C.3.2. Hershberger Bioassay in Rats (H assay) (OECD TG 441) (including OECD GD 115 on the Weanling Hershberger Bioassay)

Status: Assay validated by the OECD.

671. Modality detected/endpoints: androgens (weights of ventral prostate, seminal vesicles, LABC [levator ani plus bulbocavernosus muscle complex], cowpers glands, glans penis ↑); anti-androgens (weights of testosterone stimulated ventral prostate, seminal vesicles, LABC, cowpers glands, glans penis ↓); optional others (e.g. liver, paired kidney, paired adrenal and testis weights, changes in serum hormones including thyroid hormones). *Note:* weanling H assay does not include glans penis.

Background to the assay

672. This assay is a short-term *in vivo* screening assay in male rats for chemicals that interact with the androgen receptor (AR) and chemicals that inhibit the enzyme 5-alpha reductase. Route of administration of test substance is via oral gavage or subcutaneous injection. It is based on changes in weight of the accessory tissues of the male reproductive tract in response to androgens and anti-androgens in animal models where endogenous androgens are minimal as a result of castration or because the animals are immature. The surgically castrated peripubertal rat is the primary model validated for the assay and is described in OECD TG 441 (adopted in September 2007). This model is sensitive to androgens and anti-androgens. An alternative model – the intact (uncastrated) weanling rat – was also validated due to animal welfare concerns with the castration procedure, but did not seem to consistently detect weak anti-androgenic chemicals at the doses tested, although androgenic chemicals were detected. The castrated peripubertal model is therefore more commonly used because both androgenic and anti-androgenic protocols can be run in the same experiment. The use of the weanling H assay is described in a guidance document (OECD, 2009). The castrated peripubertal rat model utilises the weights of five androgen-dependent sex accessory tissues (ventral prostate, seminal vesicles, LABC, cowpers glands and glans penis) as the primary endpoints, whilst for the weanling rat model the list does not include the glans penis because the weanling male has not yet achieved preputial separation. Testis weight is an optional endpoint in the weanling model, although it should be noted that the weight changes with androgens and anti-androgens are opposite to those seen with the other sex accessory tissues. Serum hormone levels are also optional for both models. These include the thyroid hormones (T3 and T4) so that additional information on thyroid effects may also be obtained, and luteinising hormone (LH), follicle stimulating hormone (FSH) and testosterone.

673. The castrated peripubertal rat does not have an intact hypothalamic/pituitary/gonadal (HPG) axis and therefore chemicals acting through this mechanism will not be detected. The HPG axis in the weanling rat is intact and therefore it is possible that such chemicals may be detected. In practice, this has not been tested and the immaturity of the animals, plus the co-administration of testosterone in the anti-androgen test, makes this unlikely.

674. Although this assay is a "screen", some authorities may regard a decrease in the weight of sex accessory tissues as possibly adverse; for example OECD GD 43 (OECD, 2008c) states that "a significant change in absolute testis weight (increase or decrease) can indicate an adverse effect". If this occurs in immature animals at a time when this should not occur naturally, then this could represent an adverse effect in a sensitive life stage. Likewise, the castrated H assay may be regarded as a model for immature animals and therefore a decrease sex accessory tissue weights could be regarded as adverse. Interpretations of the results of this assay may vary according to region and regulation, and should always utilise all data in a weight of evidence approach. Androgenic chemicals cause growth of the sex accessory tissues whilst anti-androgenic chemicals inhibit the growth caused by co-administration of testosterone. Anti-androgens may act either via AR antagonism (e.g. flutamide) or they may act via inhibition of the enzyme 5-alpha reductase (e.g. finasteride), which converts testosterone to the more potent dihydrotestosterone. 5-alpha reductase inhibitors may be distinguished from AR antagonists in the H assay by a more pronounced effect on the ventral prostate. AR antagonists can also be distinguished from 5-alpha reductase inhibitors by the use of *in vitro* assays as 5-alpha reductase inhibitors do not generally interact with AR. At present there are no validated assays for 5-alpha reductase inhibition although literature methods are available (Lo et al., 2007).

675. The growth of the sex accessory tissues may not always be entirely of androgenic origin. High doses of other hormones may give similar responses (e.g. potent estrogens may increase the weight of seminal vesicles). Chemicals affecting steroid metabolism could also conceivably affect the anti-androgen assay.

When/why the assay may be used

676. Although OECD TG 441 can be used at any stage in the hazard assessment process, the most likely use scenario will be following a positive result in an AR transactivation assay or AR Binding Assay, in order to determine whether the positive result *in vitro* is translated into a positive result *in vivo*. It may also be used as a screen in the absence of positive *in vitro* data, when a chemical that is negative in the *in vitro* AR-interaction screens is suspected of producing androgenic metabolites *in vitro*. In this case, the first option would be to use an additional metabolising system in the *in vitro* tests, but the H assay as an *in vivo* assay will include all metabolising systems. Another possible scenario is following observation of effects in higher tier tests, for example delayed puberty onset in males, but which are not exclusively indicative of an effect on AR. In the European Union, chemicals included in REACH, Plant Protection Products and Biocides legislation may have been tested in OECD TG 421/422, TG 416 (Two-Generation Reproductive Toxicity Study) or the Extended One-Generation Reproduction Toxicity Study (EOGRTS – OECD TG 443), the H assay may then be used as a follow up to clarify the mode of action (MOA). The H assay is also likely to be carried out as part of the United States Environmental Protection Agency's Endocrine Disruptor Screening Program (US EPA EDSP) Tier 1 screening battery. The castrated peripubertal rat assay (as described in OECD TG 441) is mandatory for the US EPA EDSP Tier 1 screening battery and is most likely to be the assay of choice in other testing strategies. Selection of the most appropriate assays has to be on a case-by-case basis, but also considering the need to minimise animal testing.

677. It should be noted that the H assay was designed to be sensitive and will detect weak and strong AR modulators and 5-alpha reductase inhibitors. In the validation of the H assay, trenbolone acetate and testosterone were defined as "potent" androgens whilst finasteride was a "potent" anti-androgen. Linuron and vinclozolin were defined as "weak" anti-

androgens (OECD, 2008b), but no weak androgens were tested. Weakly acting chemicals may not always be detected as endocrine disruptors (EDs) when tested in higher level tests because the endocrine system in intact/adult animals has a greater ability to compensate than in the H assay where the HPG axis is disrupted/immature and in the case of repeat dose studies dose levels may need adjustment to lower doses in order to cope with general toxicity.

678. The route of exposure is also an important consideration for the H assay. OECD TG 441 states that the test substance may be administered by oral or subcutaneous routes, but suggests that the route most relevant for human exposure should be used. The route will have consequences for absorption, distribution, metabolism and excretion (ADME) and is an important consideration when interpreting results.

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

Introduction to the table of scenarios

680. Table C.3.2 gives guidance on a further step to take in the event of a positive (+) or negative (-) result in the H 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.

681. The results of OECD TG 441 are given in the second column. Criteria for positive results in OECD TG 441 are given in the test guideline itself, i.e. a statistically significant increase (agonism) or decrease (antagonism or 5-alpha reductase inhibition) in weights of two or more of the sex accessory tissues compared to the relevant control and all target tissues showing some change in the relevant direction. In the case of agonists, the control is only treated with vehicle for the test substance whilst for antagonists and 5-alpha reductase inhibitors, the control is treated with testosterone plus vehicle for the test substance. Negative results are no (statistically significant) changes in weights of the sex accessory tissues compared to the relevant control. Single, isolated changes would also be considered negative. The guideline suggests that combined evaluation of all sex accessory tissue responses could be achieved using appropriate multivariate data analysis. It is important that quality criteria (coeffecients of variation) for the weights of control sex

accessory tissues are demonstrated. Details are given in the test guideline. Note that in the weanling assay, testis weight decreases with agonists and increases with antagonists. Details of the criteria for positive results in this assay are given in the GD (OECD, 2009).

682. Optional endpoints may include measurement of serum LH, FSH or testosterone. These endpoints should supplement the sex accessory tissue weights and the assay should not be considered to be positive if changes in these endpoints occur in the absence of weight changes in the primary tissues. In addition, serum T3 and T4 levels may provide useful information on possible effects on the thyroid, although measurement of thyroid weight and serum TSH levels would be also useful in this case. They are not considered further here as this is not the primary use of the assay. Measurement of serum testosterone may be useful if induction of liver xenobiotic metabolising enzymes is suspected. Experience with of serum hormone determinations in rodent assays has revealed that their detection/measurement in rodent studies can be challenging. A recent workshop on "Practicability of Hormonal Measurements" was organised by the BfR (Germany) and the finding from this workshop will be published (Kucheryavenko et al., 2018). The optional endpoint of liver weight would also be very useful. In these cases, increased clearance of testosterone may lead to an apparent anti-androgenic effect on the sex accessory tissues that does not result from interaction with AR.

683. 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 sex accessory tissue weights, non AR-related changes should be taken into account and further investigations made.

Existing data to be considered

684. Existing "mechanism" *in vitro* data are assumed to be available from estrogen receptor (ER-), 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. As noted above, there is no validated assay available for 5-alpha reductase inhibitors at present and although 5-alpha reductase is present in H295R cells used in the Steroidogenesis Assay, the assay does not include the required endpoint for this (dihydrotestosterone). Although the current *in vitro* test guidelines do not incorporate metabolic activation, published information on use of metabolic activation systems is available (Jacobs et al., 2008; 2013) as is an OECD detailed review paper (OECD, 2008a). These methods, however, have not yet been validated.

685. Existing "effects" data refer to *in vivo* effects that 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) or combined repeat dose/reproductive screening assays to chronic toxicity studies and multigeneration reproductive tests. 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 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 all MOA, including endocrine modalities. If data are available from single or multigeneration studies that are adequately conducted with updated guidelines that include endpoints sensitive to EDs, then there should be no reason to conduct an H assay as the higher tier test will provide stronger evidence for hazard identification/characterisation. Multigeneration studies conducted prior to the introduction of these endpoints will still provide valuable information on reproductive and endocrine organ toxicity, reproduction and development, but may not be sufficiently sensitive to endocrine active substances (EASs), in which case the H assay would provide further valuable information. A decision about whether to conduct further animal tests would, however, need to consider whether sufficient supplementary data may be provided by *in vitro* tests. Data may also be available on effects in mammalian and non-mammalian wildlife species, although caution should be used when extrapolating between taxa. A chemical causing endocrine effects in non-mammalian environmental species (fish, for example) may also have endocrine effects in mammals, but the physiological consequences of the effects are likely to be different.

686. When considering the results of the H assay, all available data should be used in order to reach a conclusion and a weight of evidence approach taken. This may include high throughput screening data, read-across data from structural analogues and quantitative structure activity 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

687. The scenarios (A to R) presented in Table C.3.2 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 441 uses rats, the well-conserved nature of AR across taxa should be a strong indication that results in this assay are relevant to other vertebrate species. Results in laboratory mammal tests are also highly relevant for environmental mammalian species. Wherever possible, the recommended "next step which could be taken" avoids unnecessary animal testing. However, sometimes conducting an animal test will be indicated and then the relevance of species, strain, exposure route and species-specific metabolism should always be considered. The sensitivity and physiological function of the hormone under investigation in the test species should also be considered. In general, lower level tests should be conducted before higher level tests in order to avoid unnecessary animal usage, unless it is apparent that a Level 5 test will be required anyway or will be needed to establish the evidence to conclude on ED properties. Information on some endocrine-related tumours may be detected more comprehensively in carcinogenicity studies (OECD TG 451/453) (Level 4); for example, detection of certain types of thyroid tumors in the absence of reproductive or developmental effects, as well as substances causing tumors in other endocrine-sensitive tissues. At Level 5, the EOGRTS (OECD TG 443) is the most sensitive reproduction assay for detecting endocrine disruption because it includes evaluation of a number of endocrine endpoints not included in the current two-generation study (OECD TG 416) adopted in 2001. It is recognised, however, that some jurisdictions may require a two-generation study. Further considerations specific to each scenario are given in the table.

688. Scenarios A to C represent positive results in the H assay in the presence of positive *in vitro* mechanistic data and positive, negative or equivocal *in vivo* effects data. A positive

result in AR-based assays in combination with a positive H assay is strong evidence for (anti)androgenic activity that may or may not be supported by the *in vivo* effects data. There may be sufficient evidence to conclude concern for endocrine disruption and therefore no need for further screening. Positive results in the H assay may also indicate similar (anti)androgenicity in lower vertebrates. These could be followed up with partial life cycle tests such as the Fish Sexual Development Test (FSDT) or the Larval Amphibian Growth and Development Assay (LAGDA). *In vivo* assays/tests with negative results should be interpreted with caution as they may either indicate that the tests used do not have sufficient power to detect weak effects or, alternatively, that the effects do not present a concern for endocrine disruption. The possibility of other mechanisms should also not be overlooked, e.g. positive ER-based assays and a positive result H assay may indicate (anti)estrogenic effects. Other (non-E,A,T,S) mechanisms may also be considered (e.g. involving other receptors or endocrine axes).

689. Scenarios D to F represent positive results in the H assay in the presence of negative *in vitro* mechanistic data and positive, negative or equivocal *in vivo* effects data. Negative results in the *in vitro* assays should be viewed with caution in case a metabolite is responsible for the positive H assay. These scenarios may also occur if enhanced metabolism or clearance of testosterone is responsible for the positive H assay. Unless the metabolic profile of the test substance is known, then one option may be to conduct these assays with an added metabolising system. If the metabolic profile is known, then a higher level *in vivo* test may be advisable. The choice of tests will depend on the available *in vivo* effects data. Positive results in the H assay may also indicate similar (anti)androgenicity in lower vertebrates. As in Scenarios A to C, *in vivo* assays/tests with negative results should be interpreted with caution as they may either indicate that the tests used do not have sufficient power to detect weak effects or, alternatively, that the effects do not present a concern for endocrine disruption.

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

691. Scenarios J to L represent negative results in the H assay in the presence of positive *in vitro* mechanistic data and positive, negative or equivocal *in vivo* effects data. The *in vitro* mechanistic data given in the table could be any of the estrogen/androgen/thyroid/ steroidogenesis (E,A,T,S) tests (e.g. the ER binding or Steroidogenesis Assay). A weak aromatase inhibitor, for example, could give Scenario J a positive result in the Steroidogenesis Assay and a positive result in the female PP assay. All three scenarios could also arise from a chemical that binds to AR but is metabolised to a non-androgenic metabolite leading to negative results in the H assay and this should be considered first when investigating the next step. Endocrine active potency may also explain differences

between *in vitro* and *in vivo* results (e.g. a chemical with weak endocrine activity may give a positive result *in vitro* but may be negative *in vivo*). Positive *in vivo* effects data may involve other E,A,T,S, non-E,A,T,S mechanisms (e.g. involving other receptors or endocrine axes), more sensitive endpoints, greater statistical power or life stages that are more sensitive to the substance than castrated/immature animals in the H assay.

692. Scenarios M to O represent negative results in the H 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. Where there are positive *in vivo* effects data, there could still be an androgen-related mechanism. The effects may be related to length of exposure, route of exposure or exposure at different life stages. Other E,A,T,S or non-E,A,T,S mechanisms may also be involved.

693. Scenarios P to R represent negative results in the H assay in the presence of various combinations of missing or equivocal data. As with the positive result scenarios above (see Paragraph 692), the next step to take in these eventualities will have to be decided on a case-by-case basis. However, the recommended first step is generally to obtain reliable mechanistic (*in vitro*) data rather than proceed directly to *in vivo* testing. In all cases, the role of metabolism, route of exposure and data from structural analogues should be considered before deciding on the next step.

694. In all scenarios (A to R), the next step to take to strengthen weight of evidence will depend on the existing information. Table C.3.2 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.

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Table C.3.2. **Hershberger Bioassay (H assay) (OECD TG 441) (including OECD GD 115 on the Weanling Hershberger Bioassay): Guidance for scenarios of combinations of results with existing data**

This table represents possible conclusions to be drawn from assay data, and a next step which could be taken if further evidence is required about possible endocrine disrupting properties and/or effects. The guidance offered is not meant to be prescriptive, but provides science-based considerations. It encourages the use of all available data and expert judgement in a weight of evidence approach. Regional and national interpretation of results and "next steps" may vary.

The conclusions are grouped into a series of scenarios (A-R), each scenario representing a different combination of assay results, existing *in vitro* data and existing *in vivo* data. The symbol "+" indicates that the data in question represent a positive result, «-" indicates a negative result, and "Eq/0" indicates that the data are either equivocal or are not available.

Existing results: * "Mechanism (*in vitro* mechanistic data)" assumes that mechanistic data are available from estrogen receptor (ER-), androgen receptor (AR-) and steroidogenesis-based assays (Level 2). Thyroid hormone receptor (TR) and other assays concerning mechanisms of thyroid disruption may be available, but they are not in common use. In practice, data from all assays may not be available and therefore this must be taken into account when deciding on the "next step". Quantitative structure activity relationship (QSAR) predictions of estrogen and androgen binding/activation may be made for some substances.

Existing results: ** "Effects (*in vivo* effects of concern)" assumes effects have been observed in other *in vivo* screens/tests which give rise to concern that the test chemical may be an endocrine disrupter. These may be repeated dose toxicity tests (e.g. OECD TG 407, TG 408 28-day and 90-day studies), reproductive tests (e.g. reproduction screening assays or two-generation studies) or read-across from chemical analogues.

Scenarios	Result of OECD TG 441 (H assay)	Existing results		Possible conclusions	Next step which could be taken to strengthen weight of evidence if necessary	Other considerations
		Mechanism (*in vitro* mechanistic data)*	Effects (*in vivo* effects of concern)**			
A	+	+	+	Strong evidence for androgen/anti-androgen (A/anti-A) activity with (potential for) adverse effects via androgen receptor (AR) mechanism. 5-alpha reductase inhibitor with (potential for) adverse effects.	Perform assay from upper levels, e.g. male pubertal assay (Level 4) OR Extended One-Generation Reproduction Toxicity Study (EOGRTS) or two-generation assay (Level 5).	Check pattern of change across sex tissues for possible 5-alpha reductase inhibition. If existing data are from Level 4 or 5 (or less sensitive) assays, there is sufficient information to conclude evidence of concern for endocrine disruption (the EOGRTS provides the most information; however, for endocrine disrupting chemicals [EDCs] with a carcinogenic potential, OECD TG 451-3 may be more sensitive). Consider route of exposures for H assay and existing effects data and possible implications of absorption, distribution, metabolism and excretion (ADME) characteristics of the chemical. The chemical itself may give positive *in vitro* results and *in vivo* results but may also be metabolised to a metabolite that also has positive results *in vitro* and *in vivo*. A positive result could have arisen from other (E,A,T,S or non-E,A,T,S) mechanisms. A/anti-A activity possible in lower vertebrates. Consider performing a Fish Sexual Development Test (FSDT) or Larval Amphibian Growth and Development Assay (LAGDA).
B	+	+	–	Strong evidence for A/anti-A activity via AR but effects not detected in other *in vivo* studies in intact animals. 5-alpha reductase inhibitor with (potential for) adverse effects but effects not detected in other *in vivo* studies in intact animals.	Perform assay from Level 4 (e.g. male pubertal assay) OR Level 5 (e.g. EOGRTS or two-generation) assay.	Check pattern of change across sex tissues for possible 5-alpha reductase inhibition. If existing data are from an adequate Level 5 assay, there may be sufficient information to conclude absence of concern for endocrine disruption (the EOGRTS provides the most information; however, for EDCs with a carcinogenic potential, OECD TG 451-3 may be more sensitive). If existing data are from a less sensitive assay, a higher level test may be required. Consider route of exposures for H assay and existing effects data and possible implications of ADME characteristics of the chemical. Check data on chemical analogues. A positive result could have arisen from other (E,A,T,S or non-E,A,T,S) mechanisms. A/anti-A activity possible in lower vertebrates. Consider performing an FSDT or LAGDA.
C	+	+	Eq/0	Strong evidence for A/anti-A activity via AR, but no or equivocal data from other *in vivo* studies. 5-alpha reductase inhibitor with (potential for) adverse effects but no or equivocal data from other *in vivo* studies.	Perform assay from Levels 4 or 5 (e.g. EOGRTS or two-generation) assay.	Check pattern of change across sex tissues for possible 5-alpha reductase inhibition. Check data on chemical analogues. Consider route of exposures for H assay and existing effects data and possible implications of ADME characteristics of the chemical. Depending on route/kinetic and existing data considerations, may perform assay from Levels 4 or 5. A positive result could have arisen from other (E,A,T,S or non-E,A,T,S) mechanisms. A/anti-A activity possible in lower vertebrates. Consider performing an FSDT or LAGDA. Equivocal results may indicate chemical has multiple modes of action (MOA).

Scenarios	Result of OECD TG 441 (H assay)	Existing results		Possible conclusions	Next step which could be taken to strengthen weight of evidence if necessary	Other considerations
		Mechanism (*in vitro* mechanistic data)*	Effects (*in vivo* effects of concern)**			
D	+	–	+	Strong evidence for A/anti-A activity. Acts via AR mechanism, but requires metabolic activation. 5-alpha reductase inhibitor but requires metabolic activation. Acts via non-AR mechanism and may or may not require metabolic activation.	Perform AR transactivation assay or binding assay with added metabolising system.	If existing data are from Level 4 or 5 (or less sensitive) assays, there is sufficient information to conclude evidence of concern for endocrine disruption (the EOGRTS provides the most information; however, for EDCs with a carcinogenic potential, OECD TG 451-3 may be more sensitive). Check pattern of change across sex tissues for possible 5-alpha reductase inhibition. 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. A/anti-A activity possible in lower vertebrates. Consider performing an FSDT or LAGDA.
E	+	–	–	Weak evidence for A/anti-A activity via AR but requires metabolic activation. 5-alpha reductase inhibitor but requires metabolic activation. Chemical requires metabolic activation and metabolite has weak activity. Weak A/anti-A activity/5-Aapha reductase inhibition does not result in adverse effects. Acts via non-AR mechanism.	Perform AR transactivation assay or binding assay with added metabolising system OR Perform assay from Levels 4 or 5.	Check pattern of change across sex tissues for possible 5-alpha reductase inhibition. If existing data are from an adequate Level 5 assay, there may be sufficient information to conclude absence of concern for endocrine disruption (the EOGRTS provides the most information; however, for EDCs with a carcinogenic potential, OECD TG 451-3 may be more sensitive). If existing data are from a less sensitive assay, a higher level test may be required. Consider route of exposures for H assay and existing effects data and possible implications of ADME characteristics of the chemical. Check data on chemical analogues. A positive result could have arisen from other (E,A,T,S or non-E,A,T,S) mechanisms, e.g. HPG axis or liver enzyme induction. A/anti-A activity possible in lower vertebrates. Consider performing an FSDT or LAGDA.
F	+	–	Eq/0	Weak evidence for A/anti-A activity via AR but requires metabolic activation. 5-alpha reductase inhibitor but requires metabolic activation. Requires metabolic activation and metabolite has weak/equivocal activity. Acts via non-AR mechanism.	Perform AR transactivation assay or binding assay with added metabolising system OR Perform assay from Levels 4 or 5.	Check pattern of change across sex tissues for possible 5-alpha reductase inhibition. Check data on chemical analogues. Further mechanistic studies may help determine MOA. Level 4 or 5 studies will provide hazard data. A positive result could have arisen from other (E,A,T,S or non-E,A,T,S) mechanisms, e.g. HPG axis or liver enzyme induction. A/anti-A activity possible in lower vertebrates. Consider performing an FSDT or LAGDA. Equivocal results may indicate chemical has multiple MOA.

Scenarios	Result of OECD TG 441 (H 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	+	Moderate or strong evidence for A/anti-A activity via AR. May require metabolic activation. 5-alpha reductase inhibitor. May require metabolic activation. Has potential for adverse effects via AR mechanism or 5-alpha reductase inhibition. May act via non-AR mechanism and may or may not require metabolic activation.	For the "0" scenario, perform AR transactivation assay or binding assay. For the "Eq" scenario, perform AR transactivation assay or binding assay with added metabolising system.	Check pattern of change across sex tissues for possible 5-alpha reductase inhibition. If existing data are from Level 4 or 5 (or less sensitive) assays, there is sufficient information to conclude evidence of concern for endocrine disruption (the EOGRTS provides the most information; however, for EDCs with a carcinogenic potential, OECD TG 451-3 may be more sensitive). Check data on chemical analogues. Further mechanistic studies may help determine MOA. A positive result could have arisen from other (E,A,T,S or non-E,A,T,S) mechanisms, e.g. HPG axis. A/anti-A activity possible in lower vertebrates. Consider performing an FSDT or LAGDA. Equivocal results may indicate chemical has multiple MOA.
H	+	Eq/0	–	Weak evidence for A/anti-A activity. May act via AR, metabolic activation is required. 5-alpha reductase inhibitor with (potential for) adverse effects but effects not detected in other *in vivo* studies in intact animals. A/anti-A activity/5-alpha reductase does not result in adverse effects.	For the "0" scenario, perform AR transactivation assay or binding assay. For the "Eq" scenario, perform ER transactivation assay or binding assay with added metabolising system.	Check pattern of change across sex tissues for possible 5-alpha reductase inhibition. If existing data are from an adequate Level 5 assay, there may be sufficient information to conclude absence of concern for endocrine disruption (the EOGRTS provides the most information; however, for EDCs with a carcinogenic potential, OECD TG 451-3 may be more sensitive). If existing data are from a less sensitive assay, a higher level test may be required. Consider route of exposures for H assay and existing effects data and possible implications of ADME characteristics of the chemical. Check data on chemical analogues. Further mechanistic studies may help determine MOA. A/anti-A activity possible in lower vertebrates. Consider performing an FSDT or LAGDA. Equivocal results may indicate chemical has multiple MOA.
I	+	Eq/0	Eq/0	A/anti-A activity of unknown potency. May act via AR, metabolic activation is required. 5-alpha reductase inhibitor of unknown potency. Unknown potential for adverse effects.	For the "0" scenario, perform AR transactivation assay or binding assay. For the "Eq" scenario, perform AR transactivation assay or binding assay with added metabolising system, or Level 4 or 5 assay if existing data indicate this is needed.	Check pattern of change across sex tissues for possible 5-alpha reductase inhibition. Check data on chemical analogues. Further mechanistic studies may help determine MOA. A/anti-A activity possible in lower vertebrates. Consider performing an FSDT or LAGDA. Equivocal results may indicate chemical has multiple MOA.

Scenarios	Result of OECD TG 441 (H 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)**			
J	–	+	+	No evidence for A/anti-A activity via AR or 5-alpha reductase inhibition *in vivo*. Route of exposure, metabolic differences or potency explain differences between H assay and existing *in vitro*/*in vivo* studies. Effects seen in existing studies are via non-AR/5-alpha reductase mechanism.	Perform AR transactivation assay or binding assay with added metabolising system.	If existing data are from an adequate Level 5 (or less sensitive) 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). Consider route of exposure for H assay and possible implications of ADME characteristics of the chemical. Effects seen in existing studies may be in a more sensitive life stage. Check data on chemical analogues. Further mechanistic studies may help determine MOA.
K	–	+	–	No evidence for A/anti-A activity via AR or 5-alpha reductase inhibition *in vivo*. Metabolic differences or potency explain *in vitro*/*in vivo* differences.	Perform AR transactivation assay or binding assay with added metabolising system.	If existing data are from an adequate Level 5 assay, there may be sufficient information to conclude absence of concern for endocrine disruption (the EOGRTS provides the most information; however, for EDCs with a carcinogenic potential, OECD TG 451-3 may be more sensitive). If existing data are from a less sensitive assay, a higher level test may be required. Metabolic deactivation of chemical may occur *in vivo* so that potential *in vitro* activity is not realised. Consider possible routes of exposure, implications of metabolism.
L	–	+	Eq/0	No evidence for A/anti-A activity via AR or 5-alpha reductase inhibition *in vivo*. Metabolic differences or potency explain *in vitro*/*in vivo* difference. Unknown potential for adverse effects.	Perform AR transactivation assay or binding assay with added metabolising system OR Perform assay from Levels 4 or 5.	Metabolic deactivation of chemical may occur *in vivo* so that potential *in vitro* activity is not realised. Consider possible routes of exposure, implications of metabolism. Equivocal results may indicate chemical has multiple MOA.
M	–	–	+	No evidence for A/anti-A activity via AR or 5-alpha reductase inhibition in H assay or *in vitro*. Metabolic differences or route of exposure explain *in vitro*/*in vivo* existing differences. Effects seen in existing studies are via non-AR or non-endocrine mechanism.	Perform *in vitro* assays with added metabolising system.	Effects seen in existing studies may be in a more sensitive life stage. Check data on chemical analogues. Further mechanistic studies may help determine MOA.
N	–	–	–	No evidence for A/anti-A activity via AR or 5-alpha reductase inhibition *in vivo* or *in vitro*. No evidence of adverse effects.	Possibly no need for further testing. If there is uncertainty, may perform assay from Levels 4 or 5.	If existing data are from an adequate Level 5 assay, there may be sufficient information to conclude absence of concern for endocrine disruption (the EOGRTS provides the most information; however, for EDCs with a carcinogenic potential, OECD TG 451-3 may be more sensitive). Check data on chemical analogues.

Scenarios	Result of OECD TG 441 (H 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 A/anti-A activity via AR or 5-alpha reductase inhibition *in vivo* or *in vitro*. Unknown potential for adverse effects via other non-AR mechanisms.	Perform assay from Levels 4 or 5.	Consider route of exposure for H assay and possible implications for ADME characteristics of the chemical in follow-up assay.
P	–	Eq/0	+	No evidence for A/anti-A activity via AR or 5-alpha reductase inhibition *in vivo*. Unknown potential for adverse effects via other mechanisms.	For the "0" scenario, perform *in vitro* E,A,T,S assays, otherwise Eq result available.	Consider route of exposure for H assay and possible implications for differences from existing assay. Effects seen in existing studies may be in a more sensitive life stage. Check data on chemical analogues. Further mechanistic studies may help determine MOA. Equivocal results may indicate chemical has multiple MOA.
Q	–	Eq/0	–	No evidence for A/anti-A activity via AR or 5-alpha reductase inhibition *in vivo*. No evidence of adverse effects.	For the "0" scenario, perform *in vitro* E,A,T,S assays, otherwise Eq result available.	If existing data are from an adequate Level 5 assay, there may be sufficient information to conclude absence of concern for endocrine disruption (the EOGRTS provides the most information; however, for EDCs with a carcinogenic potential, OECD TG 451-3 may be more sensitive). If existing data are from a less sensitive assay, a higher level test may be required. Check data on chemical analogues. Further mechanistic studies may help determine MOA.
R	–	Eq/0	Eq/0	No evidence for A/anti-A activity via AR or 5-alpha reductase inhibition activity *in vivo*.	For the "0" scenario, perform *in vitro* E,A,T,S assays , otherwise Eq result available OR Perform Level 5 assay.	Consider route of exposure for H assay and possible implications for differences from existing assay. Check data on chemical analogues. Further mechanistic studies may help determine MOA. Equivocal results may indicate chemical has multiple MOA.