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OECD GUIDELINE FOR THE TESTING OF CHEMICALS

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

(Version 2010 Nov.25)

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 Transcriptional Activation (TA) assay described in this Test Guideline is a level 2 "*in vitro* assay, providing mechanistic information". The validation study of the Stably Transfected Transactivation Assay (STTA) by Chemicals Evaluation and Research Institute (CERI) in Japan using the AR-EcoScreen^{TM} cell line to detect Androgenic agonist/antagonist activities mediated through human androgen receptor demonstrated the relevance and reliability of the assay for its intended purpose (2).

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 receptor (AR) (3)(4)(5)(6). They have been proposed for the detection of nuclear receptor mediated transactivation (1)(3)(4). Several *in vitro* TA and receptor binding assays are currently at validation at national, European and international levels, but are not yet close to completion and full assessment of their validation status. Only the assay "Stably Transfected Transcriptional Activation (STTA) using HeLa-9903 cell line for detecting estrogenic activity of chemicals" has been adopted as OECD test guideline (TG 455) in 2009. Although the need for AR mediated *in vitro* assays are also urgent, at the present time there are no in vitro screening assays for androgenic activity to enable use for OECD regulatory purposes. The aim of this TA assay is to evaluate the ability of a chemical to function as an AR ligand and activate or inhibit androgenic responses, for screening and prioritisation purposes but can also provide mechanistic information that can be used in a

weight of evidence approach.

3. Definitions and abbreviations used in this Test Guideline are described in Annex 1.

INITIAL CONSIDERATIONS AND LIMITATIONS

4. Androgen agonists and antagonist act as ligands for AR, 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 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 agonist binding to the hAR, and therefore it should not be directly extrapolated to the complex *in vivo* situation of androgen regulation of cellular processes.

5. This test method is specifically designed to detect hAR-mediated transcriptional activation and inhibition by measuring chemiluminescence as the endpoint.

PRINCIPLE OF THE TEST

6. The TA assay using a reporter gene technique is an *in vitro* tool that provides mechanistic data. The assay is used to signal binding of the androgen receptor with a ligand. Following 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 increased cellular expression of 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.

7. The test system provided in this Test Guideline utilises the AR-EcoScreen^{TM} cell line, which is derived from Chinese hamster ovary derived cell line (CHO-K1), with two stably inserted constructs: (i) the human AR expression construct (encoding the full-length human receptor), 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.

Where C3 gene derived responsive element is selected to minimize GR mediated responses among known androgen responsive elements.

8. Data interpretation for **AR agonist assay** is based upon whether or not the maximum response level induced by a test chemical equals or exceeds an agonist response equal to 10% of that induced by a maximally inducing (10 nM) concentration of the positive control (PC) 5α-dihydrotestosterone (DHT) (*i.e.* the PC10). And that **for AR antagonist assay** is based on 30% inhibitory response against 500 pM DHT by a test chemical. Data analysis and interpretation are discussed in greater detail in paragraphs 36- 52.

PROCEDURE

Cell Lines

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

10. Only cells characterized as mycoplasma-free should be used in testing. RT PCR (Real Time Polymerase Chain Reaction) is the method of choice for a sensitive detection of mycoplasma infection (8) (9) (10).

Stability of the cell line

11. To monitor the stability of the cell line **for agonist assay**, DHT, Methyltrienolone (R1881) and Di(2-ethylhexyl)phthalate (DEHP) should be used as the reference chemicals and a complete concentration-response curve in the test concentration range provided in Table 1 should be measured at least once each time the assay is performed, and the results should be in agreement with the results provided in Table 1-1.

12. To monitor the stability of the cell line **for antagonist assay**, Hydroxyflutamide (HF), Bisphenol A (BisA) and Di(2-ethylhexyl)phthalate (DEHP) should be used as the reference chemicals and a complete concentration response curve in the test concentration range provided in Table 1-2 should be measured in at least one run each day the assay is performed, and the results should be in agreement with the results provided in Table 1-2.

Cell Culture and Plating Conditions

13. 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% fetal bovine serum, Zeocin (200 μg/mL), Hygromycin (100 μg/mL) , Penicillin (100 units /m L), and Streptomycin (100 ug/ml).

Medium for the assay plate: Phenol Red Free D-MEM/F-12 supplemented with 5% DCC-FCS, Penicillin (100 units /m L), and Streptomycin (100 ug/ml).

14. Cell 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, cells can be subcultured at 20 mL of 1.5 – 3.0 x 10^4 cells/mL for 75 cm^2 cell culture flask. To prepare the assay plate, cells should be suspended with the Medium for the assay plate and then plated into wells of a microplate at 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 3 hours before the chemical exposure.

15. 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 and should not be cultured for more than 40 passages. For the AR-EcoScreen™ cell line, this will be less than two months.

16. The DCC-FBS can be prepared as described in Annex 2, or obtained from commercial sources. The selection of FBS is some time critical for the assay performance; therefore, the appropriate FBS should carefully be selected based on the cell response, as generally considered.

Acceptability Criteria

Positive and Negative Reference Chemicals

17. Prior to and during the study, the responsiveness of the test system should be verified using the appropriate concentrations of known reference chemicals as provided in Table 1-1 for AR agonist assay and Table 1-2 for AR antagonist assay. Acceptable range values derived from the validation study are given in Table 1-1 and Table 1-2 (2). These 3 concurrent reference chemicals for each AR agonist/antagonist assays should be included with each AR agonist/antagonist experiments and the results should fall within the given acceptable limits. 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, to ensure minimum variability of EC50, PC50, PC10, linearIC30, linearIC50 and logIC50 values, consistent use of materials for cell culturing is essential. The three concurrent reference chemicals, which should be included in each experiment (conducted under the same conditions including the materials, passage level of cells and technicians), can ensure

the sensitivity of the assay because the PC10s or linear IC30 of the two positive reference chemicals should fall within the acceptable range, as should the PC50s and EC50s, or linear IC50 and IC50 where they can be calculated (see Table 1).

Table 1-1 Reference chemicals for AR agonist assay

Fold-induction	> = 6.4				
PC10 value	Greater than 1 (fold-induction of VC) +2SD				
Chemical Name [CAS No.]	logPC10	logPC50	logEC50	Hill Slope	Test range
5α-Dehydrotestosterone (DHT) [521-18-6]	-9.87 ~-12.08	-9.00 ~ -11.03	-9.13 ~ -11.02	0.577 ~ 4.358	10^{-6} ~ 10^{-12}M
Methyltrienolone (R1881) [965-93-5]	-10.57 ~ -11.07	-9.10 ~ -10.86	-9.37 ~ -10.83	3.996 ~ 0.599	10^{-5} ~ 10^{-11}M
Di(2-ethylhexyl)phthalate (DEHP) [117-81-7]	-	-	-	-	10^{-5} ~ 10^{-10}M

Table 1-2 Reference chemicals for AR antagonist assay

Fold induction of spike-in [Spike-in of 500 pM DHT] /[Vehicle Control]	> = 5.0				
PC_{ATG} inhibitory ratio	= <0.46				
Chemical Name [CAS No.]	log linearIC30	log linearIC50	logIC50	Hill Slope	Test range
Hydroxyflutamide (HF) [52806-53-8]	-6.41 ~ -8.37	-6.17 ~ -7.80	-6.26 ~ -7.71	-2.503 ~ -0.652	10^{-5} ~ 10^{-10}M
Bisphenol A (BisA) [80-05-7]	-4.48 ~ -7.52	-4.29 ~ -7.05	-4.38 ~ -6.89	-2.973 ~ -0.598	10^{-5} ~ 10^{-10}M
Di(2-ethylhexyl)phthalate (DEHP) [117-81-7]	-	-	-	-	10^{-5} ~ 10^{-10}M

Positive and Vehicle Controls

18. **For agonist assay**, positive control (PC) wells (n=6) treated with a natural ligand (10 nM of DHT) and vehicle control (VC) wells (n=6) treated with vehicle alone, should be prepared in each assay plate. **For antagonist assay**, vehicle control (no spike-in, n=3), positive control for agonistic activity (PCago, 10 nM of DHT, n=3), positive control for antagonistic activity (PC_{ATG}, 0.1 μM of HF, n=3), positive control for cytotoxicity (PC_{CT}, 10 μg/mL of cycloheximide, n=3) and spike-in control (SPK, 500 pM of DHT, n=12) should be set in each assay plate. As for the inhibitory ratio of PC_{ATG}, it should be greater than 0.46.

Fold-induction

19. The mean luciferase activity of the PC (10 nM DHT) should be at least 6.4-fold that of the

mean VC on each plate for agonist assay, and at least 5.0-fold for antagonist assay. These criterion was established based on the reliability of the endpoint values from the validation study .

20. With respect to the quality control of the assay, the fold-induction corresponding to the PC10 value of the concurrent PC (10 nM DHT) should be greater than 1+2SD of the fold-induction value (=1) of the concurrent VC. For prioritization purposes, the PC10 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 concentration-based potential, and so is less useful for prioritization purposes.

Chemicals to Demonstrate Laboratory Proficiency

21. 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 by independent testing of the 10 proficiency chemicals listed in Table 2-1 and 2-2 for AR agonist and antagonist, respectively. This should be done at least in duplicate, on different days, and the results should be comparable to Table 2 and any deviations should be justified.

Table 2-1 List of Proficiency Chemicals for agonist assay

No.	Chemical name	CAS No.	Class
1	Flutamide	13311-84-7	N
2	4-*tert*-Octylphenol	140-66-9	N
3	Bisphenol A	80-05-7	N
4	Dexamethasone	50-02-2	P
5	Medroxyprogesterone acetate	71-58-9	P
6	Testosterone	58-22-0	P
7	4-Androstenedione	63-05-8	P
8	Spironolactone	52-01-7	P
9	Progesterone	57-83-0	P
10	17a-Methyltestosterone	58-18-4	P

Table 2-2 List of proficiency chemicals for antagonist assay

No.	Chemical name	CAS No.	Class
1	Methyltrienolone (R1881)	965-93-5	N
2	Fluoxymestrone	76-43-7	N
3	Medroxyprogesterone acetate	71-58-9	N

4	Cyproterone acetate	427-51-0	P
5	Flutamide	13311-84-7	P
6	Spironolactone	52-01-7	P
7	4- tert -Octylphenol	140-66-9	P
8	Procymidone	32809-16-8	P
9	Progesterone	57-83-0	P
10	Vinclozolin	50471-44-8	P

Vehicle

22. Dimethyl sulfoxide (DMSO), or appropriate solvent, at the same concentration used for the different positive and negative controls and the test chemicals should be used as the concurrent VC. Test substances should be dissolved in a solvent that solubilizes that test substance and is miscible with the cell medium. Water, ethanol (95% to 100% purity) and DMSO are suitable vehicles. If DMSO is used, the level should not exceed 0.1% (v/v). For any vehicle, it should be demonstrated that the maximum volume used is not cytotoxic and does not interfere with assay performance.

Preparation of Test Chemicals

23. Generally, the test chemicals should be dissolved in DMSO or other suitable solvent, and serially diluted with the same solvent at a common ratio of 1:10 in order to prepare solutions for dilution with media.

Solubility and Cytotoxicity: Considerations for Range Finding.

24. A preliminary test should be carried out to determine the appropriate concentration range of chemical to be tested, and to ascertain whether the test chemical may have any solubility and cytotoxicity problems. Initially, chemicals are tested up to the maximum concentration of 1 µl/ml, 1 mg/ml, or 1 mM, whichever is the lowest. Based on the extent of cytotoxicity or lack of solubility observed in the preliminary test, the first definite run should test the chemical at log-serial dilutions starting at the maximum acceptable concentration (*e.g.* 1 mM, 100 µM, 10 µM, etc.) and the presence of cloudiness or precipitate or cytotoxicity noted. Concentrations in the second, and if necessary third run should be adjusted as appropriate to better characterize the concentration-response curve and to avoid concentrations which are found to be insoluble or to induce excessive cytotoxicity.

25. For AR agonists, 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 testing methods that can provide information regarding 80% cell

viability should be used, utilising an appropriate assay based upon laboratory experience. Cytotoxicity can be evaluated with renilla luciferase activity in AR-EcoScreen^{TM} cell line, which originally established to expresses renilla luciferase constitutively. Accordingly, AR-mediated transcriptional activity and cytotoxicity can be evaluated simultaneously in same assay plate.

26. Should the results of the cytotoxicity test show that the concentration of the test substance has reduced the cell number 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.

Chemical Exposure and Assay Plate Organisation

27. For AR agonist assay, the procedure for chemical dilutions (Steps-1 and 2) and exposure to cells (Step-3) can be conducted as follows:

Step-1: Each test chemical should be serially diluted in DMSO, or appropriate solvent, and added to the wells of a microtitre plate to achieve final serial concentrations as determined by the preliminary range finding test (typically in a series of, for example 1 mM, 100 μM, 10 μM, 1 μM, 100 nM, 10 nM, 1 nM, 100 pM, and 10 pM (10^{-3}-10^{-11} M)) for triplicate testing.

Step-2: Chemical dilution: First dilute 10 μL of the test chemical in the solvent into 90 μL of media.

Step-3: Then 10μL of the diluted chemical prepared in Step-2 should be diluted into 90 μL of the media for dilution.

Step-4: Chemical exposure of the cells: Add 10 μL of diluted chemical solution (prepared in Step-3) 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.

Test samples and reference chemicals can be assigned as shown in Table 3-1.

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

Row	DHT			R1881			DEHP			Chemical-1		
	1	2	3	4	5	6	7	8	9	10	11	12
A	1 μM	→	→	10 μM	→	→	10 μM	→	→	10 μM	→	→
B	100 nM	→	→	1 μM	→	→	1 μM	→	→	1 μM	→	→
C	10 nM	→	→	100 nM	→	→	100 nM	→	→	100 nM	→	→
D	1 nM	→	→	10 nM	→	→	10 nM	→	→	10 nM	→	→
E	100 pM	→	→	1 nM	→	→	1 nM	→	→	1 nM	→	→
F	10 pM	→	→	100 pM	→	→	100 pM	→	→	100 pM	→	→
G	1 pM	→	→	10 pM	→	→	10 pM	→	→	10 pM	→	→
H	VC	→	→	→	→	→	PC	→	→	→	→	→

VC: Vehicle control (DMSO);
PC: Positive control (10 nM of DHT)

28. For AR antagonist assay, the procedure for chemical dilutions (Steps-1 and 2) and exposure to cells (Step-3) can be conducted as follows:

Step-1: Each test chemical should be serially diluted in DMSO, or appropriate solvent, and added to the wells of a microtitre plate to achieve final serial concentrations as determined by the preliminary range finding test (typically in a series of, for example 1 mM, 100 μM, 10 μM, 1 μM, 100 nM, 10 nM, 1 nM and 100 pM (10^{-3}-10^{-10} M)) for triplicate testing.

Step-2: Chemical dilution: First dilute 10 µL of the test chemical in the solvent to a concentration of 90 µL of media containing 56nM DHT/0.1% DMSO.

Step-3: Then 10µL of the diluted chemical prepared in Step-2 should be diluted into 90 µL of the media for dilution.

Step-4: 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.

29. Test samples and reference chemicals can be assigned as shown in Table 3-2.

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

Row	HF			Bisphenol A			DEHP			DEHP		
	1	2	3	4	5	6	7	8	9	10	11	12
A	10 µM	→	→	10 µM	→	→	10 µM	→	→	10 µM	→	→
B	1 µM	→	→	1 µM	→	→	1 µM	→	→	1 µM	→	→
C	100 nM	→	→	100 nM	→	→	100 nM	→	→	100 nM	→	→
D	10 nM	→	→	10 nM	→	→	10 nM	→	→	10 nM	→	→
E	1 nM	→	→	1 nM	→	→	1 nM	→	→	1 nM	→	→
F	100 pM	→	→	100 pM	→	→	100 pM	→	→	100 pM	→	→
G	SPK	→	→	→	→	→	→	→	→	→	→	→
H	VC	→	→	PC_{ago}	→	→	PC_{ATG}	→	→	PC_{CT}	→	→

VC: Vehicle control (DMSO);
PC_{ago}: Positive control (10 nM of DHT);
PC_{ATG}-: Positive control (0.1 µM of HF) ;
PC_{CT}: Cytotoxicity control (10 µg/mL of cycloheximide);
SPK (DMSO at 0.1% spiked with $5x10^{-10}$ M DHT)
** Gray colored wells are spiked with $5x10^{-10}$M DHT

30. The reference chemicals (DHT, R1881 and DHEP for agonist assay; HF, BisA and DEHP for antagonist assay) should be tested in every run (as exampled in Table 3-1 and 3-2). PC wells treated with 10 nM of DHT that can produce maximum induction of DHT and VC wells treated with DMSO (or appropriate solvent) alone should be included in each test assay plate for agonist assay (Table 4-1). In case of antagonist assay, PC_{ATG}: Antagonist positive control (0.1 µM of HF), PC_{CT}: Cytotoxicity control (10 µg/mL of cycloheximide) and SPK-in control (DMSO at 0.1% spiked with 500 pM DHT) should be prepared additionally (Table 4-2). If cells from different sources (*e.g.* different passage number, different lot, etc.,) are used in the same experiment, the reference chemicals should be tested for each cell source.

Table 4-1.: Example of plate concentration assignment of test and plate control chemicals 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	→	→	→	→	→

Plate controls = VC: Vehicle control (DMSO); PC: Positive control (10 nM of DHT)

Table 4-2.: Example of plate concentration assignment of test and plate control chemicals 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	SPK	→	→	→	→	→	→	→	→	→	→	→
H	VC	→	→	PC_{ago}	→	→	PC_{ATG}	→	→	PC_{CT}	→	→

VC: Vehicle control (DMSO);
PC_{ago}: Positive control (10 nM of DHT);
PC_{ATG}-: Positive control (0.1 µM of HF) ;
PC_{CT}: Cytotoxicity control (10 µg/mL of cycloheximide);
SPK (DMSO at 0.1% spiked with $5x10^{-10}$ M DHT)
** Gray colored wells spiked with $5x10^{-10}$M DHT

31. 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.

32. 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.

33. Special considerations will need to be applied to those compounds 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.

34. Repeat definitive tests for the same chemical should be conducted on different days, to ensure independence.

Luciferase assay

35. A commercial luciferase assay reagent, Steady-Glo Luciferase Assay System (Promega, E2510 and its equivalents) or a standard luciferase assay system (Promega, E1500 and its equivalents) can be used for the agonism detection. And Dual-Glo (Promega, E2920 and its equivalents) can be used for the antagonism detection, as long as the acceptability criteria is met. The assay reagents should be selected based on the sensitivity of the luminometer to be used. When using the standard luciferase assay system, Cell Culture Lysis Reagent (Promega, E1531, or equivalents) should be used before adding the substrate. In case of using Steady-Glo Luciferase Assay System (Promega, E2510) in the agonist assay, 40μL of prepared reagent should be directly added into the assay wells. In case of using Dual-Glo system (Promega, E2920) in the antagonist assay, 40μL of the first substrate should be added into the assay wells after removing 60 μL of supernatant to detect Firefly luciferase activity. Then 40μL of the second substrate should be added into the assay wells to detect Renilla luciferase activity.

ANALYSIS OF DATA

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

    Step 1. Calculate mean value for the VC.

    Step 2. Subtract the mean value of the VC from each well value to normalize the data.

    Step 3. Calculate the mean for the normalised PC.

    Step 4. Divide the normalized value of each well in the plate by the mean value of the normalized PC (PC=100%).

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

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).

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

Step 1. Calculate mean value for the VC.

Step 2. Subtract the mean value of the VC from each well value to normalize the data.

Step 3. Calculate the mean for the normalized SPK.

Step 4. Divide the normalized value of each well in the plate by the mean value of the normalized mean SPK (SPK=100%).

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

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).

EC50, PC50, PC10, IC50, lin.IC50 and lin.IC30 induction considerations

38. The full concentration-response curve is required for the calculation of the EC50 (IC50), but this may not always be achievable or practical due to limitations of the test concentration range (for example due to cytotoxicity or solubility problems). However, as the EC50 (IC50) and maximum induction level (corresponding to the top value of the Hill-equation) are informative parameters, these parameters should be reported where possible. For the calculation of EC50 (IC50) and maximum induction level, appropriate statistical software should be used (*e.g.* Graphpad Prism statistical software).

39. To evaluate cytotoxicity in the antagonist assay, cell viability should be expressed as the percentage of renilla luciferease activity of the chemical treated wells to the mean renilla luciferase activity of the VC wells.

40. If the Hill's logistic equation is applicable to the concentration response data, the EC50 (IC50) should be calculated by the following equation (15):

Y=Bottom + (Top-Bottom) / (1+10 exp ((log EC50 (IC50) -X) x Hill slope))

Where:

X is the logarithm of concentration; and,

Y is the response and Y starts at the Bottom and goes to the Top in a sigmoid curve.

Bottom is fixed at zero in the Hill's logistic equation.

41. For each test chemical, the following should be provided:

(i) The RPCMax which is the maximum level of response induced by a test chemical, expressed as a percentage of the response induced by 10 nM DHT on the same plate, as well as the PCMax (concentration associated with the RPCMax); and

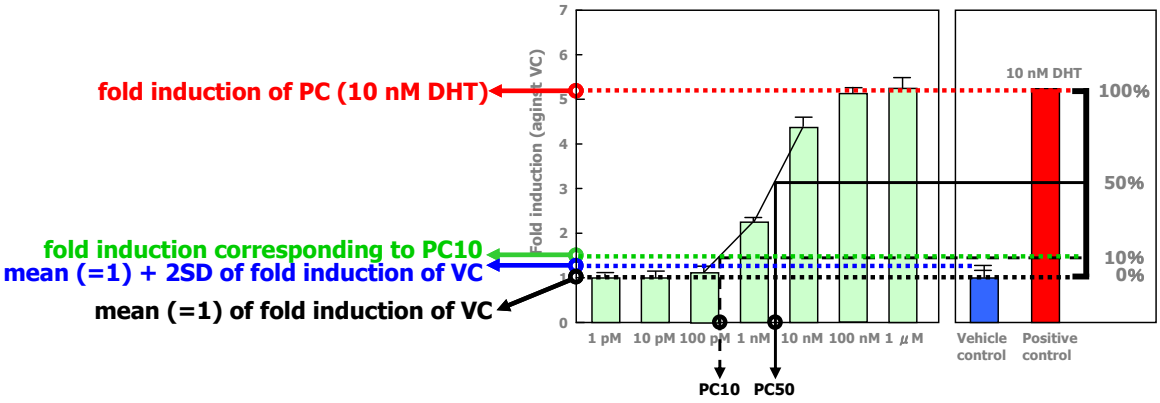
(ii) For positive chemicals, the concentrations that induce the PC10 and, if appropriate, the PC50.

42. The PCx value can be calculated by interpolating between 2 points on the X-Y coordinate, one immediately above and one immediately below a PCx value. Where the data points lying immediately above and below the PCx value have the coordinates (a,b) and (c,d) respectively, then the PCx value may be calculated using the following equation:

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

43. Descriptions of PC values are provided in Figure 1 below.

Figure 1: Example of how to derive PC-values.
The PC (Positive control; 10 nM of DHT) is included on each assay plate

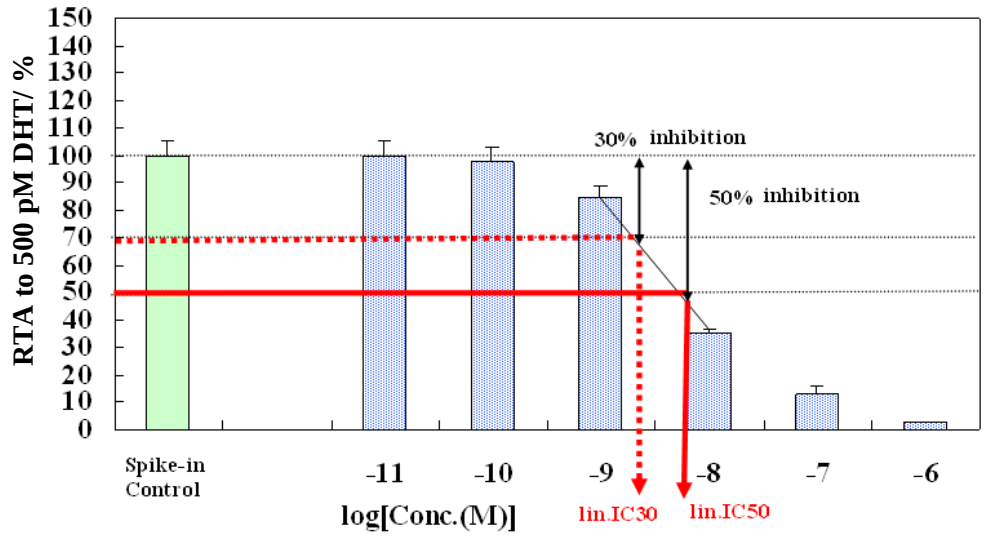


44. In case of antagonist assay, (i) The RICMax which is the maximum inhibition level of luciferase induced by a test chemical, expressed as a percentage of the response induced by 500 pM DTH on the same plate, as well as the PCMax (concentration associated with the RPCMax); and if the RICmax is exist, the lin.IC50 and lin.IC30 values should be calculated. These lin.IC50 and lin.IC30 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 that can be calculated same as PC values. Each lin.ICx

value can be calculated by a simple linear regression using two variable data points in the transcription activity same as PCx values.

45. Descriptions of lin.ICx values are provided in Figure 2 below.

<u>Figure 2.:</u> Example of how to derive lin.IC-values.
The SPK-in control (DMSO at 0.1% spiked with 500 pM DHT) is included on each assay plate.



46. To distinguish pure antagonism and cytotoxicity related decrease of luciferase activity, AR-EcoScreen is designed to express two kinds of luciferase, one is firefly luciferase inducibly expressed by AR response element and other is Renilla luciferase stably and non-inducibly expressed.

47. By using Dual-luciferase reporter assay technology, both cell viability and the antagonism can be evaluated with the same cells in a single plate run. PCct (10μg/mL of cycloheximide) is made for adjusting renilla activity by taking PCct values "renilla activities" away from that of all sample wells. To evaluate the true cytotoxicity of chemicals with AR Ecoscreen cell, such revised cell viability should be used. RICMax and lin.ICx values can be accepted if cell viability is recorded as more than 80% at the test concentration.

48. The results should be based on two (or three) independent runs. If two runs give comparable and therefore reproducible results, it is not necessary to conduct a third run. To be acceptable, the results should:

- Meet the performance standard requirements

  <for AR agonist assay >:

  - The mean luciferase activity of the PC (10 nM DHT) should be at least 6.4-fold

that of the mean VC on each plate

- The fold induction corresponding to the PC10 value of the concurrent PC(10 nM DHT) should be greater than 1+2SD of the fold induction value (=1) of the VC.
- The results of 3 reference chemicals should be within the acceptable range (Table 1-1).

<for AR antagonist assay>

- Fold induction of spike-in ([Spike-in of 500 pM DHT]/[Vehicle Control]) should be at least 5.0.
- The PC_{ATG} (0.1 μM of HF) inhibitory ratio should be greater than 0.46.
- The results of 3 reference chemicals should be within the acceptable range (Table 1-2).

● Be reproducible.

Data Interpretation Criteria

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

Positive	If the RPCMax 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 the RPCMax fails to achieve at least 10% of the response of the positive control in two of two or two of three runs.

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

Positive	If the RICMax is obtained that is equal to or exceeds 30% inhibition against of the PC_{ATG} response (500 pM DHT) in at least two of two or two of three runs.
Negative	If the RPCMax fails to achieve at least 30% inhibition against of the PC_{ATG} response in two of two or two of three runs.

49. Data interpretation criteria are shown in Table 5-1 and 5-2. Positive results will be characterized by both the magnitude of the effect and the concentration at which the effect occurs. Expressing results as a concentration at which a 50% (PC50) or 10% (PC10) **for antagonist assay**, and 50% (lin.IC50) or 30% (lin.IC30) **for antagonist assay** are reached accomplishes both of these goals. However, a test chemical is determined to be positive, if the maximum response induction or inhibition by the test chemical (RPCMax or RICMax) is equal to or exceeds 10% or 30% of the the positive control responses in at least two of two or two of three runs, while a test chemical is considered negative if the RPCMax or RICMax fails to achieve at least 10% or 30% of the response of the positive control in two of two or two of three runs.

50. The calculations of PC10, PC50 and PCMax for antagonist assay, and lin.IC50, lin.IC30 and RICMax can be calculated by using a spreadsheet available with the Test Guideline on the OECD public website2.

51. It should be sufficient to obtain PCx or lin.ICx values at least twice. However, should the resulting base-line for data in the same concentration range show variability with an unacceptably high coefficient of variation (CV; %) the data may not be considered reliable and the source of the high variability should be identified. The CV of the raw data triplicates (*i.e.* luminescence intensity data) of the data points that are used for the calculation of PC10 should be less than 20%.

52. 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 insurance that accurate data were produced, see paragraphs 47 and 48.

53. Where more information is required in addition to the screening and prioritization purposes of this TG for positive test compounds, particularly for PC10-PC49 chemicals, it can be confirmed that the observed luciferase-activity is solely an AR-specific response, using an AR antagonist.

TEST REPORT

54. The test report should include the following information:

Test substance:

- identification data and CAS Number, if known;
- physical nature and purity;
- physicochemical properties relevant to the conduct of the study;
- stability of the test substance.

Solvent/Vehicle:

- characterisation (nature, supplier and lot);
- justification for choice of solvent/vehicle;
- solubility and stability of the test substance in solvent/vehicle, if known.

Cells:

- type and source of cells;
- number of cell passages;
- methods for maintenance of cell cultures.

Test conditions:

cytotoxicity data (and justifications for the method of choice) and solubility limitations should be reported, as well as:

- composition of media, CO_2 concentration;
- concentration of test chemical;
- volume of vehicle and test substance added;
- incubation temperature and humidity;
- duration of treatment;
- cell density during treatment;
- positive and negative reference chemicals;
- duration of treatment period;
- Luciferase assay reagents (Product name, supplier and lot);
- acceptability and data interpretation criteria.

Reliability check:

- Fold inductions for each assay plate.
- Actual logEC50, logPC50, logPC10 and Hill slope values for concurrent reference chemicals.

Results:

- Raw and normalised data of luminescent signals;
- Concentration-response relationship, where possible;
- PC10, PC50 and PCMax for antagonist assay, and lin.IC50, lin.IC30 and RICMax values for antagonist assay, as appropriate;
- EC50 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

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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 binds to the same receptor.

Androgenic activity, the capability of a chemical to mimic 5α-Dehydrotestosterone 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-Dehydrotestosterone 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.

BisA: Bisphenol A

CV: Coefficient of variation

Cytotoxicity: the harmful effects to cell structure or function ultimately causing cell death and can be a result of 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-Dehydrotestosterone

DMSO: Dimethyl sulfoxide

EC50 value, the concentration of agonist that provokes a response halfway between the baseline (Bottom) and maximum response (Top).

ER; Estrogen receptor

ERE: Estrogen Response Element

Estrogenic activity, the capability of a chemical to mimic 17β-estradiol in its ability to bind to and activate estrogen receptors.

FBS: Fetal bovine serum

hERα: Human estrogen receptor alpha

HF: Hydroxyflutamide

lin.IC50: 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 nM DHT in each plate

lin.IC30: 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 nM DHT in each plate

PC: Positive contro l (DHT at 10 nM)

PC10: the concentration of a test chemical at which the response in an agonist assay is 10% of the response induced by positive control l (DHT at 10 nM) in each plate

PC50: the concentration of a test chemical at which the response in an agonist assay is 50% of the response induced by positive control l (DHT at 10 nM)in each plate

PCMax: the concentration of a test chemical inducing the RPCMax

R1881: Methyltrienolone

RICMax: maximum level of luciferase activity inhibited by a test chemical, expressed as a percentage of the response induced by 500 pM DHT on the same plate

RPCMax: maximum level of response induced by a test chemical, expressed as a percentage of the response induced by 1 nM E2 on the same plate

RT PCR: Real Time polymerase chain reaction

SD: Standard deviation

STTA: Stably Transfected Transcriptional Activation Assay.

TA: Transcriptional activation

Validation, a process based on scientifically sound principles by which the reliability and relevance of a particular test, approach, method, or process are established for a specific purpose.

Reliability is defined as the extent of reproducibility of results from a test within and among laboratories over time, when performed using the same standardized protocol. The relevance of a test method describes the relationship between the test and the effect in the target species and whether the test method is meaningful and useful for a defined purpose, with the limitations identified. In brief, it is the extent to which the test method correctly measures or predicts the (biological) effect of interest, as appropriate (16).

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

ANNEX 2

Preparation of Serum treated with Dextran Coated Charcoal (DCC)

1. The treatment of serum with dextran-coated charcoal (DCC) is a general method for removal of estrogenic compounds from serum that is added to cell medium, in order to exclude the biased response associated with residual estrogens in serum. 500 mL of fetal bovine serum (FBS) can be treated by this procedure.

Components

2. The following materials and equipment will be required:

Materials

Activated charcoal
Dextran
Magnesium chloride hexahydrate ($MgCl_2 \cdot 6H_2O$)
Sucrose
1 M HEPES buffer solution (pH 7.4)
Ultrapure water produced from a filter system

Equipment

Autoclaved glass container (size should be adjusted as appropriate)
General Laboratory Centrifuge (that can set temperature at 4°C)

Procedure

3. The following procedure is adjusted for the use of 50 mL centrifuge tubes:

[Day-1] Prepare dextran- coated charcoal suspension with 1 L of ultrapure water containing 1.5 mM of $MgCl_2$, 0.25 M sucrose, 2.5 g of charcoal, 0.25 g dextran and 5 mM of HEPES and stir it at 4°C, overnight.

[Day-2] Dispense the suspension in 50 mL centrifuge tubes and centrifuge at 10000 rpm at 4°C for 10 minutes. Remove the supernatant and store half of the charcoal sediment at 4°C for the use on Day-3. Suspend the other half of the charcoal with FBS that has been gently thawed to avoid precipitation, and heat-inactivated at 56°C for 30 minutes, then

transfer into an autoclaved glass container such as an Erlenmeyer flask. Stir this suspension gently at 4°C, overnight.

[Day-3] Dispense the suspension with FBS into centrifuge tubes for centrifugation at 10000 rpm at 4°C for 10 minutes. Collect FBS and transfer into the new charcoal sediment prepared and stored on Day-2. Suspend the charcoal sediment and stir this suspension gently in an autoclaved glass container at 4°C, overnight.

[Day-4] Dispense the suspension for centrifugation at 10000 rpm at 4°C for 10 minutes and sterilise the supernatant by filtration through 0.2 μm sterile filter. This DCC treated FBS should be stored at -20°C and can be used for up a year.