added metabolising system. For the "Eq" scenario, H assay or fish screen (OECD TG 229/230/234 or AFSS or JMASA) (Level 3) if existing data indicate this is needed.	Binding to mammalian AR indicates strong probability of binding to AR 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 AR. Effects on ER/T/S and potential for adverse effects via E,A,T,S mechanisms.	Perform AR Binding Assay or AR transactivation assay with added metabolising system or Perform assay from Levels 3-4, e.g. Uterotrophic (UT) Assay or fish screen OECD TG 229/230/234 or TG 231) (Level 3) or male PP assay (Level 4).	Lack of binding to mammalian AR indicates binding to 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. 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 an H Assay or AFSS or JMASA, then a Level 4 mammalian assay or fish screen (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 interaction with AR. Effects on ER/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 229/230/234) (Level 3) or male or female PP assay (Level 4).	Lack of binding to mammalian AR indicates binding to 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 a Level 4 vertebrate wildlife assay, then a Level 5 assay should provide more 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 an H assay or AFSS or JMASA, then a Level 4 mammalian assay or fish screen (OECD TG 229/230/234) will provide data on multiple modalities. Metabolic deactivation of chemical may occur *in vivo* so that potential *in vitro* E,T,S activity is not realised. Consider possible routes of exposure, implications of metabolism.

Scenarios	Result of AR 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)**			
L	–	+	Eq/0	No evidence for interaction with AR. 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,T,S differences.	Perform assay from Levels 3-4, e.g. fish screen (OECD TG 229/230/234) (Level 3), or male or female PP (Level 4).	Lack of binding to mammalian AR indicates binding to AR in other taxa is unlikely. Metabolic deactivation of chemical may occur *in vivo* so that potential *in vitro* E,T,S activity is not realised. Consider possible routes of exposure, implications of metabolism. Check data on chemical analogues. Equivocal results may occur if chemical has multiple MOA.
M	–	–	+	No evidence for interaction with AR. 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 (OECD TG 229/230/234) (Level 3) or male or female PP assay (Level 4).	Lack of binding to mammalian AR indicates binding to 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. 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 an H assay or AFSS or JMASA, then Level 4 mammalian assays or 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 interaction with AR. 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 or JMASA) (Level 3), or male or female PP assay (Level 4).	Lack of binding to mammalian AR indicates binding to 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, although some Level 4 assays (e.g. TG 234 [FSDT]) may be sufficient for this purpose. If existing data are from an H assay or AFSS or JMASA, then Level 4 mammalian assays or fish screens (OECD TG 229/230/234) will provide data on multiple modalities. Check data on chemical analogues.

Scenarios	Result of AR 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)**			
O	–	–	Eq/0	No evidence for interaction with AR. Metabolic differences may explain *in vitro*/*in vivo* differences. Unknown potential for adverse effects via other mechanisms.	Perform AR STTA with added metabolising system or Fish screen (OECD TG 229/230/234) (Level 3) or male or female PP assay (Level 4) if existing data indicate this is needed.	Lack of binding to mammalian AR indicates binding to AR in other taxa is unlikely. Consider possible routes of exposure, implications of metabolism. Check data on chemical analogues.
P	–	Eq/0	+	No evidence for interaction with AR. Metabolic differences may explain *in vitro*/*in vivo* differences. Unknown potential for adverse effects via other mechanisms.	Perform AR STTA with added metabolising system.	Lack of binding to mammalian AR indicates binding to 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. 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 an H assay or AFSS, then Level 4 mammalian assays or 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 occur if chemical has multiple MOA.
Q	–	Eq/0	–	No evidence for interaction with AR. No evidence of adverse effects.	Perform AR STTA with added metabolising system.	Lack of binding to mammalian AR indicates binding to 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, although some Level 4 assays (e.g. TG 234 [FSDT]) may be sufficient for this purpose. If existing data are from an H assay or AFSS, then Level 4 mammalian assays or fish screens (OECD TG 229/230) 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 AR. Unknown potential for adverse effects via other mechanisms.	For the "0" scenario, perform AR STTA with added metabolising system or Perform assay from Levels 3-4, e.g. H assay or fish screen (OECD TG 229/230/234) (Level 3) or male PP assay (Level 4).	Lack of binding to mammalian AR indicates binding to 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 occur if chemical has multiple MOA.

C.1.6. Aromatase Assay (US EPA OPPTS 890.1200)

Status: Assay validated at national level.

Modality detected/endpoints: Inhibition of aromatase (CYP19) enzyme activity.

Background to the assay

259. The Aromatase Assay is an *in vitro* screening assay to detect substances that inhibit aromatase – the cytochrome P450 enzyme complex (CYP 19) responsible for the conversion of androgens to estrogens during steroidogenesis. Inhibition of aromatase enzyme activity alters the levels of circulating estrogens in males and females, which may lead to effects on reproductive organs and other targets such as the mammary gland. Aromatase is found in many vertebrate taxa, including mammals and fish, and therefore the results of this assay are applicable to both human health and mammalian and non-mammalian wildlife populations (US EPA, 2007).

260. The assay determines the conversion of radiolabeled [1-^{3}H]-androstenedione to estrone. The progress of the reaction can be followed by measuring the formation of either of the reaction products: estrone or water. The most common assay in usage determines the formation of tritiated water as the end product of the reaction (US EPA OPPTS 890.1200, published in October 2009; US EPA, 2009). This assay was chosen to be one of the suite of assays comprising United States Environmental Protection Agency's (US EPA) Endocrine Disruptor Screening Program "Tier 1" and has been validated in that context. Aromatase enzyme may be obtained from a number of sources (e.g. human placenta or rat ovary), but human recombinant aromatase has recently become available and this is the preferred source as it is directly relevant to humans, is easily obtained and does not require the use of laboratory animals. Guideline OPPTS 890.1200 utilises the human recombinant enzyme.

261. Inhibition of aromatase may also be determined in the H295R Steroidogenesis Assay. This assay detects substances that affect production of estradiol and testosterone, but the Steroidogenesis Assay contains all the enzymes involved in steroidogenesis, from cholesterol to estradiol and testosterone. Aromatase is the final enzyme in this pathway. Chemicals causing aromatase inhibition will be detected in the Steroidogenesis Assay by causing reduced production of estradiol from the H295R cells, but as the assay is not specific for aromatase it would not be possible to discern which enzyme(s) activity is altered. The H295R Steroidogenesis Assay, as an intact cell system, will also detect chemicals that induce aromatase enzyme activity whilst the aromatase assay itself is not capable of detecting inducers.

262. The aromatase assay may be subject to variability, for example due to degradation of the enzyme, and therefore performance criteria are specified in guideline OPPTS 890.1200 in order to demonstrate that the assay is functioning correctly. An adequate response with the proficiency chemicals econazole, fenarimol, nitrofen (inhibitors) and atrazine (non-inhibitor) should be demonstrated and the inhibitor

4-hydroxyandrostenedione is used as a positive control chemical in each experiment. Compliance with the performance criteria should be checked before evaluating results from this assay. A positive result in guideline OPPTS 890.1200 requires demonstration of inhibition of aromatase activity that fits a four-parameter non-linear regression model and such that the concentration response curve crosses 50% inhibition. The concentration response curve allows the determination of potency, i.e. IC50 (concentration at which the activity of aromatase is reduced to 50% of control values). In some cases, variability may be due to limited solubility of a chemical. The maximum concentration of chemical to be used in the assay is 1mM.

When/why the assay may be used

263. Although the aromatase 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. androgen receptor [AR], estrogen receptor [ER] and the Steroidogenesis Assay) 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 available, but they are not in common use. If the *in vitro* assays are not conducted at the same time then positive results in the Steroidogenesis Assay could be followed by an aromatase assay to confirm and clarify a mode of action (MOA). The aromatase 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, 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 aromatase 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 Aromatase and Steroidogenesis Assays in combination with AR- and ER-based assays.

Introduction to the table of scenarios

264. Table C.1.6 gives guidance on a further step to take in the event of a positive (+) or negative (-) result in the aromatase 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.

265. The results of the aromatase assay are given in the second column. Criteria for positive, negative and equivocal results are given in guideline OPPTS 890.1200. A result is judged positive if the average concentration response curve crosses 50% of control activity (“inhibitor”). A negative result is obtained if the average lowest portion of concentration response curve is greater than 75% of control activity or data do not fit the regression model (“non-inhibitor”). “Equivocal” results lie between these limits. It is

important that quality and proficiency criteria are demonstrated for both positive and negative results.

266. Equivocal results for the guideline 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 indicate solubility issues.

Existing data to be considered

267. Existing "mechanism" *in vitro* data are assumed to be available from ER- and AR-based and Steroidogenesis 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.

268. 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 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 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. The aromatase inhibitor CGS 18320B was detected by the OECD TG 407 assay, although this chemical was developed as a pharmaceutical aromatase inhibitor and therefore is a strong ED, but the ability to detect chemicals that weakly inhibit with aromatase 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 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 may also have endocrine effects in mammals, but the physiological consequences of the effects are likely to be different.

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

270. When considering the results of the aromatase assay, all available data should be used in order to reach a conclusion and a weight of evidence approach taken. This may include hgh 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

271. The scenarios (A to R) presented in Table C.1.6 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 mammalian aromatase is used, the enzyme is well conserved across taxa and therefore results in this assay are likely to be 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.

272. Scenarios A to C represent positive results for aromatase inhibition in the presence of positive *in vitro* mechanistic data and positive, negative or equivocal *in vivo* effects data. A positive result is strong evidence for inhibition of aromatase 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.

273. Scenarios D to F represent positive results for aromatase inhibition 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.

274. Scenarios G to I represent positive results for aromatase inhibition 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.

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

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

277. The limitations of the aromatase 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 when negative results in the aromatase assay are obtained in the presence of various combinations of missing or equivocal data 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.

278. In all scenarios (A to R), the next step to take to strengthen weight of evidence will depend on the existing information. Table C.1.6 is meant to provide a succinct guide and may not cover all circumstances or possibilities. The scenarios may also suggest that chemicals have simple or single MOA, when in practice they may have multiple endocrine and non-endocrine MOA. In some cases, for example, two opposite modes of simultaneous action (e.g. estrogenic and anti-estrogenic) could, depending on dose, lead to a minimisation or abolition of effects, while in others two different MOA (e.g. estrogenic and anti-androgenic) could potentially reinforce effects. Endocrine pathways interact and there are

many for which no test guidelines (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.

279. 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 inhibition of aromatase (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.

280. For non-mammalian wildlife species, higher level tests with fish or amphibians (i.e TG 234 [Fish Sexual Development Test], 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 DMGT and TG 233.

281. 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.

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Table C.1.6. **Aromatase Assay (US EPA OPPTS 890.1200): 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 endocrine receptor (ER-) and androgen receptor (AR-) based assays (Level 2). It is assumed that data from the Steroidogenesis Assay are also 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 aromatase 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 of aromatase 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. TG 241 and TG 240 (Level 4/5).	A positive result indicates strong probability of aromatase inhibition in other taxa. If existing data are from adequateLevel 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 [Fish Sexual Development Test]) may be sufficient for this purpose. Compare aromatase 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 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	+	+	–	Inhibition of aromatase combined with effects on ER/AR/T/S but effects not detected in *in vivo* studies. Weak aromatase inhibition 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 aromatase 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 strong probability of aromatase inhibition 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 aromatase 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 aromatase 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 of aromatase combined with effects on ER/AR/T but no or equivocal data from *in vivo* studies. Weak aromatase inhibition may not result in adverse effects in the selected species under the conditions of the test.	Perform assays 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 strong probability of aromatase inhibition in other taxa. Compare aromatase 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 occur if chemical has multiple modes of action (MOA). Check data on chemical analogues.
D	+	–	+	Inhibition of aromatase and potential for adverse effects.	Perform assay from Levels 3-4, e.g. fish screen (OECD TG 229/230/234) (Level 3) male or female pubertal assay or (Level 4).	A positive result indicates strong probability of aromatase inhibition 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 of aromatase but effects not detected in *in vivo* studies. Weak aromatase inhibition 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 aromatase 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 strong probability of aromatase inhibition 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 aromatase 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 of aromatase but no or equivocal data from *in vivo* studies. Weak aromatase inhibition may not result in adverse effects in the selected species under the conditions of the test.	Perform assays 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 strong probability of aromatase inhibition 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 of aromatase and potential for adverse effects via aromatase inhibition or other E,A,T,S mechanisms. May act via non-aromatase inhibition 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 strong probability of aromatase inhibition 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. Equivocal results may occur if 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	–	Inhibition of aromatase but effects not detected in *in vivo* studies. Weak aromatase inhibition 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 aromatase assay with added metabolising system.	A positive result indicates strong probability of aromatase inhibition 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 non-mammalian wildlife species. Check data on chemical analogues. Further mechanistic studies may help determine MOA.

Scenarios	Result of aromatase 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	Inhibition of aromatase with unknown potential for effects in *in vivo* studies. May act via non-aromatase inhibition mechanism and may or may not require metabolic activation. Unknown potential for adverse effects.	Perform aromatase 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 strong probability of aromatase inhibition 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 occur if chemical has multiple MOA.
J	–	+	+	No evidence for aromatase inhibition. Effects on ER/AR/T/S and potential for adverse effects via EAT mechanisms.	Perform aromatase 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 aromatase inhibition 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 aromatase inhibition. Effects on ER/AR/T/S 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 aromatase inhibition 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* E,A,T,S activity is not realised. Consider possible routes of exposure, implications of metabolism.
L	–	+	Eq/0	No evidence for aromatase inhibition. Effects on ER/AR/T/S 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 aromatase inhibition 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 aromatase 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 aromatase inhibition. 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 aromatase 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 aromatase inhibition 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 aromatase inhibition. 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 aromatase inhibition 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.
O	–	–	Eq/0	No evidence for aromatase inhibition. Unknown potential for adverse effects via other mechanisms.	Perform aromatase 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 aromatase inhibition in other taxa is unlikely. Consider possible routes of exposure, implications of metabolism. Check data on chemical analogues.
P	–	Eq/0	+	No evidence for aromatase inhibition. Unknown potential for adverse effects via other mechanisms.	Perform aromatase assay with added metabolising system.	A negative result indicates that interference with aromatase inhibition 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 occur if chemical has multiple MOA.

Scenarios	Result of aromatase 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)**			
Q	–	Eq/0	–	No evidence for aromatase inhibition. No evidence of adverse effects.	Perform Steroidogenesis Assay with added metabolising system.	A negative result indicates that interference with aromatase inhibition 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 aromatase inhibition. 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 aromatase inhibition 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 occur if chemical has multiple MOA.