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OECD GUIDELINE FOR THE TESTING OF CHEMICALS

Stably Transfected Human Androgen Receptor Transcriptional Activation Assay for Detection of Androgenic Agonist and Antagonist Activity of Chemicals

INTRODUCTION

1. The OECD initiated a high-priority activity in 1998 to revise existing, and to develop new, Test Guidelines for the screening and testing of potential endocrine disrupting chemicals. The OECD Conceptual Framework for testing and assessment of potential endocrine disrupting chemicals comprises five levels, each level corresponding to a different level of biological complexity (1). The Stably Transfected (ST) human Androgen Receptor (AR) Transcriptional Activation (TA) assay for detection of androgenic agonist and antagonist activity of chemicals (AR STTA) using the AR-EcoScreen™ cell line (2) is included in level 2 for *"in vitro assays providing data about selected endocrine mechanism(s)/pathway(s) (Mammalian and non mammalian methods)"* (1).
2. *In vitro* TA assays are based upon the production of a reporter gene product induced by a chemical, following binding of the chemical to a specific receptor and subsequent downstream transcriptional activation. TA assays, using activation of reporter genes, are screening assays that have long been used to evaluate the specific gene expression regulated by specific nuclear receptors, such as the estrogen receptors (ERs) and androgen receptors (AR) (3) (4) (5) (6). They have been proposed for the detection of nuclear receptor-mediated transactivation (3) (4) (7).
3. The AR STTA test method has been validated by collaboration of the Chemicals Evaluation and Research Institute (CERI) and the National Institute of Health Sciences (NIHS) in Japan with support of the study management team from the OECD validation management group for non-animal testing (2). The AR STTA test method provides concentration-response data for substances with *in vitro* AR agonist or antagonist activity (2), which may be used for screening and prioritisation purposes and can also be used as mechanistic information in a weight of evidence approach.
4. Definitions and abbreviations used in this Test Guideline are described in Annex 1.

INITIAL CONSIDERATIONS AND LIMITATIONS

5. Androgen agonists and antagonists act as ligands for AR through AR binding, and may activate or inhibit the transcription of androgen responsive genes. This interaction may have the potential to trigger adverse health effects by disrupting androgen-regulated systems. This Test Guideline

This Guideline was adopted by the OECD Council by written procedure on 29 July 2016 [C(2016)103].

describes an assay that evaluates transcriptional activation and inhibition of AR-mediated responses. This process is considered to be one of the key mechanisms of possible endocrine disruption related health hazards, although there are also other important endocrine disruption mechanisms. These include (i) actions mediated via other nuclear receptors linked to the endocrine system and interactions with steroidogenic enzymes, (ii) metabolic activation or deactivation of hormones, (iii) distribution of hormones to target tissues, and (iv) clearance of hormones from the body. This Test Guideline exclusively addresses transcriptional activation and inhibition of an androgen-regulated reporter gene by binding to the human AR, and therefore it should not be directly extrapolated to the complex *in vivo* situation of androgen regulation of cellular processes. In addition, the assay is only likely to inform on the activity of the parent molecule bearing in mind the limited metabolising capacities of the in vitro cell systems.

6. This test method is specifically designed to detect human AR-mediated transcriptional activation and inhibition by measuring luciferase activity as the endpoint. However, substance-dependent interference with luminescence signals are known to occur due to over-activation or inhibition of the luciferase reporter gene assay system (8) (9) (10). It is therefore possible that such interference with the luciferase reporter gene may also occur in the AR STTA luciferase assay systems. This should be considered when evaluating the data.

7. This cell line has been developed to have minimal glucocorticoid receptor (GR)-mediated response, however, a limitation with respect to AR selectivity is the potential for GR cross talk (11) (12). In certain cases this may result in substances that activate GR being classified positive in this assay. When further investigation is deemed necessary, both non receptor-mediated luciferase signals and GR activation can be tested by incubating the test chemical with an AR antagonist (such as Hydroxyflutamide (HF)) to confirm whether the response by the test chemical is blocked or not (see Annex 2).

8. Considering that only single substances were used during the validation, the applicability to test mixtures has not been addressed. The test method is nevertheless theoretically applicable to the testing of multi-constituent substances and mixtures. Before use of the Test Guideline on a mixture for generating data for an intended regulatory purpose, it should be considered whether, and if so why, it may provide adequate results for that purpose. Such considerations are not needed, when there is a regulatory requirement for testing of the mixture.

PRINCIPLE OF THE TEST

9. The TA assay using a reporter gene technique is an *in vitro* tool that provides mechanistic data. The assay is used to establish signal activation or blocking of the androgen receptor caused by a ligand. Some chemicals may, in a cell type-dependent manner, display both agonist and antagonist activity and are known as selective androgen receptor modulators (SARMs). Following the ligand binding, the receptor-ligand complex translocates to the nucleus where it binds specific DNA response elements and transactivates a firefly luciferase reporter gene, resulting in an increased cellular expression of the luciferase enzyme. Luciferin is a substrate that is transformed by the luciferase enzyme to a bioluminescence product that can be quantitatively measured with a luminometer. Luciferase activity can be evaluated quickly and inexpensively with a number of commercially available test kits.

10. The test system provided in this Test Guideline utilises the AR-EcoScreen^{TM} cell line, which is derived from a Chinese hamster ovary cell line (CHO-K1), with three stably inserted constructs: (i) the human AR expression construct (encoding the full-length human receptor gene identical with Genbank ID of M20132 which has 21 times CAG trinucleotide short tandem repeat), and (ii) a

firefly luciferase reporter construct bearing four tandem repeats of a prostate C3 gene-responsive element driven by a minimal heat shock protein promoter. The C3 gene derived androgen responsive element is selected to minimise GR-mediated responses. In addition, (iii) a renilla luciferase reporter construct under the SV40 promoter, stably and non-inducibly expressed is transfected as to distinguish pure antagonism from a cytotoxicity-related decrease of luciferase activity (13) (14).

11. Data interpretation for an **AR agonistic effect** is based upon the maximum response level induced by a test chemical. If this response equals or exceeds 10% of the response induced by 10 nM 5α-dihydrotestosterone (DHT), the positive AR agonist control (PC_{AGO}) (i.e. the log PC_{10}), the test chemical is considered positive. Data interpretation for an **AR antagonistic effect** of a test chemical is based on a cut-off of a 30% inhibitory response against 500 pM DHT (i.e. the log IC_{30}). If the response exceeds this 30% AR blocking, then the chemical is considered a positive AR antagonist. Data analysis and interpretation are discussed in greater detail in paragraphs 42-56. Typical representations of the agonist and antagonist reference standard curves are shown in Figure 1.

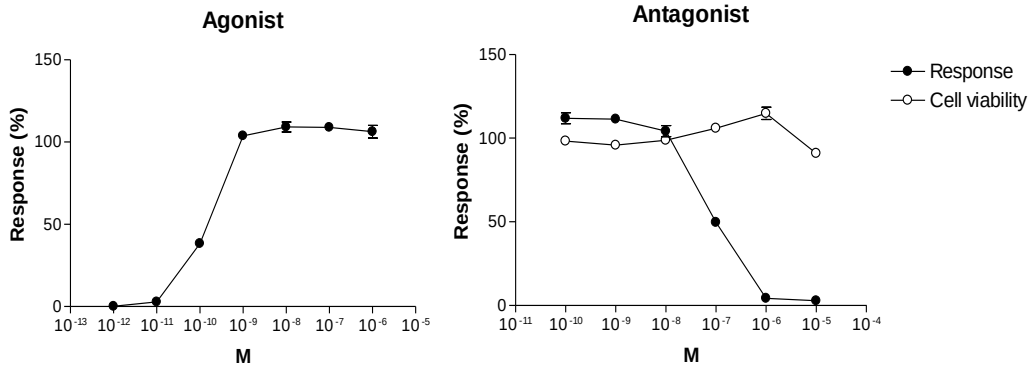

Figure 1: Typical positive control responses

PROCEDURE

Cell Lines

12. The stably transfected AR-EcoScreen™ cell line should be used for the assay. The cell line can be freely obtained from the Japanese Collection of Research Bioresources (JCRB) Cell Bank as reference No. JCRB1328, upon signing a Material Transfer Agreement (MTA).

13. Only cells characterised as mycoplasma-free (i.e. free of bacterial contamination) should be used in testing. RT PCR (Real Time Polymerase Chain Reaction) is the method of choice for a sensitive detection of mycoplasma infection (15) (16) (17).

Stability of the cell line

14. To monitor the stability of the cell line for the **agonist assay**, DHT, Mestanolone and Di(2-ethylhexyl)phthalate (DEHP) should be used as reference standards. A complete concentration response curve for all three reference standards, at the test concentration range provided in Table 1-2 and the plate concentration assignment shown in Table 3-1, should be obtained at least once each time the assay is performed, and the results should be in agreement with the results provided in Tables 1-1 and 1-2.

15. To monitor the stability of the cell line for measuring **AR antagonism**, HF, Bisphenol A (BPA) and DEHP should be used as reference standards. A complete concentration response curve for all three reference standards, at the test concentration range provided in Table 1-4 and the plate concentration assignment shown in Table 3-2, should be obtained at least once each time the assay is performed, and the results should be in agreement with the results provided in Tables 1-3 and 1-4.

Cell Culture and Plating Conditions

16. The following mediums should be prepared:
    - Medium for dilution: Phenol Red Free D-MEM/F-12.
    - Medium for cell propagation: Phenol Red Free D-MEM/F-12 supplemented with 5% v/v fetal bovine serum (FBS), Zeocin (200 µg/mL), Hygromycin (100 µg/mL), Penicillin (100 units /mL), and Streptomycin (100 µg/mL).
    - Medium for the assay plate: Phenol Red Free D-MEM/F-12 supplemented with 5%v/v Dextran-coated charcoal treated (DCC)-FBS, Penicillin (100 units/mL), and Streptomycin (100 µg/mL).
17. Cells should be maintained in a CO_2 incubator (5% CO_2) at 37±1˚C with medium for cell propagation. Upon reaching 75-90% confluency (i.e. every 3-4 days), cells are subcultured to 10 mL at a density of 0.4-0.8 x 10^5 cell/mL in 10cm^3 cell culture dishes. To prepare the assay plate (96-well plate), cells should be suspended in the medium for the assay plate and then plated into wells of a microplate containing 90 µL/well at a density of 1 x 10^5 cells/mL. Next, the cells should be pre-incubated in a 5% CO_2 incubator at 37˚±1˚C for 24 hours before chemical exposure.
18. To maintain the integrity of the response, the cells should be grown for more than one passage from the frozen stock in the conditioned media for cell propagation and should not be cultured for more than 40 passages. For the AR-EcoScreen™ cell line, this will be stable up to three month under suitable culture condition.
19. The DCC-FBS can be obtained from commercial sources. The selection of DCC-FBS is critical for the assay performance; therefore, the appropriate DCC-FBS should be selected based on the proliferative capacity and confirmation of effect on assay performance with the reference standards.

Acceptability criteria

Positive and negative reference standards

20. Prior to, and during the study, the responsiveness of the test system should be verified using the appropriate concentrations of known reference standards provided in Table 1-2 and 1-4, with DHT and Mestanolone as the positives for agonist assay, HF and BPA as the positives for antagonist assay, and DEHP as the negative for the agonist and antagonist assay. Acceptable range values derived from the validation study are also given in Table 1-2 and Table 1-4 (2). These three concurrent reference standards for each AR agonist/antagonist assay should be included in every AR agonist/antagonist experiment (conducted under the same conditions including the materials, passage level of cells and by the same technicians), and the results should fall within the given acceptable limits and the shape of concentration-response curve of positive reference standards should be sigmoidal. If this is not the case, the cause for the failure to meet the acceptability criteria should be determined (e.g. cell handling, and serum and antibiotics for quality and concentration) and the assay repeated. Once the acceptability criteria have been achieved, it is

essential in order to ensure minimum variability of log PC_{50}, log PC_{10}, log IC_{30}, log IC_{50} values, that use of materials for cell culturing is consistent.

21. The acceptability criteria of three concurrent reference standards can ensure the accuracy of quantitative sensitivity of the assay, but for the purposes of qualitative assessment, deviations from acceptable ranges of the reference standards (as specified in tables 1-2 and 1-4) could be allowed if the quality criteria (see tables 1-1 or 1-3) are met, however the reference standards should be included with each experiment and the results should be judged according to the parameters indicated in tables 1-2 and 1-4 and the concentration-response curve of the positive reference standards should be sigmoidal.

Table 1-1: Quality criteria for AR agonist assay

Fold-induction of PC_{AGO} (10 nM DHT)	≥ 6.4
FI PC_{10}	Greater than 1 +2SD (fold-induction of VC)

FI PC_{10}: fold-induction corresponding to the PC_{10} (10%) of Positive control (PC_{AGO}:10 nM of DHT)
SD: Standard Deviation, VC: Vehicle Control

Fold-induction of PC_{AGO} is calculated by the following equation:

$$\text{Fold-induction of } PC_{AGO} = \frac{\text{Mean RLU of } PC_{AGO} \text{ (10 nM DHT)}}{\text{Mean RLU of VC}}$$

RLU: Relative Light Units

Table 1-2: Acceptable range of the reference standards for AR agonist assay

Substance Name [CAS RN]	Judgment	$logPC_{10}$	$logPC_{50}$	Test range
5α-Dihydrotestosterone (DHT)[521-18-6]	Positive: PC_{10} should be calculated	-12.08 ~- 9.87	-11.03 ~ -9.00	10^{-12} ~ 10^{-6}M
Mestanolone[521-11-9]	Positive: PC_{10} should be calculated	-10.92 ~- 10.41	-10.15 ~ -9.26	10^{-12} ~ 10^{-6}M
Di(2-ethylhexyl)phthalate (DEHP) [117-81-7]	Negative: PC_{10} should not be calculated	-	-	10^{-11} ~ 10^{-5}M

Table 1-3: Quality criteria for AR antagonist assay

Fold induction of AG ref[1]	≥ 5.0
RTA of PC_{ATG} (%)[2]	≤46

AG ref = Agonist reference (500 pM DHT) in the antagonist assay
RTA : Relative Transcriptional Activity
PC_{ATG} = Antagonist control (500pM DHT, 0.1 µM HF)

[1]: Fold induction of AG ref is calculated by the following equation:

$$\text{Fold-induction of AG ref} = \frac{\text{Mean RLU of AG ref (500 pM DHT)}}{\text{Mean RLU of VC}}$$

VC: Vehicle Control, RLU: Relative Light Units

[2]: RTA of PC_{ATG} (%) is calculated by the following equation;

$$\text{RTA of } PC_{ATG} (\%) = \text{Mean}\left(\frac{\text{RLU of } PC_{ATG} - \text{Mean RLU of VC}}{\text{Mean RLU of AG ref - Mean RLU of VC}} \right) \times 100$$

Table 1-4: Acceptable range of the reference standards for AR antagonist assay

Substance Name [CAS RN]	Judgment	log IC_{30}	log IC_{50}	Test range
Hydroxyflutamide (HF) [52806-53-8]	Positive: IC30 should be calculated	-8.37 ~ -6.41	-7.80 ~ -6.17	10^{-10} ~ 10^{-5}M
Bisphenol A (BPA) [80-05-7]	Positive: IC30 should be calculated	-7.52 ~ -4.48	-7.05 ~ -4.29	10^{-10} ~ 10^{-5}M
Di(2-ethylhexyl)phthalate (DEHP) [117-81-7]	Negative: IC30 should not be calculated	-	-	10^{-10} ~ 10^{-5}M

Positive and vehicle controls

22. **For the agonist assay**, positive control (PC_{AGO}) wells (n=4) treated with an endogenous ligand (10 nM of DHT), vehicle control (VC) wells (n=4) treated with vehicle alone and positive control for cytotoxicity (PC_{CT}, 10 μg/mL of cycloheximide) wells (n=4) should be prepared on each assay plate in accordance with the plate design indicated in Table 3-1 and Table 4-1. **For the antagonist assay**, vehicle control (n=3), positive control for agonistic activity (PC_{AGO}, 10 nM of DHT, n=3), positive control for antagonistic activity (PC_{ATG}, 500 pM DHT and 0.1 μM of HF, n=3), positive control for cytotoxicity (PC_{CT}, 10 μg/mL of cycloheximide, n=3) and agonist reference (AG ref, 500 pM of DHT, n=12) should be set-up at each assay plate in accordance with the plate design indicated in Table 3-2 and Table 4-2.

Quality criteria for AR agonist assay

23. The mean luciferase activity of the PC_{AGO} (10 nM DHT) should be at least 6.4-fold higher than that of the mean VC on each plate for the agonist assay. These criteria were established based on the reliability of the endpoint values from the validation study.
24. With respect to the quality control of the assay, the fold-induction corresponding to the logPC_{10} (10%) of positive control (PC_{AGO}: 10 nM of DHT) (FI PC_{10}) should be greater than 1+2SD of the induction value (=1) of the concurrent VC. For prioritisation purposes, the log PC_{10} value can be useful to simplify the data analysis required compared to a statistical analysis. Although a statistical analysis provides information on significance, such an analysis is not a quantitative parameter with respect to a concentration-based potential, and so is less useful for prioritisation purposes.

Quality criteria for AR antagonist assay

25. The mean luciferase activity of the AG ref (500 pM DHT) should be at least 5.0-fold for antagonism assay. These criteria were established based on the reliability of the endpoint values from the validation study.
26. RTA of PC_{ATG} (500 pM DHT and 0.1 μM HF) should be less than 46%.

In summary:

27. Acceptability Criteria are the following:

For AR agonist assay:
- The mean luciferase activity of the PC_{AGO} (10 nM DHT) should be at least 6.4-fold higher than the mean VC on each plate
- The fold induction corresponding to the log PC_{10} value of the concurrent PC_{AGO} (10 nM DHT) should be greater than 1+2SD of the fold induction value of the VC.
- The shape of concentration-response curve of positive reference standards should be sigmoidal.
- The results of the three reference standards should be within the acceptable range (Table 1-2).

For AR antagonist assay:
- Fold induction of AG ref ([500 pM DHT]/[Vehicle Control]) should be at least 5.0.
- RTA of PC_{ATG} (%) should be less than 46.
- The shape of concentration-response curve of positive reference standards should be sigmoidal.
- The results of the three reference standards should be within the acceptable range (Table 1-4).

Substances to demonstrate laboratory proficiency

28. Prior to testing unknown chemicals in the AR STTA assay, the responsiveness of the test system should be confirmed by each laboratory, at least once for each newly prepared batch of cell stocks taken from the frozen stock. This is done by independently testing 10 proficiency substances listed in Tables 2-1 and 2-2 for AR agonism and antagonism, respectively. This should be done at least in duplicate, on different days, and the results should be comparable to Tables 2-1 and 2-2, and any deviations should be justified. Dependent on cell type, some of these proficiency substances may behave as SARMs and display activity as both agonists and antagonists. However, the proficiency substances are classified in Tables 2-1 and 2-2 by their known predominant activity which should be used for proficiency evaluation.

Table 2-1: List of Proficiency substances for agonist assay

Substance Name	CAS RN.	Class[1]	log PC_{10} [1] (M)	log PC_{50} [1] (M)	Chemical Class [2]	Product Class [3]
5α-Dihydrotestosterone	521-18-6	P	-12.08 ~ -9.87	-11.03 ~ -9.00	Steroid, nonphenolic	Pharmaceutical
Mestanolone	521-11-9	P	-10.92 ~ -10.41	-10.15 ~ -9.26	Steroid, nonphenolic	Pharmaceutical
Testosterone	58-22-0	P	-10.42 ~ -9.73	-9.46 ~ -8.96	Steroid, nonphenolic	Pharmaceutical
17β-estradiol	50-28-2	P	-7.74 ~ -6.75	-5.34 ~ -4.88	Steroid, phenolic	Pharmaceutical
Medroxyprogesterone 17-acetate	71-58-9	P	-9.64 ~ -8.89	-8.77 ~ -8.37	Steroid, nonphenolic	Pharmaceutical
17α-ethinyl estradiol	57-63-6	N	-		Steroid, phenolic	Pharmaceutical
Butylbenzyl phthalate	85-68-7	N	-		Phthalate	Plasticiser
Di(2-ethylhexyl)phthalate	117-81-7	N	-		Phthalate	Chemical intermediate; Plasticiser
Hydroxyflutamide	52806-53-8	N	-		Anilide	Pharmaceutical metabolite
Bisphenol A	80-05-7	N	-		Bisphenol	Chemical intermediate

Abbreviations: CAS RN: Chemical Abstracts Service Registry Number, M: molar, P: Positive, N: Negative

[1] Validation report of Androgen Receptor (AR)-Mediated Stably Transfected Transcriptional Activation (AR STTA) Assay to Detect Androgenic and Anti-androgenic Activities (2)

[2] Substances were assigned to one or more chemical classes using the U.S. National Library of Medicine's Medical Subject Headings (MeSH), an internationally recognised standardised classification scheme (available at http://www.nlm.nih.gov/mesh).

[3] Substances were assigned to one or more product classes using the U.S. National Library of Medicine's Hazardous Substances Data Bank (available at http://toxnet.nlm.nih.gov/cgi-bin/sis/htmlgen?HSDB).

Table 2-2: List of Proficiency substances for antagonist assay

Substance Name	CAS RN	Class[1]	log IC_{30} [1] (M)	log IC_{50} [1] (M)	Chemical Class [2]	Product Class [3]
Hydroxyflutamide	52806-53-8	P	-8.37 ~ -6.41	-7.80 ~ -6.17	Anilide	Pharmaceutical metabolite
Bisphenol A	80-05-7	P	-7.52 ~ -4.48	-7.05 ~ -4.29	Bisphenol	Chemical intermediate
Flutamide	13311-84-7	P	-6.20 ~ -5.69	-5.66 ~ -5.43	Anilide	Pharmaceutical
Prochloraz	67747-09-5	P	-5.77 ~ -5.47	-5.44 ~ -5.12	Imidazole	Pesticide
Vinclozolin	50471-44-8	P	-6.83 ~ -6.32	-6.47 ~ -5.85	Organochlorine	Pesticide
5α-Dihydrotestosterone	521-18-6	N	-		Steroid, nonphenolic	Pharmaceutical
Mestanolone	521-11-9	N	-		Steroid, nonphenolic	Pharmaceutical
Di(2-ethylhexyl)phthalate	117-81-7	N	-		Phthalate	Chemical intermediate; Plasticiser
Atrazine	1912-24-9	N	-		Triazine; Aromatic amine	Pesticide
6-Propyl-2-thiouracil	51-52-5	N	-		Pyrimidines	Pharmaceutical

Abbreviations: CAS RN: Chemical Abstracts Service Registry Number, M: molar, P: Positive, N: Negative

[1] Validation report of Androgen Receptor (AR)-Mediated Stably Transfected Transcriptional Activation (AR STTA) Assay to Detect Androgenic and Anti-androgenic Activities (2)

[2] Substances were assigned to one or more chemical classes using the U.S. National Library of Medicine's Medical Subject Headings (MeSH), an internationally recognised standardised classification scheme (available at http://www.nlm.nih.gov/mesh).

[3] Substances were assigned to one or more product classes using the U.S. National Library of Medicine's Hazardous Substances Data Bank (available at http://toxnet.nlm.nih.gov/cgi-bin/sis/htmlgen?HSDB).

Vehicle

29. An appropriate solvent should be used as the concurrent VC at the same concentration for the different positive and negative controls and the test chemicals. Test chemicals should be dissolved in a solvent that solubilises the test chemical and is miscible with the cell medium. Water, ethanol (95% to 100% purity) and dimethyl sulfoxide (DMSO) may be suitable vehicles accepted by the cells. Generally DMSO is used. In this case, the final level in the well should not exceed 0.1% (v/v). For any other vehicle (e.g. ethanol), it should be demonstrated that the maximum concentration used is not cytotoxic and does not interfere with the assay performance (as confirmed by response of renilla luciferase).

Preparation of test chemicals

30. The test chemicals should be dissolved in an appropriate solvent (see paragraph 29) and serially diluted with the same solvent at a common ratio of 1:10. In order to define the highest soluble concentration of the test chemical, a solubility test should be carried out following the flow diagram shown in Figure 2.

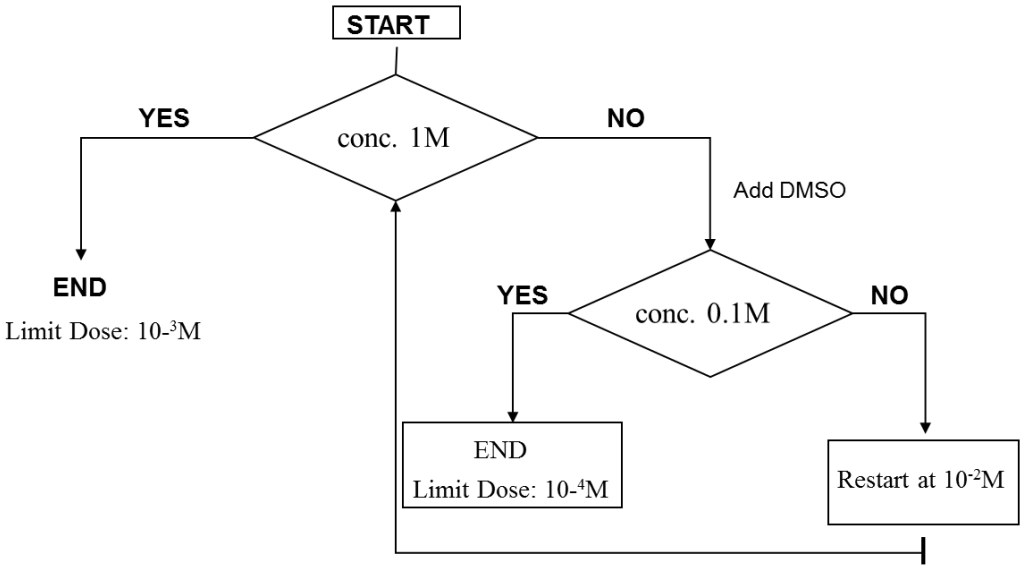

Figure 2: Diagram for solubility test

Limit dose: the highest concentration to be tested as the assay concentration.
YES: No precipitation, NO: Precipitation

31. A solubility test is a very important step to determine the maximum concentration for the assay and it may affect the sensitivity of the assay. Maximum concentration should be selected based on the avoidance of precipitation at highest concentration ranges in culture media. Precipitation observed at any concentration should be noted, but these data should not be included in the dose-response analysis.

32. For AR antagonists, the presence of increasing levels of cytotoxicity can significantly alter or eliminate the typical sigmoidal response and should be considered when interpreting the data. Cytotoxicity can be evaluated with renilla luciferase activity in the AR-EcoScreen^{TM} cell line, which was originally established to express renilla luciferase constitutively. Accordingly, AR-mediated transcriptional activity and cytotoxicity should be evaluated simultaneously in the same assay plate. For AR agonists, cytotoxicity can also affect the shape of a concentration response curve. In such case, evaluation of cytotoxicity should be performed or evaluated from the results of antagonist assay conducted for same test chemical.

33. Should the results of the cytotoxicity test show that the concentration of the test chemical has reduced renilla luciferase activity by 20% or more, this concentration is regarded as cytotoxic, and the concentrations at or above the cytotoxic concentration should be excluded from the evaluation. The maximum concentration should be considered to be reduced when intrinsic cytotoxic effect is observed at the result of initial run of the test chemical. Cytotoxicity (%) of each well is calculated by the following equations and the mean of triplicate wells of same concentration is calculated for the cytotoxicity (%) of each concentration of test chemicals.

For the agonist assay;

$$\text{Cytotoxicity (\%)} = 100 - \left(\frac{\text{RLU of each well-Mean RLU of } PC_{CT}}{\text{Mean RLU of VC - Mean RLU of } PC_{CT}} \right) \times 100$$

For the antagonist assay;

$$\text{Cytotoxicity (\%)} = 100 - \left(\frac{\text{RLU of each well-Mean RLU of } PC_{CT}}{\text{Mean RLU of AG ref - Mean RLU of } PC_{CT}} \right) \times 100$$

Chemical Exposure and Assay Plate Organisation

34. For the AR agonist assay, each test chemical should be serially diluted in DMSO, or appropriate solvent, and added to the wells of a microtiter plate to achieve final serial concentrations in the assay, as determined by the preliminary range finding test (typically a series of, for example 1 mM, 100 μM, 10 μM, 1 μM, 100 nM, 10 nM and 1 nM [10^{-3}-10^{-9} M]) for triplicate testing.

    For each test concentration of the test chemical, the procedure for chemical dilutions (Steps 1 and 2) and for exposing the cells (Step 3) can be conducted as follows:

    Step 1: Chemical dilution: First dilute 10 μL of the test chemical in solvent into 90 μL of media.

    Step 2: Then 10 μL of the diluted chemical prepared in Step 1 should be diluted into 90 μL of the media.

    Step 3: Chemical exposure of the cells: Add 10 μL of diluted chemical solution (prepared in Step 2) to an assay well containing 9×10^3 cells/90 μL/well.
    The recommended final volume of media required for each well is 100 μL.

    Reference standards and test samples can be assigned as shown in Table 3-1 and Table 4-1.

Table 3-1: Example of plate concentration assignment of the reference standards in the assay plate for agonist assay

Row	DHT			Mestanolone			DEHP			Test Chemical#		
	1	2	3	4	5	6	7	8	9	10	11	12
A	1 μM	→	→	1 μM	→	→	10 μM	→	→	1 mM	→	→
B	100 nM	→	→	100 nM	→	→	1 μM	→	→	100 μM	→	→
C	10 nM	→	→	10 nM	→	→	100 nM	→	→	10 μM	→	→
D	1 nM	→	→	1 nM	→	→	10 nM	→	→	1 μM	→	→
E	100 pM	→	→	100 pM	→	→	1 nM	→	→	100 nM	→	→
F	10 pM	→	→	10 pM	→	→	100 pM	→	→	10 nM	→	→
G	1 pM	→	→	1 pM	→	→	10 pM	→	→	1 nM	→	→
H	VC	→	→	→	PC_{AGO}	→	→	→	PC_{CT}	→	→	→

VC: Vehicle control (DMSO);
PC_{AGO}: Positive control (10 nM of DHT);
PC_{CT}: Cytotoxicity control (10 μg/mL of cycloheximide);
#: concentration of test chemical is an example

35. For the AR antagonist assay, each test chemical should be serially diluted in DMSO, or appropriate solvent, and added to the wells of a microtiter plate to achieve final serial concentrations in the assay, as determined by the preliminary range finding test (typically a series of, for example 1 mM, 100 μM, 10 μM, 1 μM, 100 nM, and 10 nM [10^{-3}-10^{-8} M]) for triplicate testing.

    For each test concentration of the test chemical the procedure for chemical dilutions (Steps 1 and 2) and for exposing cells (Step 3) can be conducted as follows:

Step 1: Chemical dilution: First dilute 10 µL of the test chemical in the solvent to a volume of 90 µL media containing 56 nM DHT/DMSO*.
Step 2: Then 10 µL of the diluted chemical prepared in Step 1 should be diluted into 90 µL of the media.
Step 3: Chemical exposure of the cells: Add 10 µL of diluted chemical solution (prepared in Step 2) to an assay well containing 9×10^3 cells/90 µL/well.
The recommended final volume of media required for each well is 100 µL.

*56 nM DHT/DMSO is added to achieve 500 pM DHT, 0.1% DMSO after dilution.

Reference standards and test samples can be assigned as shown in Table 3-2 and Table 4-2.

Table 3-2: Example of plate concentration assignment of the reference standards in the assay plate for antagonist assay

Row	HF			Bisphenol A			DEHP			Test chemical#		
	1	2	3	4	5	6	7	8	9	10	11	12
A	10 µM	→	→	10 µM	→	→	10 µM	→	→	1 mM	→	→
B	1 µM	→	→	1 µM	→	→	1 µM	→	→	100 µM	→	→
C	100 nM	→	→	100 nM	→	→	100 nM	→	→	10 µM	→	→
D	10 nM	→	→	10 nM	→	→	10 nM	→	→	1 µM	→	→
E	1 nM	→	→	1 nM	→	→	1 nM	→	→	100 nM	→	→
F	100 pM	→	→	100 pM	→	→	100 pM	→	→	10 nM	→	→
G	AG ref	→	→	→	→	→	→	→	→	→	→	→
H	VC	→	→	PC_{AGO}	→	→	PC_{ATG}	→	→	PC_{CT}	→	→

VC: Vehicle control (DMSO);
PC_{AGO}: Positive AR agonist control (10 nM of DHT);
AG ref: AR agonist reference (500 pM DHT, 0.1% DMSO)
PC_{ATG}: Positive AR antagonist control (500 pM DHT, 0.1 µM of HF);
PC_{CT}: Cytotoxicity control (10 µg/mL of cycloheximide);

** Gray colored wells are spiked with 500pM DHT
#: concentration of test chemical is an example

36. The reference standards (DHT, Mestanolone and DEHP for the agonist assay; HF, BPA and DEHP for the antagonist assay) should be tested in every experiment (as indicated in Table 3-1 and 3-2). Wells treated with 10 nM of DHT that can produce a maximum induction of DHT (PC_{AGO}), and wells treated with DMSO (or appropriate solvent) alone (VC) should be included in each test assay plate for the agonist assay as well as a cytotoxicity control (10 µg/mL of cycloheximide called PC_{CT}) (Table 4-1). In the case of the antagonist assay, a positive AR agonist control (10 nM of DHT called PC_{AGO}), an AR agonist reference (500 pM DHT, 0.1% DMSO called AG ref), a positive AR antagonist control (500 pM DHT, 0.1 µM of HF called PC_{ATG}) and cytotoxicity control (10 µg/mL of cycloheximide called PC_{CT}) should be prepared additionally (Table 4-2). If cells from different sources (e.g. different passage number, different lot numbers, etc.,) are used in the same experiment, the reference standards should be tested for each cell source.

Table 4-1: Example of plate concentration assignment of test chemicals and plate control substances in the assay plate for agonist assay

Row	Test Chemical 1			Test Chemical 2			Test Chemical 3			Test Chemical 4		
	1	2	3	4	5	6	7	8	9	10	11	12
A	conc 1 (10 µM)	→	→	1 mM	→	→	1 µM	→	→	10 nM	→	→
B	conc 2 (1 µM)	→	→	100 µM	→	→	100 nM	→	→	1 nM	→	→
C	conc 3 (100 nM)	→	→	10 µM	→	→	10 nM	→	→	100 pM	→	→
D	conc 4 (10 nM)	→	→	1 µM	→	→	1 nM	→	→	10 pM	→	→
E	conc 5 (1 nM)	→	→	100 nM	→	→	100 pM	→	→	1 pM	→	→
F	conc 6 (100 pM)	→	→	10 nM	→	→	10 pM	→	→	0.1 pM	→	→
G	conc 7 (10 pM)	→	→	1 nM	→	→	1 pM	→	→	0.01 pM	→	→
H	VC	→	→	→	PC_{AGO}	→	→	→	PC_{CT}	→	→	→

VC: Vehicle control (DMSO);
PC_{AGO}: Positive AR agonist control (10 nM of DHT);
PC_{CT}: Cytotoxicity control (10 µg/mL of cycloheximide);

The concentration of test chemicals is provided as an example.

Table 4-2: Example of plate concentration assignment of test chemicals and plate control substances in the assay plate for antagonist assay

Row	Test Chemical 1			Test Chemical 2			Test Chemical 3			Test Chemical 4		
	1	2	3	4	5	6	7	8	9	10	11	12
A	conc 1 (10 µM)	→	→	1 mM	→	→	1 µM	→	→	10 nM	→	→
B	conc 2 (1 µM)	→	→	100 µM	→	→	100 nM	→	→	1 nM	→	→
C	conc 3 (100 nM)	→	→	10 µM	→	→	10 nM	→	→	100 pM	→	→
D	conc 4 (10 nM)	→	→	1 µM	→	→	1 nM	→	→	10 pM	→	→
E	conc 5 (1 nM)	→	→	100 nM	→	→	100 pM	→	→	1 pM	→	→
F	conc 6 (100 pM)	→	→	10 nM	→	→	10 pM	→	→	100 pM	→	→
G	AG ref	→	→	→	→	→	→	→	→	→	→	→
H	VC	→	→	PC_{AGO}	→	→	PC_{ATG}	→	→	PC_{CT}	→	→

VC: Vehicle control (DMSO);
PC_{AGO}: Positive AR agonist control (10 nM of DHT);
AG ref: AR agonist reference (500 pM DHT, 0.1% DMSO)
PC_{ATG}-: Positive AR antagonist control (500 pM DHT, 0.1 µM of HF) ;
PC_{CT}: Cytotoxicity control (10 µg/mL of cycloheximide);

** Gray colored wells are spiked with 500pM DHT

The concentration of test chemicals is provided as an example.

37. The lack of edge effects should be confirmed, as appropriate, and if edge effects are suspected, the plate layout should be altered to avoid such effects. For example, a plate layout excluding the edge wells can be employed.

38. After adding the chemicals, the assay plates should be incubated in a 5% CO_2 incubator at 37±1ºC for 20-24 hours to induce the reporter gene products.

39. Special considerations will need to be applied to those substances that are highly volatile. In such cases, nearby control wells may generate false positives, and this should be considered in light of expected and historical control values. In the few cases where volatility may be of concern, the use of "plate sealers" may help to effectively isolate individual wells during testing, and is therefore recommended in such cases.

40. Repetition of definitive tests for the same chemical should be conducted on different days using freshly prepared assay reagents and dilutions of the test chemicals, to ensure independence. In cases where multiple chemicals are concurrently tested within a single run, maintaining the same plate design, while changing the order in which chemicals are added to the test wells, would be preferable.

Luciferase activity measurements

41. A commercial dual-reporter assay system (e.g. Promega, E2920 or its equivalents) is preferable to detect both of the AR response (firefly luciferase activity) and cytotoxicity (renilla luciferase activity) simultaneously, as long as the acceptability criteria are met. The assay reagents should be selected based on the sensitivity of the luminometer to be used. Procedure is according to the manufacturer's instructions basically. For instance, when using Dual-Glo Luciferase Assay system (Promega, E2920), cell Culture Lysis Reagent (Promega, E1531, or equivalents) should be used before adding the substrate. 40 μL of the first substrate should be directly added into the assay wells; then measure the firefly luciferase signal; then remove 60 μL of supernatant to detect firefly luciferase activity; and finally add 40 μL of the second substrate into the assay wells of the original plate to detect renilla luciferase activity. A luciferase assay reagent [e.g. Steady-Glo® Luciferase Assay System (Promega, E2510, or equivalents)] or a standard luciferase assay system (Promega, E1500, or equivalents) can be used to detect only for the AR response (firefly luciferase activity). When using Steady-Glo Luciferase Assay System (Promega, E2510), after adding the Cell Culture Lysis Reagent (Promega, E1531, or equivalents), 40 μL of prepared reagent should be directly added into the assay wells.

ANALYSIS OF DATA

42. **For the Agonist assay**, to obtain the relative transcriptional activity to the positive control (10 nM DHT), the luminescence signals from the same plate can be analysed according to the following steps (other equivalent mathematical processes are also acceptable):

Step 1. Calculate the mean value for the vehicle control (VC).

Step 2. Subtract the mean value of the VC from each well value in order to subtract any vehicle-driven effect or noise.

Step 3. Calculate the mean for the corrected PC_{AGO} (=the normalised PC_{AGO}).

Step 4. Divide the corrected value of each well in the plate by the mean value of the normalised PC_{AGO} (PC_{AGO} is set to 100%).

The final value of each well is the relative transcriptional activity for that well compared to the PC_{AGO} response.

Step 5. Calculate the mean value of the relative transcriptional activity for each concentration of the test chemical. There are two dimensions to the response: the averaged transcriptional activity (response) and the concentration at which the response occurs (see following section).

43. **For the Antagonist assay**, to obtain the relative transcriptional activity, the luminescence signals from the same plate can be analysed according to the following steps (other equivalent mathematical processes are also acceptable):

Step 1. Calculate the mean value for the VC.

Step 2. Subtract the mean value of the VC from each well value in order to subtract any vehicle-driven effect or noise.

Step 3. Calculate the mean for the corrected AG ref (=the normalised AG ref).

Step 4. Divide the corrected value of each well in the plate by the mean value of the normalised the AG ref (AG ref is set to 100%).

The final value of each well is the relative transcriptional activity for that well compared to the maximum response of the AG ref.

Step 5. Calculate the mean value of the relative transcriptional activity for each concentration group of the test chemical. There are two dimensions to the response: the averaged transcriptional activity (response) and the concentration at which the response occurs (see following section).

log PC50, log PC10, log IC50 and log IC30 induction considerations

44. To evaluate cytotoxicity, cell viability should be expressed as the percentage of renilla luciferase activity of the chemically-treated wells to the mean renilla luciferase activity of the wells of the vehicle control for the agonist assay or the mean renilla luciferase activity of the wells of AG ref (500 pM DHT) for the antagonist assay, in accordance with equations indicated in paragraph 33.

45. In the case of the agonist assay, the following information should be provided for each test chemical:

(i) The maximum level of response induced by a test chemical, expressed as a percentage against the response induced by PC_{AGO}(10 nM DHT) on the same plate (RPC_{max}).

(ii) For positive chemicals, the concentrations that induce an effect corresponding to that of a 10% effect for the positive control (log PC_{10}) and, if appropriate, to 50% effect for the positive control (log PC_{50}).

46. Descriptions of log PC_x values, "x" is a selected response like 10% or 50% induction compared to PC_{AGO}, are provided in Figure 3. log PC_{10} and log PC_{50} values can be defined as the test chemical concentrations estimated to elicit either a 10% or a 50% induction of transcriptional activity induced by PC_{AGO} (Positive control; 10 nM of DHT). Each log PC_x value can be calculated by a simple linear regression using two variable data points for the transcriptional activity. Where the data points lying immediately above and below the log PC_x value have the coordinates (a,b) and (c,d) respectively, then the log PC_x value is calculated using the following equation and Figure 4:

$$\log[PC_x] = c+[(x-d)/(b-d)](a-c)$$

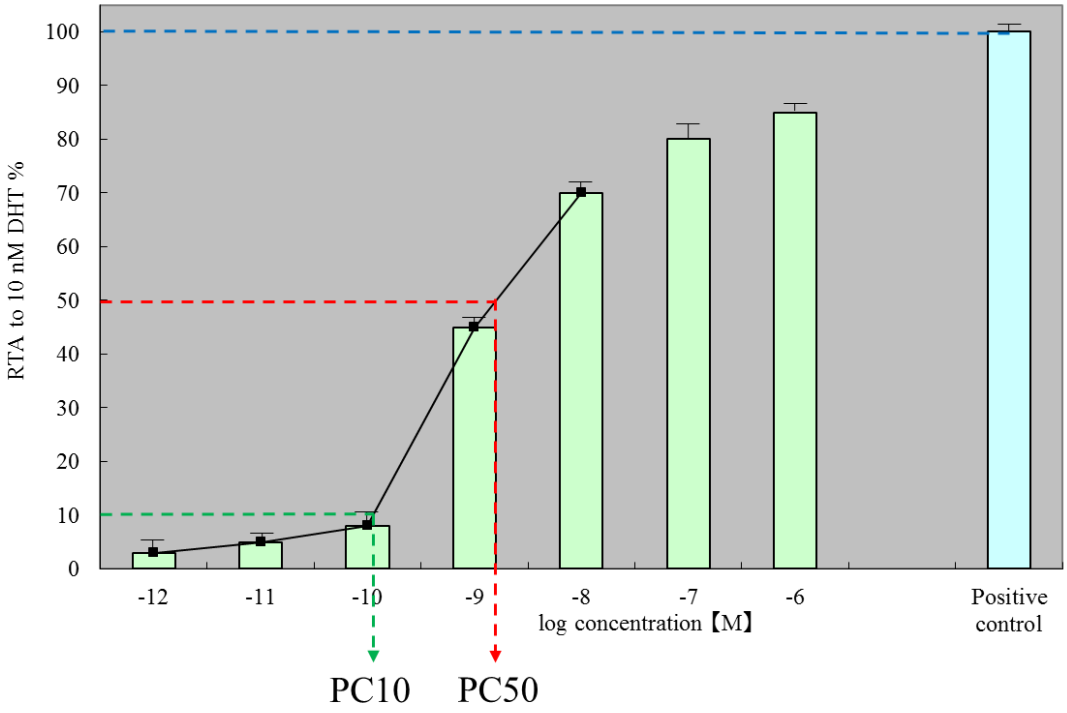

Figure 3: Schematic descriptions of log PC_x values

The PC_{AGO} (Positive control; 10 nM of DHT) is included on each assay plate in agonist assay.
RTA: relative transcriptional activity

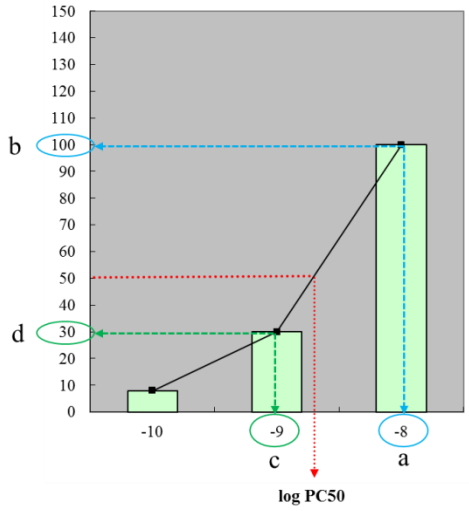

Figure 4: Example for calculation of log PC_{50}.

47. In the case of the antagonist assay, the following information should be provided for each positive test chemical: the concentrations of 30% inhibition of transcriptional activity induced by 500 pM DHT (log IC30) and, if appropriate, to 50% inhibition of activity of 500 pM DHT (log IC50).

48. Descriptions of log IC_x values, "x" is a selected response like 30% or 50% inhibition compared to DHT controls, are provided in Figure 5. log IC_{50} and log IC_{30} values can be defined as the test chemical concentrations estimated to elicit either a 50% or a 30% inhibition of transcriptional activity induced by 500 pM DHT. These values can be calculated in the same way as the log PC

values. Each log IC_x value can be calculated by a simple linear regression using two variable data points for the transcriptional activity. Where the data points lying immediately above and below the log IC_x value have the coordinates (c,d) and (a,b) respectively, then the log IC_x value is calculated using the following equation and Figure 6:

$$\log [IC_x] = a-[(b-(100-x)) /(b-d)] (a-c)$$

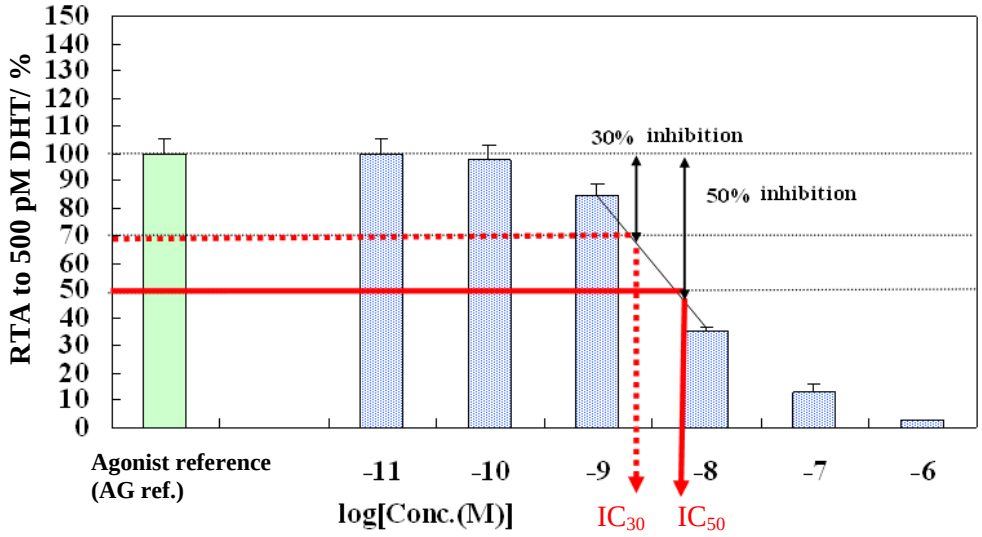

Figure 5: Schematic descriptions of log IC values.

The AG ref (DMSO at 0.1% spiked with 500 pM DHT) is included on each assay plate in antagonist assay.
RTA: relative transcriptional activity

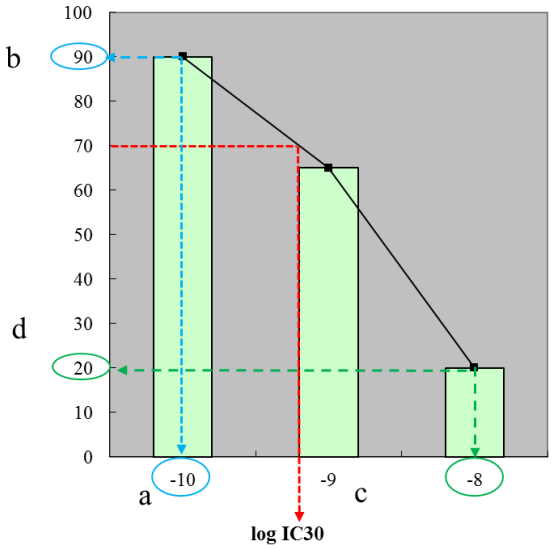

Figure 6: Examples for calculation of log IC_{30}.

49. To distinguish pure antagonism from a cytotoxicity-related decrease of luciferase activity, AR-EcoScreen™ is designed to express two kinds of luciferase: firefly luciferase inducibly expressed by the AR response element and renilla luciferase stably and non-inducibly expressed.

50. By using dual reporter assay system, both cell viability and the antagonism can be evaluated in the same cells in a single plate run. The response for the positive cytotoxic control (10μg/mL of cycloheximide called PC_{CT}) is used to adjust renilla activity by subtracting the PC_{CT} values – the so-called "renilla activities" - from those of all sample wells. To evaluate the true cytotoxicity of chemicals with the AR Ecoscreen™ assay, such revised cell viability should be used. If the cell viability is lower than 80% at the specific concentration of a test chemical, this/these data point(s) is/are left out of the calculations.

51. The results, i.e. positive or negative judgment of test chemical, should be based on a minimum of two or three independent runs. If two runs give comparable and reproducible results, it may not be necessary to conduct a third run. To be acceptable, the results should:
    - Meet the acceptability criteria (see paragraphs 20-27)
    - Be reproducible in triplicate wells (CV<20%).

Data Interpretation Criteria

52. **For the agonist assay,** data interpretation criteria are shown in Table 5-1. Positive results will be characterised by both the magnitude of the effect and the concentration at which the effect occurs. Expressing results as a concentration at which a 50% (log PC_{50}) or 10% (log PC_{10}) are reached accomplishes the goal. However, a test chemical is determined to be positive if the maximum response induction by the test chemical (RPC_{max}) is equal to or exceeds 10% of the positive control responses in at least two of two or two of three runs, whereas a test chemical is considered negative if the RPC_{max} fails to achieve at least 10% of the positive control in two of two or two of three runs.

Table 5-1: Positive and negative decision criteria for agonist assay

Positive	If a RPC_{max} is obtained that is equal to or exceeds 10% of the response of the positive control in at least two of two or two of three runs.
Negative	If a RPC_{max} fails to achieve at least 10% of the response of the positive control in two of two or two of three runs.

53. **For the antagonist assay,** data interpretation criteria are shown in Table 5-2. Positive results will be characterised by both the magnitude of the effect and the concentration at which the effect occurs. Expressing results as a concentration at which a 50% (log IC_{50}) or 30% (log IC_{30}) are reached, accomplishes this goal. However, a test chemical is determined to be positive if the log IC_{30} could be calculated in at least two of two or two of three runs, whereas a test chemical is considered as negative if the log IC_{30} could not be calculated in two of two or two of three runs.

Table 5-2: Positive and negative decision criteria for antagonist assay

Positive	If the log IC_{30} is calculated in at least two of two or two of three runs.
Negative	If the log IC_{30} fails to calculate in two of two or two of three runs.

54. The calculations of log PC_{10}, log PC_{50} and RPC_{max} for agonist assay, and log IC_{50} and log IC_{30} for antagonist assay can be calculated by using a spreadsheet available with the Test Guideline on the OECD public website.

55. It should be sufficient to obtain log PC_x or log IC_x values at least twice. However, should the resulting base-line for data in the same concentration range show variability with high coefficient

of variation (%CV), it should be considered that the reliability of the data is low and the source of the high variability should be identified. The %CV of the raw data triplicate wells (i.e. luminescence intensity data) of the data points on the same assay plate that are used for the calculation of log PC_x or log IC_x should be less than 20%. When an equivocal or inconclusive result is suspected, an additional run or check can be considered.

56. Meeting the acceptability criteria indicates the assay system is operating properly, but it does not ensure that any particular run will produce accurate data. Duplicating the results of the first run is the best assurance that accurate data were produced.

TEST REPORT

57. The test report should include the following information:

Control/Reference standards/Test chemical

- Source, lot number, expiry date, if available
- Stability of the test chemical itself, if known;
- Solubility and stability of the test chemical in solvent, if known.
- Measurement of pH, osmolality and precipitate in the culture medium to which the test chemical was added, as appropriate.

Mono-constituent substance:

- Physical appearance, water solubility, and additional relevant physicochemical properties;
- Chemical identification, such as IUPAC or CAS name, CAS number, SMILES or InChI code, structural formula, purity, chemical identity of impurities as appropriate and practically feasible, etc.

Multi-constituent substance, UVCBs and mixtures:

- Characterised as far as possible by chemical identity (see above), quantitative occurrence and relevant physicochemical properties of the constituents.

Solvent/Vehicle:

- Characterisation (nature, supplier and lot number);
- Justification for choice of solvent/vehicle;
- Solubility and stability of the test chemical in solvent/vehicle, if known.

Cells:

- Type and source of cells;
- Number of cell passages;
- Methods for maintenance of cell cultures.

Test conditions:

Solubility limitations should be reported, as well as:

- Composition of media, CO_2 concentration;
- Concentration of test chemical;
- Volume of vehicle and test chemical added;
- Incubation temperature and humidity;
- Duration of treatment;

- Cell density during treatment;
- Positive and negative reference standards;
- Duration of treatment period;
- Luciferase assay reagents (Product name, supplier and lot);
- Acceptability and data interpretation criteria.

Acceptability check:

- Fold inductions for each assay plate.
- Actual log PC_{50} and log PC_{10} (or log IC_{50} and log IC_{30}) values for concurrent reference standards.

Results:

- Raw and normalised data of luminescent signals;
- The maximum fold induction level;
- Cytotoxicity data;
- Concentration-response relationship, where possible;
- Log PC_{10}, log PC_{50} and PC_{max} for agonist assay, and log IC_{50} and log IC_{30} values for antagonist assay, as appropriate;
- EC_{50} values, if appropriate;
- Statistical analyses, if any, together with a measure of error (e.g. SD, %CV or 95% confidence interval) and a description of how these values were obtained.

Discussion of the results

Conclusion

LITERATURE

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ANNEX 1

Definitions and abbreviations

Agonist: A substance that binds to a specific receptor and triggers a response in the cell. It mimics the action of an endogenous ligand that binds to the same receptor.

AG ref: Agonist reference (500 pM of DHT) in the antagonist assay.

Androgenic activity: the capability of a chemical to mimic 5α-Dihydrotestosterone in its ability to bind to and activate androgen receptors. AR-mediated specific androgenic activity can be detected in this Test Guideline.

Antagonist: A type of receptor ligand or chemical that does not provoke a biological response itself upon binding to a receptor, but blocks or dampens agonist-mediated responses.

Anti-androgenic activity: the capability of a chemical to suppress the action of 5α-Dihydrotestosterone-mediated through androgen receptors. AR-mediated specific anti-androgenic activity can be detected in this Test Guideline.

AR: Androgen receptor

ARTA: Androgen Receptor Transcriptional Activation Assay.

BPA: Bisphenol A

%CV: Coefficient of variation

Cytotoxicity: the harmful effects to cell structure or function ultimately causing cell death. It can be reflected by a reduction in the number of cells present in the well at the end of the exposure period or a reduction of the capacity for a measure of cellular function when compared to the concurrent vehicle control.

DCC-FBS: Dextran-coated charcoal treated fetal bovine serum.

DEHP: Di(2-ethylhexyl)phthalate

DHT: 5α-Dihydrotestosterone

DMSO: Dimethyl sulfoxide

EC_{50} value: the concentration of agonist that provokes a response halfway between the baseline (Bottom) and maximum response (Top).

ER: Estrogen receptor

FBS: Fetal bovine serum

GR: Glucocorticoid recoptor

HF: Hydroxyflutamide

IC_{50}: the concentration of a test chemical at which the measured activity in an antagonist assay inhibits at level of 50% of the maximum activity induced by 500 pM DHT in each plate.

IC_{30}: the concentration of a test chemical at which the measured activity in an antagonist assay inhibits at level of 30% of the maximum activity induced by 500 pM DHT in each plate.

PC_{AGO}: Positive AR agonist control (DHT at 10 nM)

PC_{ATG} : Positive AR antagonist control (500 pM DHT and 0.1 μM of HF)

PC_{CT}: the response of the positive cytotoxic control (10µg/mL of cycloheximide)

PC_{10}: the concentration of a test chemical at which the response in an agonist assay is 10% of the response induced by positive control (DHT at 10 nM) in each plate.

PC_{50}: the concentration of a test chemical at which the response in an agonist assay is 50% of the response induced by positive control (DHT at 10 nM) in each plate.

PC_{max}: the concentration of a test chemical inducing the RPCmax.

RPC_{max}: maximum level of response induced by a test chemical, expressed as a percentage to the response induced by PC_{AGO} (10 nM DHT) on the same plate.

RLU: Relative Light Units

RTA: Relative Transcriptional Activity

RT PCR: Real Time polymerase chain reaction

SARMs : Selective androgen receptor modulators

SD: Standard deviation

STTA: Stably Transfected Transcriptional Activation Assay.

TA: Transcriptional activation

UVCBs: Chemical Substances of Unknown or Variable Composition, Complex Reaction Products and Biological Materials

Validation: The process by which the reliability and relevance of a particular approach, method, process or assessment is established for a defined purpose (18).

VC (Vehicle control): The vehicle that is used to dissolve test and control chemicals is tested solely as vehicle without dissolved chemical.

ANNEX 2

False positives: Assessment of non-AR-mediated luminescence signals

1. False positives might be generated by non-AR-mediated activation of the luciferase gene, or direct activation of the gene product or unrelated luminescence. Such effects are indicated by an incomplete or unusual dose-response curve. If such effects are suspected, the effect of an AR antagonist (e.g. Hydroxyflutamide (HF) at non-toxic concentration) on the response should be examined.

2. To ensure validity of this approach, the agonistic activity of the following needs to be tested in the same plate:

- Agonistic activity of the unknown chemical with / without 1 µM of HF (in triplicate)
- VC (in triplicate)
- 1 µM HF (in triplicate)
- 500 pM of DHT (in triplicate) as PC_{AGO}

3. Data interpretation criteria

Note: All wells should be treated with the same concentration of the vehicle.

- If the agonistic activity of the unknown chemical is NOT affected by the treatment with HF, it is classified as "Negative".
- If the agonistic activity of the unknown chemical is inhibited, apply the decision criteria (Table 5-1).
- If the agonistic activity at any concentrations tested is inhibited by the treatment with 1 µM of HF (AR antagonist), the difference in the responses between the wells non-treated with the AR antagonist and wells treated with the AR antagonist is calculated. This difference should be considered as the true response and should be used for the calculation of the appropriate parameters to enable a classification decision to be made.

True response = (Response without HF) - (Response with HF)

4. Data analysis

Check the performance standard.

Check the CV between wells treated under the same conditions.

1. Calculate the mean of the VC
2. Subtract the mean of VC from each well value **not** treated with HF
3. Calculate the mean of HF
4. Subtract the mean of the VC from each well value treated with HF
5. Calculate the mean of the PC_{AGO}
6. Calculate the relative transcriptional activity of all other wells relative to the PC_{AGO}