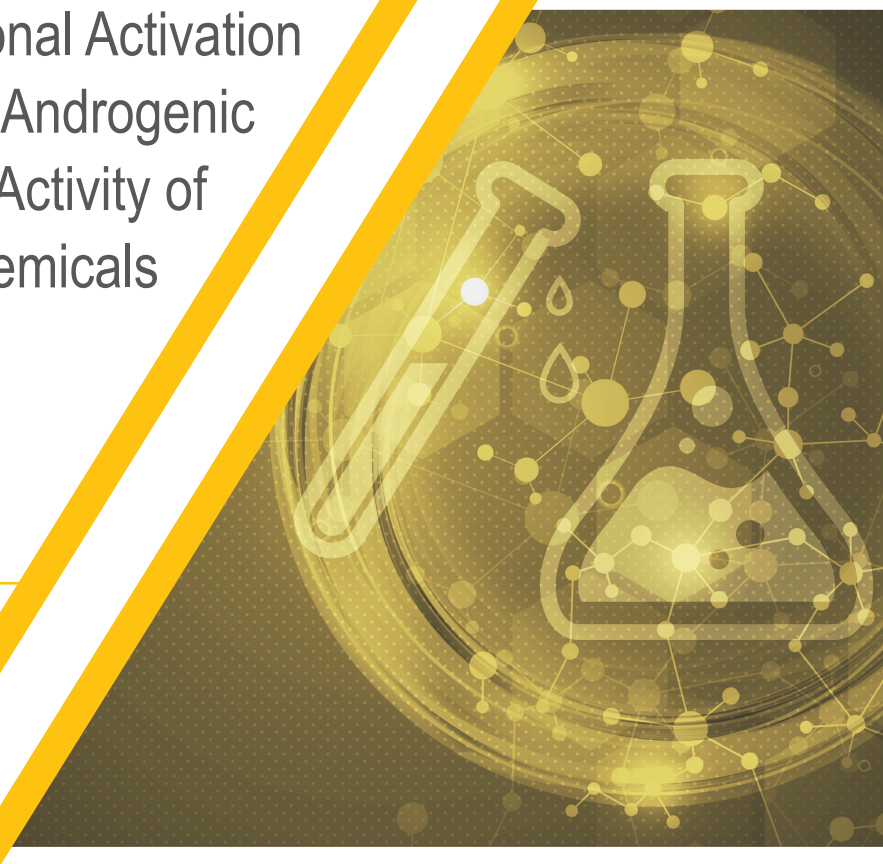
Section 4
Health effects

Test Guideline No. 458

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



4 July 2023

OECD Guidelines for the Testing of Chemicals

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OECD GUIDELINE FOR THE TESTING OF CHEMICALS

Androgen receptor transactivation (ARTA) test guideline of similar in vitro methods

1 Introduction

1. Disruption of the endocrine system may occur through a number of different mechanisms including interference with (i) hormone action mediated via nuclear receptors linked to the endocrine system (ii), hormone production via steroidogenic or other enzymes, (iii) metabolic activation or deactivation of hormones, (iv) distribution of hormones to target tissues, and (v) clearance of hormones from the body. This Test Guideline (TG) exclusively addresses transcriptional activation and inhibition of an androgen-regulated reporter gene.

2. The results of the methods in this TG should not be directly extrapolated to the complex *in vivo* situation of androgen regulation of any cellular or physiological processes.

3. This TG describes the methodology of Androgen Receptor TransActivation (ARTA) assays that detect agonist and antagonists. It comprises several mechanistically and functionally similar test methods for the identification of androgen receptor agonists and antagonists. The fully validated reference test methods described in this TG are:

- The AR-EcoScreen™ method using the AR-EcoScreen™ cell line (1) (Method 1, found in Annex C)
- The AR-CALUX® method using the AR-CALUX® cell line (2) (Method 2, found in Annex D)
- The ARTA method using the 22Rv1/MMTV_GR-KO cell line (3) (Method 3, found in Annex E)

4. These three test methods address the same endpoint, i.e. transactivation of a reporter gene by a ligand bound androgen receptor (see paragraphs 5 and 6). An overview of the similarities and differences between the test methods is given in Annex B (Tables B.1 and B.2). All three test methods are performed in 96-well plates while a high-throughput application has also been reported (but not yet validated according to OECD Guidance Document 34, 2020) for the AR-CALUX® test method (4). Method 1 includes a specificity control for the agonist detection but not for the antagonist, whereas methods 2 and 3 include a specificity control for the antagonist assay to give assurance that what is measured is a competitive antagonist. Each test method has a distinct protocol and test run acceptability criteria. Each test method has its own data interpretation criteria to conclude on agonist and antagonist activity.

Background and principles of the test methods included in this test guideline

5. *In vitro* Transactivation (TA) methods are based upon the transcription and translation of a reporter gene (e.g. luc gene) following binding of a chemical to a specific receptor and subsequent transactivation. Different reporter genes can be used in these assays. TA methods have been used to evaluate the gene expression profiles regulated by specific nuclear receptors, such as the estrogen receptors (ERs) and androgen receptors (ARs) (5) (6) (7) (8). They have been proposed for the detection of nuclear receptor-mediated transactivation (5) (6) (9).

6. Androgen agonists and antagonists act as ligands for the 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 e.g. processes necessary for cell proliferation, normal fetal development, and reproductive function.

7. The OECD initiated a high-priority activity in 1998 to revise existing, and to develop new TGs 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 (10). The 3 ARTA methods described in this TG are included in level 2 for *"in vitro assays providing data about selected endocrine mechanism(s)/pathway(s) (Mammalian and non mammalian methods)"*.

8. The test methods described in this TG cannot be used on their own for safety assessment decisions. They provide concentration-response data for chemicals with *in vitro* (anti)androgenic activity, which may be used for screening and prioritization purposes and can also be used as mechanistic information in a weight of evidence approach.

9. Validation studies of the AR-EcoScreen™ test method, the AR-CALUX® test method, and 22Rv1/MMTV_GR-KO test method have demonstrated their relevance and reliability (1, 3, 11).

10. An overview of the main characteristics, the acceptability criteria and the main abbreviations used in each test method is described in Annex B (Tables B.1 and B.2). For information purposes, Tables B.3a and B.3b of Annex B provide the results for the chemicals that were tested in common between at least 2 test methods of this TG. The classification comparison is made with the ICCVAM list of 2003 (6) (used as the reference list for the AR-EcoScreen™ which was adopted in 2016) and with the recently updated ICCVAM list of 2017 (12). For the antagonist testing, the 3 test method results were concordant, whereas results of agonist testing resulted in 4 non concordant classifications with the 22Rv1/MMTV_GR-KO method. A possible reason for this could be the different cell lines used in the 3 different test methods (1, 3, 11). The chemical 17β-Estradiol, known as an ER agonist, shows AR agonist activity with all 3 test methods, although in the AR-CALUX® method only a weak activity was observed.

11. Supplementary information on these chemicals as well as on an additional 13 chemicals tested with the AR-CALUX® method can be found in the validation study reports (1, 3, 11).

12. General and test method specific definitions and abbreviations used in the test methods in this TG can be found in Annex A.

Demonstration of laboratory proficiency

13. Each laboratory should demonstrate proficiency in using the test method of choice prior to using that method for testing chemicals with unknown activity. Proficiency is demonstrated by testing 8 proficiency chemicals for agonist activity (see Table B.4a in Annex B) and 9 proficiency chemicals for antagonist activity (see Tables B.4b and B.4c in Annex B). This testing will also confirm the responsiveness of the test system. Testing should be replicated at least twice, on different days, and the results should be consistent to the listed classifications and values in Tables B.4a and B.4a. Moreover, a historical database of data generated with the reference standards and the vehicle/solvent controls shall be maintained to confirm the reproducibility of the test method in the respective laboratory over time.

Test report

14. For reporting purposes, the template provided in Annex B, should be used for each test method.

2 Literature

1. OECD (2016), Validation report of Androgen Receptor (AR) Mediated Stably Transfected Transactivation (AR STTA) Assay to Detect Androgenic and Anti-androgenic Activities. Environment, Health and Safety Publications, Series on Testing and Assessment (No. 241), Organisation for Economic Cooperation and Development, Paris.

2. Transactivation assay for the detection of compounds with (anti)androgenic potential using AR-CALUX® cells (2019). Available at (https://tsar.jrc.ec.europa.eu/test-method/tm2010-07).

3. Validation Study Report of the 22Rv1/MMTV_GR-KO ARTA assay (2019). Available at (http://www.nifds.go.kr/brd/m_18/view.do?seq=12486&srchFr=&srchTo=&srchWord=&srchTp=&itm_seq_1=0&itm_seq_2=0&multi_itm_seq=0&company_cd=&company_nm=&page=1) .

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5. EDSTAC (1998), Endocrine Disruptor Screening and Testing Advisory Committee (EDSTAC) Final report. Available at (https://www.epa.gov/endocrine-disruption/endocrine-disruptor-screening-and-testing-advisory-committee-edstac-final).

6. ICCVAM (2003), ICCVAM Evaluation of *In Vitro* Test Methods for Detecting Potential Endocrine Disruptors: Estrogen Receptor and Androgen Receptor Binding and Transcriptional Activation Assays. Available at (https://ntp.niehs.nih.gov/iccvam/docs/endo_docs/edfinalrpt0503/edfinrpt.pdf).

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9. Gray, L.E. Jr. (1998). Tiered screening and testing strategy for xenoestrogens and antiandrogens. *Toxicol. Lett.,* 102-103, 677-680.

10. OECD (2018), Revised Guidance Document No 150 on Standardised Test Guidelines for Evaluating Chemicals for Endocrine Disruption, OECD Series on Testing and Assessment, No. 150, OECD Publishing, Paris, https://doi.org/10.1787/9789264304741-en .

11. Validation Study Report on the Performance assessment of the AR-CALUX® *in vitro* method (2019). Available at (https://tsar.jrc.ec.europa.eu/test-method/tm2010-07) .

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Annex A. Definitions and abbreviations

General definitions and abbreviations that apply to all the test methods in this TG and/or to the tables in Annex B

Acceptability criteria: Minimum standards for the performance of experimental controls and reference standards. All acceptability criteria should be met for an experiment to be considered valid

Agonist: A chemical 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

Androgen activity: The capability of a chemical to mimic a ligand in its ability to bind to and activate androgen receptors

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-androgen activity: The capability of a chemical to suppress the action of the agonist ligand mediated through androgen receptors. AR-mediated specific anti-androgen activity can be detected in this Test Guideline.

AR: Androgen Receptor

ARE: Androgen Receptor Element

ARTA: Androgen Receptor TransActivation

BDS: BioDetection Systems (The Netherlands)

BLR: Between Laboratory Reproducibility

CERI: Chemicals Evaluation and Research Institute (Japan)

CASRN: Chemical Abstracts Service Registry Number

CRISPR-Cas9: Clustered Regularly Interspaced Short Palindromic Repeats-CRISPR associated

CV: Coefficient of Variation

Cytotoxicity: 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.

DHT: 5α-DiHydroTestosterone

DMSO: DiMethyl SulfOxide

EC_{50}: The half maximal effective concentration of a stimulating (agonist) test chemical

ED: Endocrine Disruptor

ER: Estrogen Receptor

FBS: Fetal Bovine Serum

IF: Induction Factor/Fold

InhF: Inhibition Factor/Fold

GR: Glucocorticoid Receptor

IC_{50}: The half maximal effective concentration of an inhibitory (antagonist) test chemical

InChI: International Chemical Identifier

IUPAC: International Union of Pure and Applied Chemistry

KO: KnockOut

Luc: Luciferase gene

MTA: Material Transfer Agreement

MFDS: Ministry of Food and Drug Safety (Korea)

MMTV: Mouse Mammary Tumour Virus

Negative control: Separate part of a test system treated with a chemical for which it is known that the test system should not respond. The negative control provides evidence that the test system is not responsive under the actual conditions of the assay

NIHS: National Institute of Health Sciences (Japan)

PCR: Polymerase Chain Reaction

PR: Progesterone Receptor

Positive control: Separate part of the test system treated with a chemical for which it is known that the test system should respond. The positive control provides evidence that the test system is responsive under the actual conditions of the assay

R^2: Square of the correlation coefficient (criterion for the specificity control test)

Reference chemical: A chemical used to provide a basis for comparison with the test chemical

Reference standard: Used to demonstrate the adequacy of a test method. In this TG, reference standards refer to 3 chemicals of which 2 elicit a positive response (a dose response or at one fixed concentration) and one does not provide a response. One of the 2 chemicals with a positive dose response is the reference chemical

Reliability: Measure of the extent that a test method can be performed reproducibly within and between laboratories over time, when performed using the same protocol. It is assessed by calculating WLR and BLR

RI: Relative Induction

RLU: Relative Light Units

Run: An individual ex*periment* that evaluates chemical action on the biological outcome of the test method. Each run is a complete experiment performed on replicate wells of cells plated from a common pool of cells at the same time

SD: Standard Deviation

SMILES: Simplified Molecular-Input Line-Entry System

Study: The full range of experimental work performed to evaluate a single, specific chemical using a specific test method. In this TG, a study comprises all steps including tests of dilution of test chemical in the test media, runs (which can be pre-screen runs and comprehensive runs), data analysis, quality assurance, cytotoxicity assessments, etc. Completion of a study allows the classification of the test chemical activity on the toxicity target that is evaluated by the test method used and an estimate of potency relative to the positive reference chemical

TA: Transactivation. The initiation of mRNA synthesis in response to a specific chemical signal, such as a binding of an androgen to an androgen receptor

Test chemical: what is being tested and is not related to the applicability of the assay to the testing of mono-constituent chemicals, multi-constituent chemicals and/or mixtures

Test method: Within the context of the TG, a test method is one of the methodologies accepted as valid in meeting the performance criteria outlined in the TG. Components of the test method include, for example, the specific cell line with associated growth conditions, specific media in which the test is conducted, plate set up conditions, arrangement and dilutions of test chemicals along with any other required quality control measures and associated data evaluation steps

Test system: Any biological, chemical or physical system or a combination thereof used in a study. *In vitro* test systems are mainly biological systems (e.g. cells or tissues)

UN GHS: United Nations Globally Harmonized System of classification and labelling of chemicals

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

Validated test method: A test method for which a validation study has been completed to determine the relevance (including accuracy) and reliability for a specific purpose. It is important to note that a validated test method may not have sufficient performance in terms of accuracy and reliability to be found acceptable for the proposed purpose

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

Vehicle control: The solvent (vehicle) that is used to dissolve test and reference standards. It is tested solely as vehicle without dissolved chemical

WLR: Within Laboratory Reproducibility

Test method specific terminology

AR-EcoScreen™ test method

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

BPA: BisPhenol A

DCC-FBS: Dextran-Coated Charcoal treated Fetal Bovine Serum

DEHP: Di(2-EthylHexyl)Phthalate

HF: HydroxyFlutamide

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}: AR agonist control displaying a positive response with DHT at 10 nM

PC_{ATG}: AR antagonist control displaying a positive response with 500 pM DHT and 1 μM of HF

PC_{CT}: The response of the cytotoxic control (10 μg/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 the reference chemical (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 the reference chemical (DHT at 10 nM) in each plate

PC_{max}: The concentration of a test chemical inducing the RPC_{max}

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

RTA: Relative Transcriptional Activity

AR-CALUX® test method

ARE: Androgen Responsive Elements

AU: Absorbance Units

Comprehensive run: experiment carried out after the pre-screen run with a smaller dilution step (e.g. 2, 3 or 5) in order to calculate the parameters with more precision

DF: Dilution Factor

DMEM: Dulbecco's Modified Eagle's medium

EC_{10}, EC_{50}: Concentration of a test chemical at which 10% or 50% of its maximum induction response is observed

FLU: Flutamide

hAR: Human Androgen Receptor

HTS: High Throughput Screening

IATA: Integrated Approach to Testing and Assessment. IATA are pragmatic, science-based approaches for chemical hazard characterisation that rely on an integrated analysis of existing information coupled with the generation of new information using testing strategies

IC_{20}, IC_{50}: Concentration of a test chemical at which 20% or 50% inhibition is observed when compared to its maximum response

IP: Intellectual Property

LDH: Lactate Dehydrogenase

PC_{10}, PC_{50}, PC_{80}: Concentration of test chemical giving 10%, 50% or 80% induction (or inhibition) with respect to the maximum induction of the reference chemical DHT (agonist), or, the solvent control (antagonist)

PC_{max}, PC_{min}: Concentration of a test chemical where the response is maximal (corresponding to RPC_{max}) or minimal (RPC_{min})

Pre-screen run: Experiment that evaluates the dose response, usually carried out with a large dilution step (e.g. 10) in order to capture the full dose response (if possible). It serves to determine the range of concentrations to be used in a following comprehensive run.

REF RPC_{10}, REF RPC_{50}, REF RPC_{80}: Response level (as determined by relative induction (RI)) of the reference chemical DHT or Flutamide at 10%, 50% or 80%

REF EC_{50}: Concentration of the reference chemical DHT at which 50% of its maximum response is observed (in the agonist assay)

REF IC_{50}: Concentration of the reference chemical Flutamide at which 50% of its maximum response is observed (in the antagonist assay)

RPC_{max}: The maximum response level (highest induction) of the test chemical

RPC_{min}: The minimum response level (highest inhibition) of the test chemical

RI: Relative induction

Specificity control: A test which is carried out to assess if the antagonist response is the result of competitive binding to the AR

S_c: Specificity control response at a specific concentration c, expressed in relative induction.

S_c^n: Normalized specificity control response at a specific concentration c, expressed in relative induction

SC: Solvent control (agonist: assay medium plus 0.1 % solvent; antagonist: assay medium plus 0.1 % solvent and spiked with the EC_{50} concentration DHT)

VC: Vehicle control (assay medium plus 0.1% solvent, used in the antagonist assay)

Y_{ic}: Standard response at concentration c (C1-C8), expressed in relative induction) and technical replicate i (1-3)

Y_c: Average of the standard response Y_{ic} over the 3 technical replicates

ZF: Z-factor

22Rv1/MMTV_GR-KO test method

ATCC: American Type Culture Collection

Comprehensive run: experiment carried out after the pre-screen run with a smaller dilution step (e.g. 3 or 5) in order to calculate the parameters with more precision

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

DEHP: Di(2-EthylHexyl)Phthalate

GF-AFC: Glycyl phenylalanyl-aminofluorocoumarin

IC_{30}: The concentration of a chemical at which its inhibitory response equals 30% of the maximum response of the AR agonistic control (800 pM DHT) in AR antagonist assay

KTR: Korean Testing and Research Institute

NIFDS: National Institute for Food and Drug Safety Evaluation

PC_{10}: The concentration of a chemical at which its response equals 10% of the maximum response of the AR agonistic control (10 nM DHT) in AR agonist assay

PC_{50}: The concentration of a chemical at which its response equals 50% of the maximum response of the AR agonistic control (10 nM DHT) in AR agonist assay

PC_{AGO1}: Control for AR agonist assay displaying a positive response with 10 nM DHT

PC_{AGO2}: Agonist control for AR antagonist assay (800 pM DHT)

PC_{ANTA}: Antagonist control displaying a positive response with 800 pM DHT and 1 μM of Bicalutamide.

PC_{CT}: Cytotoxic control (1 mM SDS)

Pre-screen run: Experiment that evaluates the dose response, usually carried out with a large dilution step (e.g. 10) in order to capture the full dose response (if possible). It serves to determine the range of concentrations to be used in a following comprehensive run.

RTA: Relative Transcriptional Activity

SDS: Sodium Dodecyl Sulfate

Specificity control: A test which is carried out to assess if the antagonist response is the result of competitive binding to the AR

Sc: The relative induction of a test chemical at concentration c when 100 nM DHT is used in the antagonist assay (specificity control)

Yc: The relative induction of a test chemical at concentration c when 800 pM DHT is used in the antagonist assay

Annex B. Information relevant to the three test methods.

Overview tables and list of proficiency chemicals

Table B.1. Overview of the characteristics of the 3 test methods in this TG

Test method name	AR-EcoScreen™	AR-CALUX®	22Rv1/MMTV_GR-KO
Developer	Otsuka Pharmaceuticals Co., Ltd., CERI and NIHS.	BDS	MFDS, Korea Univ. and Dongguk Univ.
Cell line	AR-EcoScreen™	AR-CALUX®	22Rv1/MMTV_GR-KO
Cell type	Chinese hamster ovarian cancer cell	Human osteo-sarcoma cell	Human prostate carcinoma epithelial cell
Genetic modification	• Human AR cDNA • heat shock protein promoter -4 C3 ARE-firefly luc (*Photinus pyralis*) • SV40 promoter-renilla luc (*Renilla reniformis*) (for simultaneous measurement of cytotoxicity)	• Human AR cDNA • TATA promoter -3xARE -firefly luc (*Photinus pyralis*)	• Endogenous AR • MMTV LTR promoter containing ARE-firefly luc (*Photinus pyralis*) • Knocked out GR by CRISPR-Cas9
Special feature	• Minimal GR crosstalk due to the selection of an appropriate androgen responsive element • High throughput applicability	• No or little GR, ER and PR expression • High throughput applicability	• No ER and PR expression • GR knock-out

Availability	Material transfer agreement (MTA) including a licence agreement with Japanese Collection of Research Bioresources (JCRB) Cell Bank and cell owner	Licence agreement with BDS	Material transfer agreement (MTA) Korean Collection for Type Cultures

Table B.2a. Overview of reference standards and acceptability criteria for the three test methods for AGONIST properties.

Test method name	AR-EcoScreen™	AR-CALUX®	22Rv1/MMTV_GR-KO
AGONIST			
Reference chemical	5α-Dihydrotestosterone (DHT)	5α-Dihydrotestosterone (DHT)	5α-Dihydrotestosterone (DHT)
Criteria	Range logPC_{50} -11.03/-9.00 (log[M])	Range EC_{50} 1.10^{-10}/1.10^{-9}M	Range logPC_{50} -10.6/-9.0 (log[M])
	Range logPC_{10} -12.08/-9.87 (log[M])		Range logPC_{10} -12.2/-9.7 (log[M])
	Sigmoidal curve	Sigmoidal curve	Sigmoidal curve
	IF PC_{AGO} > 6.4 (PC_{AGO}: DHT 1.0 x 10^{-8} M)	IF DHT 1.0 x 10^{-7} M > 20	IF PC_{AGO} ≥ 13 (PC_{AGO}: DHT 1.0 x 10^{-8} M)
	IF PC_{10} > 1 + 2SD (induction of VC)		IF PC_{10} > 1 + 2SD (induction of VC)
	CV < 20% in triplicate wells	|CV| logEC_{50} < 1.5%	
		ZF > 0.5	
Positive control	Mestanolone	17α -Methyltestosterone	Mestanolone
Criteria	Range logPC_{50} -10.15/-9.26 (log[M])	RI > 30%	Range logPC_{50} -10.2/-8.6 (log[M])
	Range logPC_{10} -10.92/-10.41 (log[M])		Range logPC_{10} -12.3/-9.8 (log[M])
	Sigmoidal curve		
	CV < 20% in triplicate wells		

Test method name	AR-EcoScreen™	AR-CALUX®	22Rv1/MMTV_GR-KO
Negative control	Di(2-ethylhexyl)phthalate (DEHP)	Corticosterone	Di(2-ethylhexyl)phthalate (DEHP)
Criteria	PC_{10} cannot be calculated	RI <10%	PC_{10} cannot be calculated
Specificity control (agonist)		NA	NA
Criteria	Confirmation by adding potent AR antagonist (1 µM HF) to clarify the non-AR mediated induction of luciferase.		

Table B.2b. Overview of reference standards and acceptability criteria for the three test methods for ANTAGONIST properties.

Test method name	AR-EcoScreen™	AR-CALUX®	22Rv1/MMTV_GR-KO
ANTAGONIST			
Reference chemical	Hydroxyflutamide (HF)	Flutamide (FLU)	Bicalutamide
Criteria	Range logIC_{50} -7.80/-6.17 (log[M])	IC_{50} range 1.0 x 10^{-7}/1.0 x 10^{-6}M	Range logIC_{50} -7.0/-5.8 (log[M])
	Range logIC_{30} -8.37/-6.41 (log[M])		Range logIC_{30} -7.5/-6.2 (log[M])
	RTA of PC_{ATG} < 46% (PC_{ATG}: 500 pM DHT + HF 1 µM))	InhF FLU 3.10^{-5}M > 10	RTA of PC_{ATG} ≤ 53.6 % (PC_{ATG}: 800 pM DHT + Bicalutamide 1 µM)
	Sigmoidal curve	Sigmoidal curve	Sigmoidal curve
	CV< 20% in triplicate wells	|CV| logIC_{50} < 3%	
		ZF > 0.5	
Positive control	Bisphenol A	Linuron	Bisphenol A
Criteria	Range log IC_{50} -7.05/-4.29 (log[M])	RI < 60%	Range log IC_{50} -6.2/-5.0 (log[M])
	Range logIC_{30} -7.52/-4.48 (log[M])		Range logIC_{30} -6.6/-5.4 (log[M])
	CV< 20% in triplicate wells		

Test method name	AR-EcoScreen™	AR-CALUX®	22Rv1/MMTV_GR-KO
Negative control	DEHP	Levonorgestrel	DEHP
Criteria	IC_{30} cannot be calculated	RI > 85%	IC_{30} cannot be calculated
Other control	IF AG_{ref} > 5 (AG_{ref}: 500 pM DHT)	NA	IF AG_{ref} ≥ 10 (AG_{ref}: 800 pM DHT)
Specificity control (antagonist)		DHT	DHT
Criteria	NA	R^2 test chemical ≤ 0.9	R^2 test chemical < 0.9
		R^2 FLU ≤ 0.7	

NA: not applicable

Note: 1) Different mathematical techniques are used in the three methods for the calculation of IC_{50}, IC_{30} (interpolation for AR-EcoScreen™ test method and 22Rv1/MMTV_GR-KO test method; curve fitting for AR-CALUX®test method); 2) Different spiking concentrations of DHT were used in the antagonist assay: 500 pM in AR-EcoScreen™ test method; 300 pM in AR-CALUX® test method; 800 pM in 22Rv1/MMTV_GR-KO test method.

Table B.3a. Overview of results from the three test methods in this TG. Chemicals were tested in two or three methods for AGONIST properties

		Expected outcome[1]		AR-EcoScreen™			AR-CALUX®			22Rv1/MMTV_GR-KO			Chemical Class[5]	Product Class[6]
Chemical Name	CASRN	Ref. (2003)	Ref. (2017)	Outcome Validation[2]	log PC_{10}[2] (M)	log PC_{50}[2] (M)	Outcome Validation[3]	log PC_{10}[3] (M)	log EC_{50}[3] (M)	Outcome Validation[3]	log PC_{10}[4] (M)	log PC_{50}[4] (M)		
5α-Dihydrotestosterone	521-18-6	P	P	P	-12.08/-9.87	-11.03/-9.00	P	-10.64/-10.14	-9.98/-9.42	P	-10.60/-9.83	-9.73/-8.95	Steroid, nonphenolic	Pharmaceutical
Mestanolone (Methyldihydrotestosterone)	521-11-9	P		P	-10.92/-10.41	-10.15/-9.26	P	-10.26/-9.99	-9.53/-9.39	P	-10.36/-9.66	-9.65/-8.39	Steroid, nonphenolic	Pharmaceutical
Testosterone	58-22-0	P	P	P	-10.42/-9.73	-9.46/-8.96	P	-9.81/-9.60	-9.25/-8.80	P	-10.28/-9.91	-9.67/-8.66	Steroid, nonphenolic	Pharmaceutical

17β-Estradiol	50-28-2	P		P	-7.74/-6.75	-5.34/-4.88	P	-6.70/-5.85	-	P	-8.76/-8.49	-7.19/-6.03	Steroid, phenolic	Pharmaceutical
Medroxyprogesterone 17-acetate	71-58-9	P	P	P	-9.64/-8.89	-8.77/-8.37	P	-9.91/-8.32	-9.23/-7.75	P	-8.77/-8.20	-7.64/-6.01	Steroid, nonphenolic	Pharmaceutical
17α-Ethinyl estradiol	57-63-6	N		N	-		N	-		P	-6.21/-5.27	-	Steroid, phenolic	Pharmaceutical
Butylbenzyl phthalate	85-68-7	N	N	N	-		N	-		N	-		Phthalate	Plasticiser
Di(2-ethylhexyl)phthalate	117-81-7	N		N	-		N	-		N	-		Phthalate	Chemical intermediate; Plasticiser
Hydroxyflutamide	52806-53-8	N		N	-		N	-		P	-5.54/-5.04	-	Anilide	Pharmaceutical metabolite
Bisphenol A	80-05-7	N		N	-		N	-		N	-		Bisphenol	Chemical intermediate
Methyl testosterone	58-18-4	P	P		NT	NT	P	-9.73/-9.57	-9.11/-8.95	P	-10.39/-9.99	-9.63/-9.28	Steroid, nonphenolic	Pharmaceutical
Progesterone	57-83-0	P			NT	NT	N			P	-7.13/-6.19	-5.50/-5.01	Steroid, nonphenolic	Pharmaceutical
Corticosterone	50-22-6	N			NT	NT	N			P	-7.16/-5.47	,	Steroid, nonphenolic	Pharmaceutical
Levonorgestrel	797-63-7	P	P		NT	NT	P	-9.42/-9.26	-8.91/-8.61	P	-10.28/-9.73	-9.06/-8.46	Steroid, nonphenolic	Pharmaceutical
Vinclozolin	50471-44-8	N			NT	NT	N	-		N	-		Organochlorine	Pesticide
Prochloraz	67747-09-5		N		NT	NT	N	-		N	-		Imidazole	Pesticide
Atrazine	1912-24-9	N	N		NT	NT	N	-		N	-		Triazine; Aromatic amine	Pesticide
6-Propyl-2-thiouracil	51-52-5	N			NT	NT	N	-		N	-		Pyrimidines	Pharmaceutical
o,p-DDT	789-02-6	N	N		NT	NT	N	-		N	-		Organochlorine	Pesticide
Bicalutamide	90357-06-5	N			NT	NT	N	-		N	-		Anilide	Pharmaceutical
Linuron	330-55-2	P			NT	NT	N	-		N	-		Urea	Pesticide

Table B.3b. Overview of results from the three test methods in this TG. Chemicals were tested in two or three methods for ANTAGONIST properties

		Expected outcome[1]		AR-EcoScreen™			AR-CALUX®			22Rv1/MMTV_GR-KO			Chemical Class [5]	Product Class [6]
Chemical Name	CASRN	Ref. (2003)	Ref. (2017)	Outcome validation[1]	log IC_{30} [2] (M)	log IC_{50} [2] (M)	Outcome validation[3]	log PC_{80} [3] (M)	log IC_{50} [3] (M)	Outcome validation[4]	log IC_{30} [4] (M)	log IC_{50} [4] (M)		
Hydroxyflutamide	52806-53-8	P	P	P	-8.37/ -6.41	-7.80/-6.17	P	-8.63/-8.01	-7.80/-7.54	P	-8.17/-7.45	-7.79/-7.11	Anilide	Pharmaceutical metabolite

Bisphenol A	80-05-7	P	P	P	-7.52/ -4.48	-7.05/-4.29	P	-6.75/-6.12	-5.93/-5.81	P	-5.92/-5.56	-5.68/-5.29	Bisphenol	Chemical intermediate
Flutamide	13311-84-7	P		P	-6.20/ -5.69	-5.66/-5.43	P	-7.51/-6.71	-6.60/-6.23	P	-7.11/-6.62	-6.70/-6.26	Anilide	Pharmaceutical
Prochloraz	67747-09-5	P	P	P	-5.77/ -5.47	-5.44/-5.12	P	-6.42/-6.02	-5.78/-5.59	P	-6.02/-5.30	-5.47/-4.95	Imidazole	Pesticide
Vinclozolin	50471-44-8	P	P	P	-6.83/ -6.32	-6.47/-5.85	P	-7.91/-7.00	-7.50/-6.75	P	-7.22/-6.74	-6.94/-6.44	Organochlorine	Pesticide
5α-Dihydrotestosterone	521-18-6	N		N	-		N	-		N	-	-	Steroid, nonphenolic	Pharmaceutical
Mestanolone	521-11-9	N		N	-		N	-		N	-	-	Steroid, nonphenolic	Pharmaceutical
Di(2-ethylhexyl)phthalate	117-81-7	N		N	-		N	-		N	-	-	Phthalate	Chemical intermediate; Plasticiser
Atrazine	1912-24-9	N	N	N	-		N	-		N	-	-	Triazine; Aromatic amine	Pesticide
6-Propyl-2-thiouracil	51-52-5	N		N	-		N	-		N	-	-	Pyrimidines	Pharmaceutical
17β-Estradiol	50-28-2	P			NT	NT	P	-9.05/-8.04	-8.40/-7.64	P	-7.98/-7.20	-	Steroid, phenolic	Pharmaceutical
17α-Ethinyl estradiol	57-63-6	N			NT	NT	P	-8.42/-7.75	-7.57/-7.26	P	-7.91/-7.29	-7.54/-6.88	Steroid, phenolic	Pharmaceutical
Butylbenzyl phthalate	85-68-7	N			NT	NT	P	-6.13/-5.46	-5.81/-5.11	P	-5.10/-4.57	-4.86/-4.28	Phthalate	Plasticiser
Progesterone	57-83-0	P			NT	NT	P	-8.78/-8.57	-8.07/-8.03	P	-7.40/-6.30	-6.88/-5.97	Steroid, nonphenolic	Pharmaceutical
Corticosterone	50-22-6	N			NT	NT	P	-6.85/-6.77	-6.35/-6.33	P	-6.36/-6.11	-5.91-5.51	Steroid, nonphenolic	Pharmaceutical
o,p-DDT	789-02-6	P	P		NT	NT	P	-7.33/-6.84	-6.36/-6.24	P	-5.82/-5.48	-5.56/-5.21	Organochlorine	Pesticide
Bicalutamide	90357-06-5	P	P		NT	NT	P	-8.18/-7.19	-7.23/-6.69	P	-6.92/-6.37	-6.39/-6.10	Anilide	Pharmaceutical
Linuron	330-55-2	P	P		NT	NT	P	-6.64/-6.38	-5.85/-5.70	P	-5.64/-5.33	-5.33/-5.11	Urea	Pesticide
Medroxyprogesterone 17-acetate	71-58-9	N			NT	NT	N	-		N	-		Steroid, nonphenolic	Pharmaceutical
Levonorgestrel	797-63-7	N			NT	NT	N	-		N	-		Steroid, nonphenolic	Pharmaceutical

Abbreviations for Tables B.3a and B.3b: M: molar, P: Positive, N: Negative, NT: not tested

[1]Expected outcome: Classifications reported by the ICCVAM evaluation of 2003 (5) and the ICCVAM AR-reference list of 2017 (12). The 2017 reference included additional criteria for independent confirmation of reference chemical activity in at least two assays (positive) or lack of activity in at least two assays and absence of positive activity (negative) activity. Thus, some chemicals identified in the 2003 reference did not have sufficient data to meet these criteria and were excluded for the later reference.

[2]Validation report of the AR-EcoScreen™ method (minimal/maximal values of all valid runs of all participating labs).

[3]Validation report of the AR-CALUX® method (minimal/maximal values of all valid runs of all participating labs).

[4]Validation report of the 22Rv1/MMTV_GR-KO method (minimal/maximal values of all valid runs of all participating labs).
[5]Chemicals 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).
[6]Chemicals 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 B.3a. List of proficiency chemicals to demonstrate technical proficiency with each of the three methods in this test guideline (AGONIST assay).

			AR-EcoScreen™			AR-CALUX®			22Rv1/MMTV_GR-KO			Chemical Class [5]	Product Class [6]
Chemical Name	CASRN	AR ref. list [1]	Class[2]	log PC_{10}[2] (M)	log PC_{50}[2] (M)	Class[3]	log PC_{10}[3] (M)	log EC_{50}[3] (M)	Class[4]	log PC_{10}[4] (M)	log PC_{50}[4] (M)		
5α-Dihydrotestosterone	521-18-6	P	P	-12.08/ -9.87	-11.03/ -9.00	P	-10.64/-10.14	-9.98/-9.42	P	-10.60/-9.83	-9.73/-8.95	Steroid, nonphenolic	Pharmaceutical
Mestanolone (Methyldihydrotestosterone)	521-11-9		P	-10.92/ -10.41	-10.15/-9.26	P	-10.26/-9.99	-9.53/-9.39	P	-10.36/-9.66	-9.65/-8.39	Steroid, nonphenolic	Pharmaceutical
Testosterone	58-22-0	P	P	-10.42/ -9.73	-9.46/-8.96	P	-9.81/-9.60	-9.25/-8.80	P	-10.28/-9.91	-9.67/-8.66	Steroid, nonphenolic	Pharmaceutical
17β-Estradiol	50-28-2		P	-7.74/ -6.75	-5.34/-4.88	P	-6.70/-5.85	-	P	-8.76/-8.49	-7.19/-6.03	Steroid, phenolic	Pharmaceutical
Medroxyprogesterone 17-acetate	71-58-9	P	P	-9.64/-8.89	-8.77/-8.37	P	-9.91/-8.32	-9.23/-7.75	P	-8.77/-8.20	-7.64/-6.01	Steroid, nonphenolic	Pharmaceutical
Butylbenzyl phthalate	85-68-7	N	N	-		N	-		N	-		Phthalate	Plasticiser
Di(2-ethylhexyl)phthalate	117-81-7		N	-			-		N	-		Phthalate	Chemical intermediate; Plasticiser
Bisphenol A	80-05-7		N	-		N	-		N	-		Bisphenol	Chemical intermediate

Abbreviations: M: molar, P: Positive, N: Negative

[1]ICCVAM AR-reference list (2017) (12).

[2]Validation report of the AR-EcoScreen™ method (minimal/maximal values of all valid runs of all participating labs).

[3]Validation study report of the AR-CALUX® method (minimal and maximal values of all valid runs of all participating labs.

[4]Validation report of the 22Rv1/MMTV_GR-KO method (minimal/maximal values of all valid runs of all participating labs).

[5]Chemicals 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).

[6]Chemicals 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 B.4b. List of proficiency chemicals to demonstrate technical proficiency with each of the three test methods of this TG (ANTAGONIST assay).

			AR-EcoScreen™			AR-CALUX®			22Rv1/MMTV_GR-KO			Chemical Class [5]	Product Class [6]
Chemical Name	CASRN	AR ref. list [1]	Class[2]	log IC_{30} [2] (M)	log IC_{50} [2] (M)	Class[3]	log PC_{80}[3] (M)	log IC_{50} [3] (M)	Class[4]	log IC_{30} [4] (M)	log IC_{50} [4] (M)		
Hydroxyflutamide	52806-53-8		P	-8.37/-6.41	-7.80/-6.17	P	-8.63/-8.01	-7.80/-7.54	P	-8.17/-7.45	-7.79/-7.11	Anilide	Pharmaceutical metabolite
Bisphenol A	80-05-7		P	-7.52/-4.48	-7.05/-4.29	P	-6.75/-6.12	-5.93/-5.81	P	-5.92/-5.56	-5.68/-5.29	Bisphenol	Chemical intermediate
Flutamide	13311-84-7		P	-6.20/-5.69	-5.66/-5.43	P	-7.51/-6.71	-6.60/-6.23	P	-7.11/-6.62	-6.70/-6.26	Anilide	Pharmaceutical
Prochloraz	67747-09-5		P	-5.77/-5.47	-5.44/-5.12	P	-6.42/-6.02	-5.78/-5.59	P	-6.02/-5.30	-5.47/-4.95	Imidazole	Pesticide
Vinclozolin	50471-44-8		P	-6.83/-6.32	-6.47/-5.85	P	-7.91/-7.00	-7.50/-6.75	P	-7.22/-6.74	-6.94/-6.44	Organochlorine	Pesticide
Mestanolone	521-11-9		N	-		N	-		N	-		Steroid, nonphenolic	Pharmaceutical
Di(2-ethylhexyl)phthalate	117-81-7		N	-		N	-		N	-		Phthalate	Chemical intermediate; Plasticiser
Atrazine	1912-24-9		N	-		N	-		N	-		Triazine; Aromatic amine	Pesticide
6-Propyl-2-thiouracil	51-52-5		N	-		N	-		N	-		Pyrimidines	Pharmaceutical

Table B.4c. List of 2 proficiency chemicals to test the specificity of the antagonist (for the AR-CALUX® method only).

			AR-CALUX®			
Chemical Name	CAS RN.	AR ref. list[1]	Class[3]	Observation in the specificity control test when testing with a lower and higher concentration of ligand DHT	Chemical Class [5]	Product Class [6]
Ketoconazole	65277-42-1		N	Two dose responses with a shift, $R^2 \leq 0.9$	Piperazine	Pharmaceutical, Antifungal

Cycloheximide	66-81-9		N	Two dose responses with a shift , $R^2 \leq 0.9$	Piperidone	Pharmaceutical, Fungicide

Abbreviations for Tables B.4b and B.4c: M: molar, P: Positive, N: Negative

[1]ICCVAM AR-reference list (2017) (12).

[2]Validation report of the AR-EcoScreen™ method (minimal/maximal values of all valid runs of all participating labs).

[3]Validation study report of the AR-CALUX® method (minimal and maximal values of all valid runs of all participating labs.

[4]Validation report of the 22Rv1/MMTV_GR-KO method (minimal/maximal values of all valid runs of all participating labs).

[5]Chemicals 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).

[6]Chemicals 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).

Test report

1. The following template should be used for all three test methods. The result section is test method specific.
2. The test report should include the following information:

General information:

- Name and address of the sponsor, test facility and study director;
- Reference to TG 458 and to the test method;
- Reference to the solubility method.

Demonstration of proficiency:

- Statement that the testing facility has demonstrated proficiency in the use of the test method before routine use by testing of the proficiency chemicals

Reference standards (reference chemical, positive and negative control) and test chemical:

- Source, batch/lot number, expiry date, CAS number if available;
- Purity and chemical identity of impurities as appropriate and practically feasible;
- Physical appearance, water solubility, molecular weight and additional relevant physicochemical properties to the extent available;
- Treatment prior to testing if applicable (e.g. warming);
- Storage conditions and stability to the extent available;
- Choice of solvent/vehicle for each test chemical and justification.

Mono-constituent chemical:

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.

Multi-constituent chemical, UVCBs and mixtures:

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

Solvent/vehicle:

e.g DMSO, water, ethanol;
- Source, batch/lot number;
- Justification for choice of solvent/vehicle;

Test method conditions:

- Cell line used, its source, storage and maintenance conditions, passage number and level of confluence of cells used for testing;
- Cell counting method used for seeding prior to testing and measures taken to ensure

homogeneous cell number distribution;
- Luminometer used (e.g. model), including instrument settings. Luciferase substrate used (product name, supplier, lot);
- Type of plates and their supplier and code;
- Application procedure and exposure time as specified in the protocol;
- List of the acceptability criteria to be met;
- Description of any modification of the test procedure;
- Reference to the cytotoxicity procedure.

Results obtained with the AR-EcoScreen™ test method:

Tabulation of the following results for the reference standards;

- For all reference standards: normalized data of luminescent signals; results of the application of the acceptability criteria;
- Measure of error (e.g. SD, % CV or 95% confidence interval)

Tabulation of the following results for the test chemicals;

- Solubility data and stability if known;
- Measurement of precipitate in the culture medium to which the test chemical was added, as appropriate;
- For each run:
  - Cytotoxicity data;
  - Concentrations tested;
  - Normalized data of luminescent signals and a measure of error (e.g. SD, % CV or 95% confidence interval)
  - Agonist testing: PC_{max}, log PC_{10}, log PC_{50}, EC_{50} values if appropriate, the maximum fold induction level;
  - Antagonist testing: log IC_{30}, log IC_{50}, the maximum fold inhibition level;
  - The result (positive or negative) per chemical after application of the decision criteria.
  - The conclusion (positive or negative) per chemical (based on the result of two or three runs)
- Number of runs performed;
- Graphs depicting the concentration-response relationship of reference chemicals and test chemicals;
- Description of any other relevant observation.

Results obtained with the AR-CALUX® test method:

Tabulation of the following results for the reference standards;

- For all reference standards: normalized data of luminescent signals; results of the application of the acceptability criteria;
- In addition, for the reference chemicals: EC_{10} and EC_{50} values for DHT, IC_{20} and IC_{50} values for Flutamide;
- Measure of error e.g. coefficient of variation

Tabulation of the following results for the test chemicals;

- Solubility data and stability if known;
- Measurement of precipitate in the culture medium to which the test chemical was added, as

appropriate;

- For the pre-screen run:
  - Cytotoxicity data (LDH leakage and microscopy observations);
  - Concentrations tested;
    - Normalized data of luminescent signals and a measure of error (e.g. SD, % CV or 95% confidence interval);
  - For the comprehensive run:
    - Cytotoxicity data (microscopy observations);
    - Concentrations tested, including dilution factors;
    - Normalized data of luminescent signals and a measure of error (e.g. SD, % CV or 95% confidence interval);
    - Agonist testing: RPCmax, PCmax, EC10, EC50, PC10, PC50, |CV| of logEC50, if appropriate
    - Antagonist testing: RPCmin, PCmin, IC20, IC50, PC80, PC50, |CV| of logIC50, if appropriate
    - Specificity control: R2
    - The result (positive or negative) per chemical after application of the decision criteria (based on one comprehensive run).
  - Graphs depicting the concentration-response relationship of reference chemicals and test chemicals; including graphs for the specificity control;
  - Description of any other relevant observation.

Results obtained with the 22Rv1/MMTV_GR-KO test method:

Tabulation of the following results for the reference standards;

- For all reference standards: normalised data of luminescent signals; results of the application of the acceptability criteria;
- Measure of error (e.g. SD, % CV or 95% confidence interval)

Tabulation of the following results for the test chemicals;

- Solubility data and stability if known;
- Measurement of precipitate in the culture medium to which the test chemical was added, as appropriate;
- For each pre-screen run and each comprehensive run:
- Cytotoxicity data;
- Concentrations tested, including dilution factors;
- Normalized data of luminescent signals and a measure of error e.g. coefficient of variation;
- Agonist testing: log PC_{10}, log PC_{50}, the maximum fold induction level;
- Antagonist testing: log IC_{30}, log IC_{50}, the maximum fold inhibition level;
- Specificity control: R^2
- The result (positive or negative) per chemical after application of the decision criteria.
- The conclusion (positive or negative) per chemical (based on the result of two or three pre-screen runs, or, two or three comprehensive runs).
- Number of runs performed (pre-screen and comprehensive testing);
- Graphs depicting the concentration-response relationship of reference chemicals and test chemicals; including graphs for the specificity control;
- Description of any other relevant observation.

Discussion of the results

Conclusion

Annex C. (Method 1) Androgen Receptor TransActivation Assay for Detection of Androgenic Agonist and Antagonist Activity of Chemicals using the stably transfected human AR-EcoScreen™ cell line

Initial Considerations and Limitations

1. The “General Introduction” should be read before using this test method (Main body page 6-9).

2. The AR-EcoScreen™ method uses the AR-EcoScreen™ cell line to detect (anti)androgenic activity. This method exclusively addresses transactivation 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.

3. This test method is specifically designed to detect human AR-mediated transactivation and inhibition by measuring luciferase activity as the endpoint. A high-throughput assay design can be achieved by using PC values and fixed-dose format. However, chemical-dependent interference with luminescence signals are known to occur due to over-activation or inhibition of the luciferase reporter gene assay system (1) (2) (3). It is therefore possible that such interference with the luciferase reporter gene may also occur in the AR-EcoScreen™ luciferase assay systems. This should be considered when evaluating the data.

4. 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 (4) (5). In certain cases, this may result in chemicals that activate GR being classified positive in the test method. 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 Appendix 1).

5. The test method was validated using single chemicals, therefore the applicability to test mixtures has not been addressed. The test method is nevertheless theoretically applicable to the testing of multi-constituent chemicals 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.

6. Definitions and abbreviations used in this test method are described in Annex A of this TG.

7. The AR-EcoScreen™ 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 (6).

8. The previous (2016) draft of TG 458 indicated the AR EcoScreen™ test method was run with in the antagonist assay with 0.1 µM HF. This has been updated to 1.0 µM HF (see Table B.2b) to increase the sensitivity of the assay and align with concentrations used during the validation of the antagonist assay. Results of previous studies conducted using 0.1 µM HF are reliable if performance criteria were met.

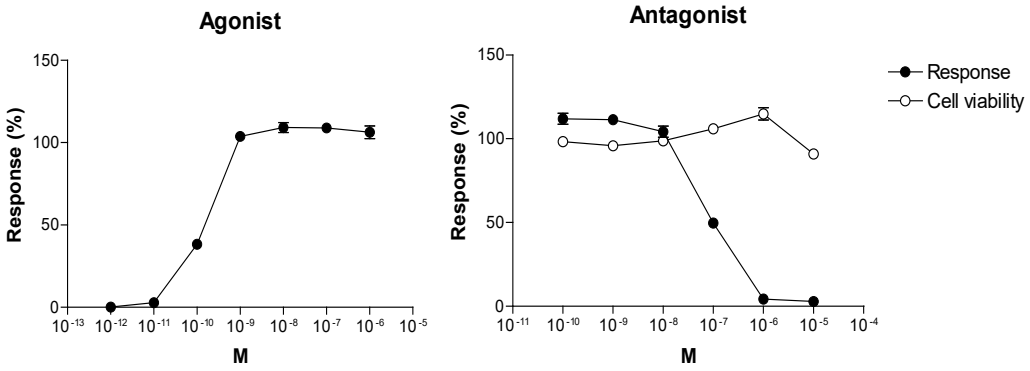
Principle of the Test Method

9. The test method using a reporter gene technique is an *in vitro* tool that provides mechanistic data. The test method is used to establish signal activation or blocking of the AR caused by a ligand. 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 Annex utilises the AR-EcoScreen™ 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 reporter 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) for cell viability assessment, 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. The two enzyme activities can be measured simultaneously in the same cell and in the same well. This feature facilitates the detection of the antagonist (7) (8). AR-Ecoscreen GR KO M1(JCRB1761), a modified cell line with the GR KO via genome editing and not yet officially validated, is available from JCRB for more stringent investigation of AR-mediated antagonism.(9).

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 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 48-60. Typical representations of the agonist and antagonist reference chemical curves (DHT and HF) are shown in Figure C.1.

Figure C.1. Typical positive control responses



Demonstration of Laboratory Proficiency

12. Prior to testing chemicals with unknown activity in the AR EcoScreen™, the responsiveness of the test system should be confirmed by each laboratory to yield the expected results, at least once for each newly prepared batch of cell stocks taken from the frozen stock. This is done by independently testing the proficiency chemicals listed in the Tables B.4a for agonism and B.4b for antagonism of Annex B in this TG. This should be done at least in duplicate, on different days, and the results should be consistent to the classifications and values of the Tables B.4a and B.4b in Annex B and any deviations should be justified. However, the proficiency substances are classified in Tables B.2a and B.2b by their known predominant activity which should be used for proficiency evaluation.

Procedure

Cell lines

13. 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 reference No. JCRB1328, upon signing a Material Transfer Agreement (MTA) including license agreement.

14. Only cells characterised as mycoplasma-free should be used in testing. PCR based methods are recommended for a sensitive detection of mycoplasma infection (10) (11) (12).

Stability of the cell line

15. 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 C.1b and the plate concentration assignment shown in Table C.a, 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 C.1a and C.1b.

16. 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 C.1d and the plate concentration assignment shown in Table C.2b, 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 C.1c and C.1d.

Cell culture and plating conditions

17. 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).

18. 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 × 10^5 cell/mL in φ100 mm 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 microtiter plate containing 90 µL/well at a density of 1.0 × 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.

19. 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. The AR-EcoScreen™ cell line will be stable up to three months under suitable culture condition.

20. 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

21. 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 Tables C.1b and C.1d, with DHT and Mestanolone as the positive reference standards for the agonist assay, HF and BPA as the positive reference standards for the antagonist assay, and DEHP as the negative reference standard for the agonist and antagonist assay. Acceptable range values derived from the validation study are also given in Table C.1b and Table C.1c (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, quality of serum and antibiotics, concentration, etc.) 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 the use of materials for cell culturing is consistent.

22. 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 C.1b and C.1c) could be allowed if the quality criteria 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 C.1b and C.1c and the

concentration-response curve of the positive reference standards (reference chemical and positive control) should be sigmoidal.

Table C.1a. Quality criteria for AR agonist assay

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

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

Induction fold of PC_{AGO} is calculated by the following equation:

$$\text{Induction fold of } PC_{AGO} = \frac{\text{Mean RLU of } PC_{AGO} \text{ (10 nM DHT)}}{\text{Mean RLU of VC}}$$

RLU: Relative Light Units

Table C.1b. Acceptable range of the reference standards for AR agonist assay

Chemical 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	1.0×10^{-12} ~ 1.0×10^{-6}M
Mestanolone[521-11-9]	Positive: PC_{10} should be calculated	-10.92 ~-10.41	-10.15 ~ -9.26	1.0×10^{-12} ~ 1.0×10^{-6}M
Di(2-ethylhexyl)phthalate (DEHP) [117-81-7]	Negative: PC_{10} should not be calculated	-	-	1.0×10^{-11} ~ 1.0×10^{-5}M

Table C.1c. Quality criteria for AR antagonist assay

Induction fold of AG ref	≥ 5.0
RTA of PC_{ATG} (%)	≤46

AG ref = Agonist reference (500 pM DHT) in the antagonist assay
RTA : Relative Transcriptional Activity
PC_{ATG} = AR Antagonist control (500pM DHT, 1 μM HF)

Induction fold of AG ref is calculated by the following equation:

$$\text{Induction fold of AG ref} = \frac{\text{Mean RLU of AG ref (500 pM DHT)}}{\text{Mean RLU of VC}}$$

VC: Vehicle Control, RLU: Relative Light Units

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 C.1d. Acceptable range of the reference standards for AR antagonist assay

Chemical 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	1.0×10^{-10} ~ 1.0×10^{-5}M
Bisphenol A (BPA) [80-05-7]	Positive: IC30 should be calculated	-7.52 ~ -4.48	-7.05 ~ -4.29	1.0×10^{-10} ~ 1.0×10^{-5}M
Di(2-ethylhexyl)phthalate (DEHP) [117-81-7]	Negative: IC30 should not be calculated	-	-	1.0×10^{-10} ~ 1.0×10^{-5}M

Vehicle control, AR agonist control and AR antagonist control

23. **For the agonist assay**, AR agonist 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 C.2a and Table C.3b.

24. **For the antagonist assay**, vehicle control (n=3), AR agonist control (PC_{AGO}, 10 nM of DHT, n=3), AR antagonist control control (PC_{ATG}, 500 pM DHT and 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 C.2b and Table C.3b.

Quality criteria for AR agonist assay

25. The mean luciferase activity of the PC_{AGO} (10 nM DHT) should be equal to or higher than 6.4-fold compared with 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.

26. With respect to the quality control of the assay, the induction fold corresponding to the log PC_{10} (10%) of the AR agonist control (PC_{AGO}: 10 nM of DHT) (IF PC_{10}) should be greater than 1+2SD of the induction value (=1) of the concurrent VC.

Quality criteria for AR antagonist assay

27. The mean luciferase activity of the AG ref (500 pM DHT) should be equal to or higher than 5.0-fold compared with the mean VC on each plate for antagonism assay. These criteria were established based on the reliability of the endpoint values from the validation study.

28. RTA of PC_{ATG} (500 pM DHT and 1 µM HF) should be less than 46%.

In summary:

29. Acceptability criteria are the following:

For AR agonist assay:

- The mean luciferase activity of the PC_{AGO} (10 nM DHT) should be equal to or higher than6.4-fold compared with the mean VC on each plate.
- The induction fold corresponding to the log PC_{10} value of the concurrent PC_{AGO} (10 nM DHT)

should be greater than 1+2SD of the induction fold 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 C.1b).

For AR antagonist assay:

- Induction fold of AG ref ([500 pM DHT]/[Vehicle Control]) should be equal to or higher than 5.0 compared with the mean VC on each plate.
- 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 C.1d).

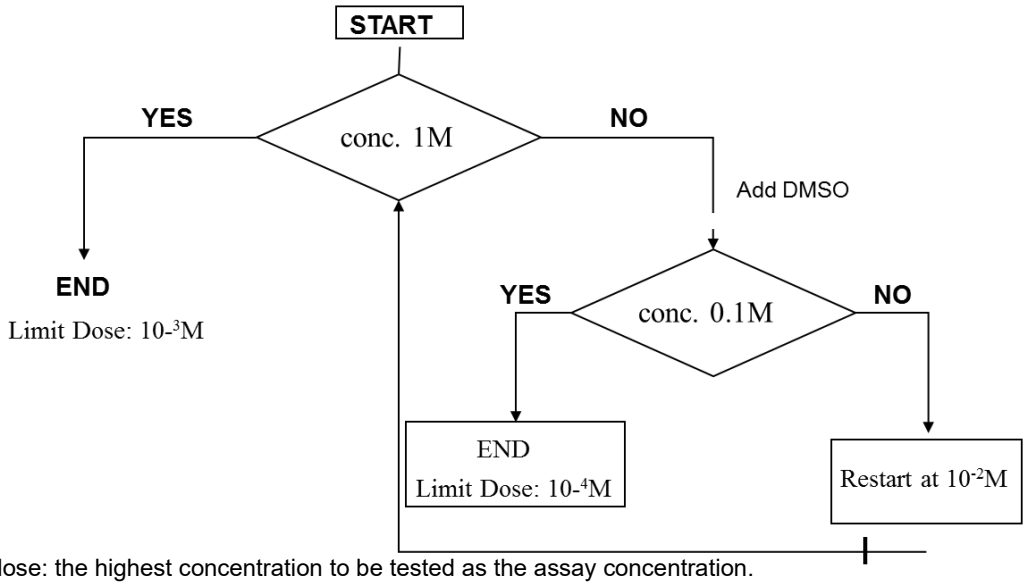
Vehicle

30. 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 concentration 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

31. The test chemicals should be dissolved in an appropriate solvent (see paragraph 30) 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 C.2.

Figure C.2. Diagram for solubility test



Limit dose: the highest concentration to be tested as the assay concentration.

YES: No precipitation, NO: Precipitation

32. 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 a medium for the assay plate. Precipitation observed at any concentration should be noted, but these concentrations should not be included in the dose-response analysis.

Cytotoxicity evaluation

33. 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™ 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.

34. 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;

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

Test chemical exposure and assay plate organisation

35. For the AR agonist assay, each test chemical should be serially diluted in DMSO or appropriate solvent, by using a single column of polypropylene plate or other appropriate item, and added to the wells of a microtiter plate to achieve final serial concentrations in the assay, from the maximum concentration determined by the solubility test with common dilution ratio of 10 (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.

36. 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.

37. Reference standards and test samples can be assigned as shown in Table C.2a and Table C.3a.

Table C.2a Example of plate concentration assignment of the reference standards in the assay plate for the 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}: AR agonist control (10 nM of DHT);
PC_{CT}: Cytotoxicity control (10 µg/mL of Cycloheximide);
#: concentration of test chemical is an example

38. For the AR antagonist assay, each test chemical should be serially diluted in DMSO or appropriate solvent by using a single column of polypropylene plate or other appropriate item, and added to the wells of a microtiter plate to achieve final serial concentrations in the assay, from the maximum concentration determined by the solubility test with common dilution ratio of 10 (for example 1 mM, 100 µM, 10 µM, 1 µM, 100 nM, and 10 nM [1.0×10^{-3}-1.0×10^{-8} M]) for triplicate testing.

39. 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.

40. Reference standards and test samples can be assigned as shown in Table C.2b and Table C.3b.

Table C.2b. Example of plate concentration assignment of the reference standards in the assay plate for the 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}: AR agonist control (10 nM of DHT);
AG ref: AR agonist reference (DMSO)
PC_{ATG}: AR antagonist control (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

41. 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 Tables C.2a and C.2b). Wells treated with 10 nM of DHT (PC_{AGO}), 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 with 10 µg/mL of Cycloheximide (PC_{CT}) (Table C.3a). In the case of the antagonist assay, a AR agonist control with 10 nM of DHT (PC_{AGO}), an AR agonist reference with DMSO (or appropriate solvent) and spiked 500 pM DHT(AG ref), a AR antagonist control with 1 µM of HF and spiked 500 pM DHT (PC_{ATG}) and cytotoxicity control with 10 µg/mL of Cycloheximide (PC_{CT}) should be included in each assay plate (Table C.3b). 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 C.3a. Example of plate concentration assignment of test chemicals 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_{AGO}	→	→	→	PC_{CT}	→	→	→

VC: Vehicle control (DMSO);
PC_{AGO}: 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 C.3b. Example of plate concentration assignment of test chemicals 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	AG ref	→	→	→	→	→	→	→	→	→	→	→
H	VC	→	→	PC_{AGO}	→	→	PC_{ATG}	→	→	PC_{CT}	→	→

VC: Vehicle control (DMSO);
PC_{AGO}: AR agonist control (10 nM of DHT);
AG ref: AR agonist reference (DMSO)
PC_{ATG}-: AR antagonist control (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.

43. 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.

44. 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.

45. Special considerations will need to be applied to those chemicals 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.

46. 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 to avoid the effects of location of chemical.

Luciferase activity measurements

47. 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 should be followed according to the manufacturer’s instructions with the following modifications For instance, when using Dual-Glo Luciferase Assay system (Promega, E2920), 60 µL of supernatant should be removed from a well of assay plate before adding the substrate, then 40 µL of the first substrate should be directly added into the assay wells and measure the firefly luciferase signal. 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), 40 µL of prepared reagent should be directly added into the assay wells. When using a standard luciferase assay system (Promega, E1500, or equivalents), the substrate should be added after adding the Cell Culture Lysis Reagent (Promega, E1531, or equivalents).

Analysis of Data

48. **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 paragraphs 51-60).

49. **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%).

50. 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 paragraphs 51-60).

Calculation of parameters: EC_{50}, log PC_{50}, log PC_{10}, log IC_{50} and log IC_{30} induction considerations

51. The full concentration-response curve is required for the calculation of the EC_{50}, 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 EC_{50} 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 EC_{50} and maximum induction level, appropriate statistical software should be used (e.g. Graphpad Prism statistical software).

52. If the Hill's logistic equation is applicable to the concentration response data, the EC_{50} should be calculated by the following equation (13):

- $Y=Bottom + (Top-Bottom) / (1+10 \exp ((\log EC_{50} -X) \times Hill\ slope))$
- 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.

53. 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 34.

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

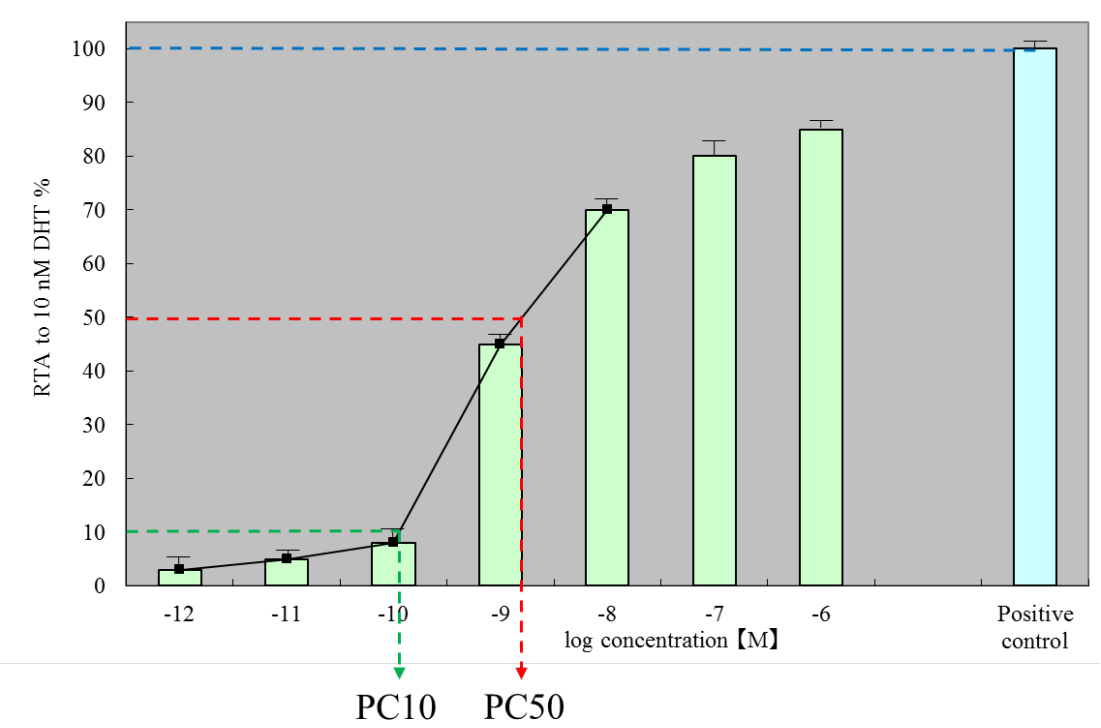
(i) The maximum level of response induced by a test chemical, expressed as a percentage against the response induced by PC_{AGO}(10 nM DHT) on the same plate (RPC_{max}).

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

55. Descriptions of log PC_x values, "x" is a selected response like 10% or 50% induction compared to PC_{AGO}, are provided in Figure C.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} (AR agonist 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 C.3:

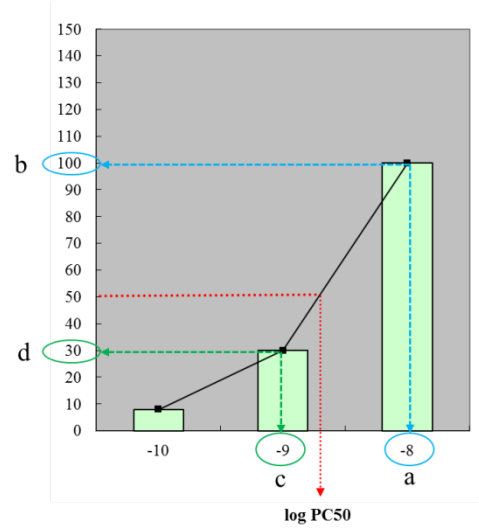
$$\log[PC_x] = c+[(x-d)/(b-d)](a-c)$$

Figure C.3. Schematic descriptions of log PC_x values



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

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

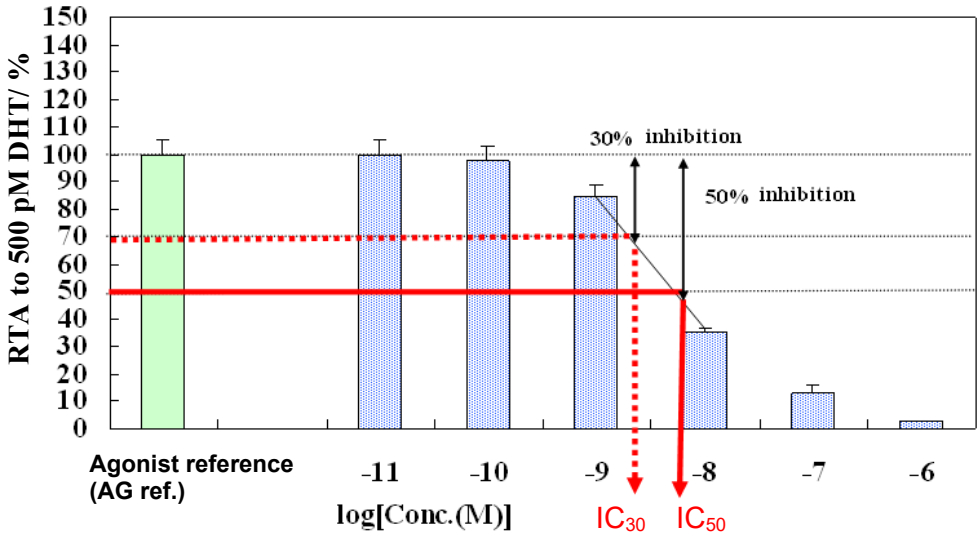


56. In the case of the antagonist assay, the following information should be provided for each positive test chemical: the concentrations of 30% inhibition of transcriptional activity induced by 500 pM DHT (log IC30) and, if appropriate, to 50% inhibition of activity of 500 pM DHT (log IC50).

57. Descriptions of log IC_x values, "x" is a selected response like 30% or 50% inhibition compared to DHT controls, are provided in Figure C.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 C.5:

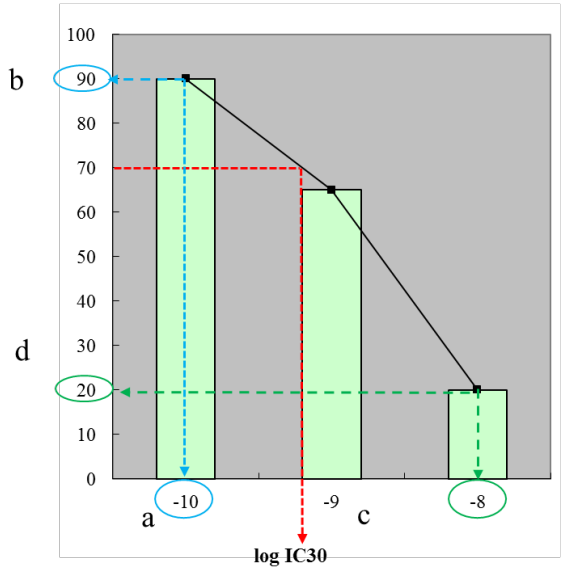
$$\log [IC_x] = a-[(b-(100-x)) /(b-d)] (a-c)$$

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



58. 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.

59. 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 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.

60. 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 21-29)
- Be reproducible in triplicate wells (CV<20%).

Data Interpretation Criteria

61. **For the agonist assay,** data interpretation criteria are shown in Table C.4a. 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 reference chemical response 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 reference chemical response in two of two or two of three runs.

Table C.4a. 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.
Negative	If a RPC_{max} fails to achieve at least 10% of the response of the positive control.

62. **For the antagonist assay,** data interpretation criteria are shown in Table C.4b. 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 C.4b. Positive and negative decision criteria for antagonist assay

Positive	If the log IC_{30} is calculated.
Negative	If the log IC_{30} cannot be calculated.

63. 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.

64. 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.

65. 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.

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Appendix C.1

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

Data interpretation criteria

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

- If the agonistic activity of the chemical is NOT affected by the treatment with HF, it is classified as “Negative”.
- If the agonistic activity of the chemical is inhibited, apply the decision criteria (Table C.5a).
- 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)

Data analysis

4. Check the performance standard.

5. Check the CV between wells treated under the same conditions.

   a Calculate the mean of the VC
   b Subtract the mean of VC from each well value **not** treated with HF
   c Calculate the mean of HF
   d Subtract the mean of the VC from each well value treated with HF
   e Calculate the mean of the PC_{AGO}
   f Calculate the relative transcriptional activity of all other wells relative to the PC_{AGO}

Annex D. (Method 2): Androgen Receptor TransActivation Assay for Detection of Androgenic Agonist and Antagonist Activity of Chemicals using the stably transfected human AR-CALUX® cell line1

Initial Considerations and Limitations

1. The “General Introduction” should be read before using this test method (Main body page 6-9).

2. The AR-CALUX®(*) transactivation assay uses the human osteosarcoma U2OS AR-CALUX® cell line to detect (anti)androgenic activity mediated through a human androgen receptor (hAR). The AR-CALUX® cell line expresses stably transfected hAR and has no or little expression of other steroid hormone receptors (1).

3. This test method is specifically designed to detect AR-mediated transactivation by measuring bioluminescence as the endpoint. Bioluminescence is commonly used as a read out in various bioassays because of the high signal-to-noise ratio (2). Chemical dependent interference with luminescence signals are reported for certain luc-transformed cell lines but were not observed with the CALUX cell lines.

4. The cell line has low metabolic activity. By combining the test method with a S9 fraction, the impact of metabolism on test chemical activity can be studied (3) and is currently (2020) being validated.

5. The test method has been used for high throughput screening purposes (4). It did not undergo a validation according to the OECD Guidance Document 34.

6. The test method is theoretically applicable to the testing of multi-constituent chemicals and mixtures. During the validation study of this test method single test chemicals were mainly used. When considering testing of mixtures or difficult-to-test chemicals (e.g. unstable) upfront consideration should be given to whether the results of such testing will yield results that are scientifically meaningful.

7. The validation study of the AR-CALUX® test method demonstrated the reliability and relevance of the assay for its intended purpose (5). The test method protocol is described in the referenced document (6).

8. Definitions and abbreviations used in this test method are described in Annex A of this TG.

[1] Note (*): "CALUX" is a registered trademark, owned by Abraham Brouwer. BioDetection Systems BV (BDS) has obtained the exclusive global right to use and sublicense this trademark.

Principle of the Test Method

9. The test method is used to assess the transactivation of a reporter gene. The AR, when bound to a ligand, is translocated to the nucleus. In the nucleus, the receptor-ligand complex binds specific DNA sequences (androgen responsive elements: AREs) and transactivates a firefly luciferase reporter gene, resulting in increased cellular expression of the luciferase enzyme. Following the addition and subsequent catalytic oxidation of the substrate luciferin, light is emitted. The light produced can easily be detected and quantified using a luminometer.

10. The test system utilises stably transfected AR-CALUX® cells. AR-CALUX® cells originated from the human osteoblastic osteosarcoma U2OS cell line. Human U2OS cells were stably transfected with 3xARE-TATA-Luc and pSG5-neo-hAR using the calcium phosphate co-precipitation method. The U2OS cell line was selected as the good candidate to serve as the androgen - (and other steroid hormone) responsive reporter cell line, based on the observation that the U2OS cell line showed little or no endogenous receptor activity. The absence of endogenous receptors was assessed using luciferase reporter plasmids only, showing no activity when receptor ligands were added. Furthermore, this cell line supported strong hormone-mediated responses when cognate receptors were transiently introduced (1).

11. Testing chemicals for (anti)-androgenic activity using the AR-CALUX® cell line includes a pre-screen run followed by a comprehensive run/specificity control test. During the pre-screen run, the solubility, the cytotoxicity and a refined concentration-range of test chemicals for comprehensive testing are determined. In the subsequent comprehensive run for agonism and antagonism, the test chemical is assessed using the refined concentration-ranges followed by data interpretation. For antagonism, the test chemical is assessed simultaneously with a comprehensive run and a specificity control test.

12. The specificity control test is included to discriminate true competitive antagonists from false positive antagonists (e.g. due to cytotoxicity, cell stress or aspecific inhibition). Cells are exposed to both the EC_{50} concentration and the 100x EC_{50} concentration of the reference agonist DHT when treated with 8 concentrations of the test chemical. This is carried out in the same plate. It will result in two dose responses of which the one generated with the higher ligand concentration (100x EC_{50}) is shifted to the right (see Figure D.6). The shift can be quantitatively measured and an acceptance criterion was developed (R^2). Criteria for data interpretation are described in detail in paragraph 70. Briefly, a test chemical is considered positive for agonism in case at least two consecutive concentrations of the test chemical show a response that is equal or higher than 10% of the maximum response of the reference standard DHT (PC_{10}). A test chemical is considered positive for antagonism in case at least two consecutive concentrations of the test chemical show a response that is equal or lower than 80% of the maximum response of the reference standard Flutamide (PC_{80}) and the specificity control criteria are met.

Demonstration of Laboratory Proficiency

13. Each laboratory should demonstrate proficiency in using this test method prior to testing chemicals with unknown activity. Proficiency is demonstrated by testing the proficiency chemicals for agonist activity and antagonist activity (see Tables B.4a and B.4b in Annex B). This testing will also confirm the responsiveness of the test system. Testing should be replicated at least twice, on different days, and the results should be consistent to the listed classifications and values in Tables B.4a and B.4b. Moreover, an historical database of data generated with the reference standards and the vehicle/solvent controls shall be maintained to confirm the reproducibility of the test method in the respective laboratory over time.

Procedure

Cell line

14. The stably transfected U2OS AR-CALUX® cell line should be used for the test method. The cell line can be obtained from BioDetection Systems BV, Amsterdam, the Netherlands with a technical licensing agreement.

15. Only mycoplasma free cell cultures should be used. Cell batches used should either be certified negative for mycoplasma contamination, or a mycoplasma test should be performed before use. A highly sensitive test, such as PCR methodologies, should be used for detection of mycoplasma infection (8, 9).

Stability of the cell line

16. To maintain the stability and integrity of the AR-CALUX® cells, the cells should be stored < -130˚C (e.g. in liquid nitrogen). Following thawing of the cells to start a new culture, cells should be sub-cultured at least twice before being used to assess the (anti)androgenic activity of chemicals. Cells should not be sub-cultured for more than 30 passages.

17. To monitor the stability of the cell line over time, its responsiveness to the reference chemicals (for agonist and antagonist testing) should be verified by evaluating the EC_{50} or IC_{50}. In addition, the relative induction of the positive control (PC) and the negative control (NC) should be monitored. The results should be in agreement with the acceptability criteria for the agonist (Table D.3) or antagonist AR-CALUX® test method (Table D.4). The reference standards, i.e. the reference chemical, positive and negative controls are given in Tables D.1 and D.2 for the agonist and antagonist mode respectively including the concentrations to be used.

Cell culture and plating conditions

18. The AR-CALUX® cells should be cultured in growth medium (DMEM/F12 (1:1)) with phenol red as pH indicator, supplemented with fetal bovine serum (7.5%), non-essential amino acids (1%), penicillin (10 Units/mL), streptomycin (10 µg/mL) and geneticin (G-418) (0.2 mg/mL) as selection marker. Cells should be placed in a CO_2 incubator (5% +/- 1% CO_2) at 37˚C +/- 1˚C and humidified. When cells reach 85-95% confluency, cells should either be subcultured or prepared for seeding in 96-well microtiter plates. In case of the latter, cells should be resuspended at $1x10^5$ cells/mL in assay medium (DMEM/F12 (1:1)) without phenol red, supplemented with Dextran-Coated Charcoal treated fetal bovine serum (5% v/v), non-essential amino acids (1% v/v), penicillin (10 Units/mL) and streptomycin (10 µg/mL) and plated into 96-well microtiter plates (100 µL of homogenised cell suspension). Cells should be pre-incubated in a CO_2 incubator (5% +/- 1% CO_2, 37˚C +/- 1˚C, humidified) for 24 hours prior to exposure.

19. Prior to starting any study, all materials (glass tubes, vessels, plastic ware) and reagents (e.g. serum, DMSO) that will be used during the testing should be investigated, as defined in the protocol (6), for any possible interference with the measurements.

Acceptability criteria

20. Agonist and antagonist activities of the test chemical(s) are tested in runs (pre-screen run and comprehensive run). Each run consists of a maximum of 6 microtiter plates. Each run contains 1 full series of dilutions of a reference chemical (C1 to C8), a fixed concentration of a positive control, a fixed concentration of a negative control, a solvent control (and vehicle control for the antagonist assay) and a

positive control for cytotoxicity. In Figures D.1 and D.2, the plate setup for agonist and antagonist runs are given.

21. In the first plate of each run,

- A complete dilution series of the reference chemical (DHT for agonism and Flutamide for antagonism) is measured (Tables D.5 and Table D.6). This reference chemical should demonstrate a sigmoidal dose-response curve. The EC_{50} or IC_{50} derived from the response of the series of dilutions of the reference chemical, and the CV of log(EC_{50}) and log(IC_{50}) for the reference chemicals should fulfil the requirements as indicated in Tables D.3 (agonism) or Table D.4 (antagonism).
- The calculated relative induction of both the positive and negative control should fulfil the requirements as indicated in Tables D.3 and D.4.

22. For each of the microtiter plates within a run, the following is calculated:

- During all measurements, the induction factor of the reference chemical should be calculated by dividing its average relative light unit (RLU) response at the highest concentration (C8) by the average solvent control RLU response. This induction factor should fulfil the minimum requirements for the induction fold as indicated in Tables D.3 and D.4.
- For each test-plate, the Z-factor is calculated according to the equation given below. This Z-factor should fulfil the minimum requirements for the Z-factor as indicated in Tables D.3 and D.4.

23. A run is considered valid when it fulfils the requirements as stated in Tables D.3 and D.4 and permits to evaluate the response of the test chemicals.

24. The acceptability criteria are applicable to both pre-screen runs and comprehensive runs.

Table D.1. Concentrations of the reference standards for the agonist testing

	Chemical	CASRN	Test range (M) in well
Reference chemical	DHT	521-18-6	1.0×10^{-11} - 1.0×10^{-07}
Positive control (PC)	17α-Methyltestosterone	58-18-4	1.0×10^{-07}
Negative control (NC)	Corticosterone	50-22-6	1.0×10^{-06}

Table D.2. Concentrations of the reference standards for the antagonist testing

	Chemical	CASRN	Test range (M) in well
Reference chemical	Flutamide	13311-84-7	1.0×10^{-08} - 3.0×10^{-05}
Positive control (PC)	Linuron	330-55-2	1.0×10^{-05}
Negative control (NC)	Levonorgestrel	797-63-7	1.0×10^{-06}

Table D.3. Acceptability criteria for agonism pre-screen and comprehensive testing

Number	Acceptability criteria	
1	Sigmoidal curve of reference chemical DHT	Yes
2	EC_{50} range reference chemical DHT	1.0×10^{-10} –1.0×10^{-09} M
3	|CV| of estimated log(EC_{50}) reference chemical DHT	< 1.5%
4	Relative induction (%) PC 17α-Methyltestosterone	> 30%
5	Relative induction (%) NC Corticosterone	< 10%
6	Minimum induction fold of the highest DHT concentration (C8), with respect to the solvent control (SC) on each plate	> 20
7	Z-factor calculated on each plate with DHT C8 and SC	> 0.5

Table D.4. Acceptance criteria for antagonism pre-screen and comprehensive testing

Number	Acceptability criteria	
1	Sigmoidal curve of reference chemical Flutamide	Yes
2	IC_{50} range reference chemical Flutamide	1.0×10^{-07} – 1.0×10^{-06} M
3	|CV| of estimated log(IC_{50}) reference chemical Flutamide	< 3%
4	Relative induction PC (Linuron)	< 60%
5	Relative induction NC (Levonorgestrel)	> 85%
6	Minimum inhibition fold of the highest Flutamide concentration (C8) with respect to the solvent control (SC) on each plate	> 10
7	Z-factor calculated on each plate with Flutamide C8 and SC	> 0.5
8	R^2 between Y_c and S_c^n for Flutamide	≤ 0.7

Solvent/vehicle control and reference standards

25. For both pre-screen and comprehensive runs, the same solvent/vehicle control and the reference standards (reference chemicals, positive controls and negative controls) should be used. In addition, the concentrations of the reference standards should be the same.

Solvent control and vehicle control

26. The solvent used to dissolve test chemicals should solubilize the test chemical completely and should be miscible with the assay medium. DMSO, water and ethanol (95% to 100% purity) are suitable solvents. DMSO (CASRN 67-68-5) is the first choice and its maximum concentration during incubation should not exceed 0.1% (v/v). Prior to the use of another solvent it should be demonstrated that it does not cause cytotoxicity of the cells and interference with the assay performance at exposure concentrations which simulate the experimental conditions.

27. The solvent used to dissolve the test chemicals should also be tested without the dissolved test chemical (solvent control (SC)).

28. For testing agonism, the SC contains assay medium plus the solvent. For testing antagonism, the SC contains the assay medium plus the solvent and a fixed concentration of the agonist reference chemical DHT (the EC_{50} concentration). The vehicle control (VC) however contains the assay medium plus the solvent but does not contain the fixed concentration of the agonist reference chemical.

Reference chemicals

29. The agonist reference chemical is DHT and comprises a series of dilutions of eight concentrations (Tables D.1 and D.5).

30. The antagonist reference chemical is Flutamide and comprises a series of dilutions of eight concentrations (Tables D.2 and D.6). Each of the concentrations of the antagonist reference chemical is spiked with a fixed concentration of the agonist reference chemical DHT (EC_{50} concentration = 3.0×10^{-10} M) in order to measure attenuation of the agonist response.

31. The antagonist reference chemical for the specificity control is Flutamide where each concentration is spiked with a 100X EC_{50} concentration of DHT. It comprises a series of dilutions of eight concentrations (Tables D.2 and D.6).

Positive control

32. The positive control for agonist studies is 17α-Methyltestosterone (Table D.1).

33. The positive control for antagonist studies is Linuron (Table D.2). This control is spiked with a fixed concentration of the agonist reference chemical DHT (3.0×10^{-10} M).

Negative control

34. The negative control for agonist studies is Corticosterone (Table D.1).

35. The negative control for antagonist studies is Levonorgestrel (Table D.2). This control is spiked with a fixed concentration of the agonist reference chemical DHT (3.0×10^{-10} M).

Preparation of the reference standards and the test chemicals

36. Reference standards (reference chemicals, positive controls, negative controls) and test chemicals are dissolved in 100% DMSO (or an appropriate solvent). Appropriate (serial) dilutions should then be prepared in the same solvent for the reference standards and the test chemicals. Before being dissolved, all chemicals should be allowed to equilibrate to room temperature. Freshly prepared stock solutions of the reference standards and the test chemicals should not have noticeable precipitate or cloudiness.

37. Stock solutions of the reference chemicals (DHT and Flutamide) may be prepared in bulk and stored as aliquots at -20˚C +/- 1˚C for up to 3 months. Once an aliquot is thawed, it can be stored at -20˚C +/- 1˚C and re-used (thawing/freezing) for up to 3 weeks. Stock solutions of test chemicals should be prepared fresh before each experiment.

38. Final dilutions of the reference standards and the test chemicals (i.e. working solutions) should be prepared fresh for each experiment and used within 24 hours of preparation.

Solubility, cytotoxicity and range finding

39. Test chemicals shall be assessed at a maximum concentration of 0.1 M (stock solution). When the molecular weight of a test chemical cannot be calculated such as for multi constituent chemicals, polymers, mixtures, UVCBs etc, the gravimetric method should be used starting from 50 mg/mL.

40. The solubility protocol, as used in the validation study, can be found in referenced document (10). Other protocols can be used as long as it is shown that they are suitable by e.g. testing the proficiency chemicals. The solubility of the test chemicals in the solvent of choice should be determined starting from a maximum stock concentration of 0.1 M. In case this concentration shows solubility problems, lower concentrations of stock solutions should be prepared until the test chemicals are fully solubilized. Subsequently, solubility of the test chemical should be assessed in assay medium at exposure concentrations (exposure concentration is 0.1% of the stock concentration, i.e. 0.1 mM).

41. During the pre-screen run, 1:10 serial dilutions of the test chemical are tested. An appropriate refined concentration range for test chemicals is derived from the pre-screen results, to be tested during the comprehensive run. The dilution factor (DF) to be used for comprehensive testing should be as follows: in case a positive response is observed (RI ≥10% for agonism or RI ≤80% for antagonism), an alternating

DF 3/3.3 is applied; in case only the highest tested concentration is above the 10% threshold (in agonism testing) or below the 80% threshold (in antagonism testing), DF 2 is applied; in case no response is observed, DF 5 is applied (see Tables D.5 and D.6).

42. Cytotoxicity testing is included in the agonist and antagonist test method protocols, and, is incorporated in both the pre-screen run and comprehensive runs. Following exposure to test chemicals, in pre-screen runs cytotoxicity is assessed with both the lactate dehydrogenase (LDH) leakage test and qualitative visual inspection (i.e. microscopic observation of the cells for morphological changes). The visual inspection is considered as an important evaluation tool given that the LDH leakage test reports on cell death only (cell lysis). For comprehensive runs, the qualitative visual inspection to score cytotoxicity is sufficient. With respect to the LDH leakage test, the concentration of the test chemical is regarded as cytotoxic when the percentage LDH leakage is higher than 15% with respect to the positive control for cytotoxicity (0.01% of Triton X-100). Other cytotoxicity tests can be used as long as it is shown that they are suitable e.g. by testing the proficiency chemicals.

Test chemical exposure and assay plate organisation

43. Following trypsination of a flask of confluent cultured cells, cells are re-suspended in assay medium at $1x10^5$ cells/mL. 100 µL of re-suspended cells are plated in the wells B1-G11 of a 96-well microtiter plate. The remaining wells are filled with 200 µL of Phosphate Buffered Saline (PBS) (see Figures D.1 and D.2). The plated cells are pre-incubated for 24 +/- 8 hours in a CO_2 incubator (5% +/- 1% CO_2, 37°C+/- 1°C, humidified).

44. After pre-incubation, the condition of the cells is verified visually (cytotoxicity, contamination and confluence (microscopy)). Only plates that show no visual cytotoxicity or contamination and have a minimum of 85% confluence in a representative portion of all wells are used for testing. Cells in the wells B1-G11 are exposed by the addition of 100 µL of assay medium containing appropriate dilution series of the reference standards, the test chemicals, the solvent controls and cytotoxicity controls (Table D.5: agonist testing; Table D.6: antagonist testing).

45. All reference standards, test chemicals and solvent controls are tested in triplicate whereas the cytotoxicity control Triton X-100 is tested in six replicate wells. In Figure D.1, the plate layout for agonist testing is given which is identical for pre-screen testing and comprehensive testing. In Figure D.2, the plate layout for antagonist pre-screen testing is given. All exposed wells, except for the vehicle control wells (VC), contain a fixed concentration of agonist reference chemical DHT (3.0×10^{-10} M (EC_{50})). In Figure D.3, the plate layout for antagonist comprehensive testing is shown, including the specificity control test with 100x EC_{50} of DHT. This corresponds to C(1-8)100 in the plate layout.

46. The 96-well microtiter plates should be incubated for another 24 +/- 2 hours in a CO_2 incubator (5% +/- 1% CO_2, 37°C +/-1°C, humidified). After incubation, the plates are visually inspected for cytotoxicity and contamination. For pre-screen testing, 100 µl of the exposure medium from each well is transferred to another plate to be used for the cytotoxicity test (paragraph 42). The remaining 100 µl of exposure medium in the well is removed in order to expose the cells in the wells to lysis substrate (paragraph 47) for measuring luminescence.

Figure D.1. Plate layout of the 96-well microtiter plates for agonist pre-screen and comprehensive testing and for assessment of agonist effects.

Plate 1

	1	2	3	4	5	6	7	8	9	10	11	12
A												
B	Triton X-100	SC	C1 DHT	C2 DHT	C3 DHT	C4 DHT	C5 DHT	C6 DHT	C7 DHT	C8 DHT	PC	
C	Triton X-100	SC	C1 DHT	C2 DHT	C3 DHT	C4 DHT	C5 DHT	C6 DHT	C7 DHT	C8 DHT	PC	
D	Triton X-100	SC	C1 DHT	C2 DHT	C3 DHT	C4 DHT	C5 DHT	C6 DHT	C7 DHT	C8 DHT	PC	
E	Triton X-100	SC	C1	C2	C3	C4	C5	C6	C7	C8	NC	
F	Triton X-100	SC	C1	C2	C3	C4	C5	C6	C7	C8	NC	
G	Triton X-100	SC	C1	C2	C3	C4	C5	C6	C7	C8	NC	
H												

Subsequent plates

	1	2	3	4	5	6	7	8	9	10	11	12
A												
B	Triton X-100	SC	C1	C2	C3	C4	C5	C6	C7	C8	C8 DHT	
C	Triton X-100	SC	C1	C2	C3	C4	C5	C6	C7	C8	C8 DHT	
D	Triton X-100	SC	C1	C2	C3	C4	C5	C6	C7	C8	C8 DHT	
E	Triton X-100	SC	C1	C2	C3	C4	C5	C6	C7	C8	C8 DHT	
F	Triton X-100	SC	C1	C2	C3	C4	C5	C6	C7	C8	C8 DHT	
G	Triton X-100	SC	C1	C2	C3	C4	C5	C6	C7	C8	C8 DHT	
H												

C(1-8) DHT = series of dilutions (1-8, low-to-high concentrations) of reference chemical DHT
C(1-8) = series of dilutions (1-8, low-to-high concentrations) of test chemical.
SC = solvent control of the test chemical / reference standards (the same solvent as in C (1-8)).
PC = positive control 17α-Methyltestosterone.
NC = negative control Corticosterone
Grey cells: = outer wells, filled up with 200 µL of PBS.
Triton X-100 = positive control for cytotoxicity

Figure D.2. Plate layout of the 96-well microtiter plates for antagonist pre-screen testing and assessment of antagonist effects.

Plate 1

	1	2	3	4	5	6	7	8	9	10	11	12
A												
B	Triton X-100	SC	C1 FLU	C2 FLU	C3 FLU	C4 FLU	C5 FLU	C6 FLU	C7 FLU	C8 FLU	VC	
C	Triton X-100	SC	C1 FLU	C2 FLU	C3 FLU	C4 FLU	C5 FLU	C6 FLU	C7 FLU	C8 FLU	VC	
D	Triton X-100	SC	C1 FLU	C2 FLU	C3 FLU	C4 FLU	C5 FLU	C6 FLU	C7 FLU	C8 FLU	VC	
E	Triton X-100	NC	C1	C2	C3	C4	C5	C6	C7	C8	PC	
F	Triton X-100	NC	C1	C2	C3	C4	C5	C6	C7	C8	PC	
G	Triton X-100	NC	C1	C2	C3	C4	C5	C6	C7	C8	PC	
H												

Subsequent plates

	1	2	3	4	5	6	7	8	9	10	11	12
A												
B	Triton X-100	SC	C1	C2	C3	C4	C5	C6	C7	C8	C8 FLU	
C	Triton X-100	SC	C1	C2	C3	C4	C5	C6	C7	C8	C8 FLU	
D	Triton X-100	SC	C1	C2	C3	C4	C5	C6	C7	C8	C8 FLU	
E	Triton X-100	SC	C1	C2	C3	C4	C5	C6	C7	C8	C8 FLU	
F	Triton X-100	SC	C1	C2	C3	C4	C5	C6	C7	C8	C8 FLU	
G	Triton X-100	SC	C1	C2	C3	C4	C5	C6	C7	C8	C8 FLU	
H												

C(1-8) FLU = series of dilutions (1-8, low-to-high concentrations) of reference chemical Flutamide (spiked with EC_{50} concentration DHT)
C(1-8) = series of dilutions (1-8, low-to-high concentrations) of test chemical (spiked with EC_{50} concentration DHT)
NC = negative control Levonorgestrel (spiked with EC_{50}concentration DHT)
PC = positive control Linuron (spiked with EC_{50} concentration DHT)
SC = solvent control of the test chemical/ reference standards (the same solvent as in C (1-8) (spiked with

EC_{50}concentration DHT).
VC = vehicle control (solvent control without the spiking with DHT).
Grey cells = outer wells, filled up with 200 µL of PBS.
Triton X-100 = positive control for cytotoxicity

Figure D.3. Plate layout of the of the 96-well microtiter plates for antagonist comprehensive testing and assessment of antagonist effects, including specificity control test.

Plate 1

	1	2	3	4	5	6	7	8	9	10	11	12
A												
B	Triton X-100	SC	C1 FLU	C2 FLU	C3 FLU	C4 FLU	C5 FLU	C6 FLU	C7 FLU	C8 FLU	VC	
C	Triton X-100	SC	C1 FLU	C2 FLU	C3 FLU	C4 FLU	C5 FLU	C6 FLU	C7 FLU	C8 FLU	VC	
D	Triton X-100	SC	C1 FLU	C2 FLU	C3 FLU	C4 FLU	C5 FLU	C6 FLU	C7 FLU	C8 FLU	VC	
E	Triton X-100	NC	C1 FLU 100	C2 FLU 100	C3 FLU 100	C4 FLU 100	C5 FLU 100	C6 FLU 100	C7 FLU 100	C8 FLU 100	PC	
F	Triton X-100	NC	C1 FLU 100	C2 FLU 100	C3 FLU 100	C4 FLU 100	C5 FLU 100	C6 FLU 100	C7 FLU 100	C8 FLU 100	PC	
G	Triton X-100	NC	C1 FLU 100	C2 FLU 100	C3 FLU 100	C4 FLU 100	C5 FLU 100	C6 FLU 100	C7 FLU 100	C8 FLU 100	PC	
H												

Subsequent plates

	1	2	3	4	5	6	7	8	9	10	11	12
A												
B	Triton X-100	SC	C1	C2	C3	C4	C5	C6	C7	C8	C8 FLU	
C	Triton X-100	SC	C1	C2	C3	C4	C5	C6	C7	C8	C8 FLU	
D	Triton X-100	SC	C1	C2	C3	C4	C5	C6	C7	C8	C8 FLU	
E	Triton X-100	SC	C1 100	C2 100	C3 100	C4 100	C5 100	C6 100	C7 100	C8 100	C8 FLU	
F	Triton X-100	SC	C1 100	C2 100	C3 100	C4 100	C5 100	C6 100	C7 100	C8 100	C8 FLU	
G	Triton X-100	SC	C1 100	C2 100	C3 100	C4 100	C5 100	C6 100	C7 100	C8 100	C8 FLU	
H												

C(1-8) FLU =series of dilutions (1-8, low-to-high concentrations) of reference chemical Flutamide (spiked with EC_{50} concentration DHT)
C(1-8) FLU 100 =series of dilutions (1-8, low-to-high concentrations) of reference chemical Flutamide (spiked with 100X EC_{50} concentration DHT)
NC = negative control Levonorgestrel (spiked with EC_{50} concentration DHT)
PC = positive control Linuron (spiked with EC_{50} concentration DHT)
SC = solvent control of the test chemical/ reference standards (the same solvent as in C (1-8) (spiked with EC_{50} concentration DHT).
VC = vehicle control (solvent control without the spiking with DHT).
C(1-8) = series of dilutions (1-8, low-to-high concentrations) of test chemical (spiked with EC_{50} concentration DHT)
C(1-8) 100 = series of dilutions (1-8, low-to-high concentrations) of test chemical ((spiked with 100x EC_{50} concentration DHT) (specificity test)
Grey cells = outer wells, filled up with 200 µL of PBS.
Triton X-100 = positive control for cytotoxicity

Measurement of luminescence

47. There are several options for the measurement of luminescence. The methods used during the validation of the AR-CALUX® test method included either the use of a commercial kit, which could be either a flash or a glow luminescence kit, or the preparation of the luminescence substrate in-house. In any case, the medium from the wells should be removed and the cells should be lysed following 24 +/- 2 hours of incubation to measure luciferase activity.

48. For measuring the luminescence, a luminometer is required. When transparent plates are used, the luminometer has to be equipped with 2 injectors. The luciferase reaction is started by injection of the substrate luciferin. The reaction is stopped by addition of an appropriate solvent (e.g. 0.2 M NaOH or 25% v/v acetic acid depending on the luminometer) to prevent carry over of luminescence from one well to the

other. When a commercial kit is used, the specific instructions supplied with the kit must be followed. White plates allow the use of a luminometer without or with one injector depending on the kit used.

49. Light emitted from each well is expressed as Relative Light Units (RLUs) per well.

Pre-screen run for (ant)agonist testing

50. The pre-screen analysis results are used to determine a refined concentration-range of the test chemicals for the comprehensive testing. Evaluation of pre-screen analysis results and the determination of the refined concentration-range of test chemicals for comprehensive testing is described in depth in the agonist and antagonist test method protocol (6). Here, a brief summary of the procedures for determining the concentration range of the test chemicals for agonist and antagonist testing is given. See Tables D.5 and D.6 for guidance of serial dilution design.

51. During the pre-screen run, test chemicals should be tested using the dilution series as indicated in Tables D.5 (agonism) and D.6 (antagonism). All concentrations should be tested in triplicate wells according to the plate layout as indicated in Figures D.1 (agonism) or D.2 (antagonism).

52. A pre-screen run shall always be followed by a comprehensive run, regardless if the response observed during the pre-screen is positive or negative. One comprehensive run shall suffice for drawing a conclusion following the decision criteria in Table D.7.

Selection of concentrations for assessment of (ant)agonist effects

53. Only results that fulfil the acceptability criteria (Tables D.3 and D.4) are considered valid and allow evaluating the response to test chemicals. In case one or more microtiter plates in a run fail to fulfil the acceptability criteria, the respective microtiter plates should be tested again. In case the first plate containing the complete series of dilutions of the reference chemical fails the acceptability criteria, the complete run (6 plates) has to be tested again.

54. Determine the (lowest) concentration at which maximum induction (agonism) or inhibition (antagonism) is observed and does not show cytotoxicity. The highest concentration of the test chemical to be tested in the comprehensive run should be 3-times this selected concentration or a maximum exposure concentration of 0.1 mM or 50 µg/ml for chemicals where the molarity is not known.

55. A complete refined dilution series of the test chemical should be prepared with dilutions steps as indicated in Tables D.5 and D.6, starting with the highest concentration as determined above

56. A test chemical that does not elicit any (ant)agonist effect, should be tested in the comprehensive run starting with the highest, non-cytotoxic concentration identified during the pre-screen with dilutions steps as indicated in Tables D.5 and D.6.

Comprehensive run for agonist testing

57. Following the selection of the refined concentration ranges, test chemicals should be tested comprehensively using the dilution series indicated in Table D.5 (agonism). All concentrations should be tested in triplicate wells according to the plate layout as indicated in Figure D.1 (agonism) (see paragraph 45).

58. Only results that fulfil the acceptability criteria (Table D.3) are considered valid and allow evaluating the response to test chemicals. In case one or more microtiter plates in a run fail to fulfil the acceptability criteria, the chemicals tested in the respective microtiter plates should be tested again in a repeated run.

In case the first plate containing the complete series of dilutions of the reference chemical fails the acceptability criteria, the complete run (6 plates) has to be tested again.

Comprehensive run and specificity control test for antagonist testing

59. Following the selection of the refined concentration ranges, a test chemical shall be tested simultaneously with a comprehensive test and a specificity control test (on the same plate), using the dilutions series indicated in Table D.6 (antagonism) (see paragraph 45). All concentrations should be tested in triplicate wells according to the plate layout as indicated in Figure D.3 (antagonism).

60. Only results that fulfil the acceptability criteria (Table D.4) are considered valid and allow evaluation of the response of the test chemicals. In case one or more microtiter plates in an analysis series fail to fulfil the acceptance criteria, the respective microtiter plates should be tested again. In case the first plate containing the complete series of dilutions of the reference chemicals fails the acceptability criteria, the complete run (6 plates) has to be tested again.

Table D.5. Concentration and dilutions of reference standards and test chemicals used for agonist testing

Reference DHT conc. (M) in well		Controls conc. (M) in well		Test chemical	Pre-screen	Comprehensive		
					DF 10	DF 5	DF 3/ 3.33	DF 2
C1	1.0×10^{-11}	PC	1.0×10^{-07}	C1	10,000,000 x	78125 x	3,000 x	128 x
C2	3.0×10^{-11}	NC	1.0×10^{-06}	C2	1,000,000 x	15625 x	1,000 x	64 x
C3	1.0×10^{-10}	SC	0	C3	100,000 x	3125 x	300 x	32 x
C4	3.0×10^{-10}			C4	10,000 x	625 x	100 x	16 x
C5	1.0×10^{-09}			C5	1,000 x	125 x	30 x	8 x
C6	3.0×10^{-09}			C6	100 x	25 x	10 x	4 x
C7	1.0×10^{-08}			C7	10 x	5 x	3 x	2 x
C8	$1.0 \times *10^{-07}$			C8	1 x	1 x	1 x	1 x

PC - positive control (17α-Methyltestosterone)
NC - negative control (Corticosterone)
SC - test chemical solvent control

Table D.6. Concentration and dilutions of reference standards and test chemicals used for antagonist testing

Reference Flutamide conc. (M) in well		Controls conc. (M) in well		Test chemical	Pre-screen DF 10	Comprehensive DF 5	DF 3/3.33	DF 2
C1	1.0×10^{-08}	PC	1.0×10^{-05}	C1	10,000,000 x	78125 x	3,000 x	128 x
C2	3.0×10^{-08}	NC	1.0×10^{-06}	C2	1,000,000 x	15625 x	1,000 x	64 x
C3	1.0×10^{-07}	SC	0	C3	100,000 x	3125 x	300 x	32 x
C4	3.0×10^{-07}	VC	0	C4	10,000 x	625 x	100 x	16 x
C5	1.0×10^{-06}			C5	1,000 x	125 x	30 x	8 x
C6	3.0×10^{-06}			C6	100 x	25 x	10 x	4 x
C7	1.0×10^{-05}			C7	10 x	5 x	3 x	2 x
C8	3.0×10^{-05}			C8	1 x	1 x	1 x	1 x
		Supplemented agonist conc. (M in well)						
		DHT 3.0×10^{-10} (=EC_{50})						
		Supplemented agonist conc. Specificity control (M in well)						
		DHT 3.0×10^{-8}(=100x EC_{50})						

PC - positive control (Linuron)
NC - negative control (Levonorgestrel)
SC - solvent control
VC - vehicle control (does not contain fixed concentration of the agonist reference chemical)l

Analysis of Data

Normalisation of the data

61. Raw data derived from the luminometer are expressed as RLUs. When the acceptability criteria are met, as indicated in Tables D.3 and D.4, the following calculation steps are performed to determine the required parameters. The raw data should be transferred to a data analysis spreadsheet designed for pre-screen or comprehensive runs.

For the agonist assay:

62. For each test chemical and the reference chemical DHT calculate

- the relative induction at concentration *c*, technical replicate *i* , (Y_{ic}) as follows:

$$Y_{ic} = \frac{RLU\ of\ test\ chemical\ (i\ replicate) - average\ RLU\ of\ SC}{average\ RLU\ of\ DHT_{C8} - average\ RLU\ of\ SC} \times 100, \quad i = 1,2,3$$

- the average of the relative inductions over the 3 technical replicates (Y_c),

$$Y_c = average\ Y_{ic}$$

For the antagonist assay:

63. For each test chemical, and reference chemical FLU calculate

- the relative induction at concentration *c*, technical replicate *i*, (Y_{ic}) as follows: .

$$Y_{ic} = \frac{RLU\ of\ test\ chemical\ (i\ replicate) - average\ RLU\ of\ FLU_{C8}}{average\ RLU\ of\ SC - average\ RLU\ of\ FLU_{C8}} \times 100, \quad i = 1,2,3$$

- the average of the relative inductions over the 3 technical replicates (Y_c)

$$Y_c = average\ Y_{ic}$$

Note: SC in the antagonist assay is assay medium spiked with DHT EC_{50} concentration

For the specificity control test

- To calculate the test chemical's specificity control at concentration *c*, replicate *i*, S_{ic}, apply the same formula as given above for the antagonist assay but the RLU of test chemical (i replicates) shall be those obtained in the specificity control test (spiking with 100x EC_{50} DHT).
- In addition calculate the test chemical's normalized specificity control (S_c^n) by setting the C1 concentration of the test chemical's specificity control (S_c) at 100%, i.e.

$$S_c^n = 100 \times \frac{S_c}{S_{c_1}}, \qquad c = c_1, \ldots, c_8$$

Cytotoxicity

64. For all test chemical concentrations evaluated during the pre-screen analysis, calculate the percentage LDH leakage with respect to the cytotoxicity positive control 0.01% Triton X-100 (percentage set at 100%), according to the following equation:

$$\%\ LDH\ leakage = \frac{average\ AU\ test\ chemical\ - average\ AU\ SC}{average\ AU\ positive\ control\ - average\ AU\ SC} \times 100$$

Note: AU= absorbance unit

65. In addition, qualitative visual inspection of the cells following exposure to the test chemicals shall be carried out. The test chemical is regarded cytotoxic at a specific concentration when:

- either the average percentage LDH leakage of the triplicate sample is higher than 15% with respect to the positive control
- or, cytotoxicity is observed with a microscope.

Calculation of parameters

66. After the normalization of the data, apply a non-linear regression (variable slope, 4 parameters) to the Y_{ic} data using the following equation:

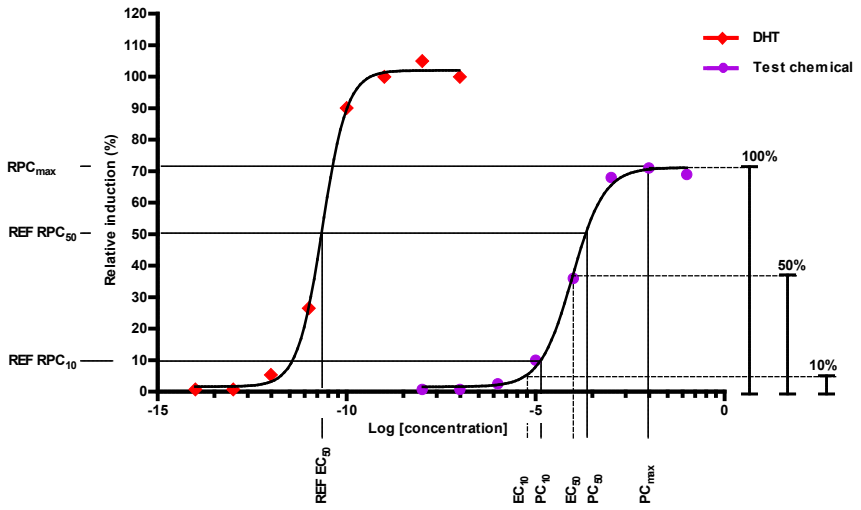
$$y = Bottom + \frac{(Top - Bottom)}{\left(1 + 10^{((LogEC_{50} - x) * HillSlope)}\right)}$$

x =	Log of dose or concentration
y =	Response (relative induction (%))
Top =	Maximum induction (%)
Bottom =	Minimum induction (%)
$LogEC_{50}$ =	Log of concentration at which 50% of maximum response is observed
HillSlope =	Slope factor of Hill slope

67. For agonist testing, determine the EC_{10} and EC_{50} of the reference chemical, and, determine the EC_{10}, EC_{50}, PC_{10}, and PC_{50} of the test chemicals. For antagonist testing, determine IC_{50} and IC_{20} of the reference chemical, and, determine the IC_{20}, IC_{50}, PC_{80}, and PC_{50} of the test chemicals. To further characterise the potency of a test chemical, the magnitude of the effect (agonism: RPC_{max}; antagonism: RPC_{min}) and the concentration at which the effect occurs (agonism: PC_{max}; antagonism: PC_{min}) should be

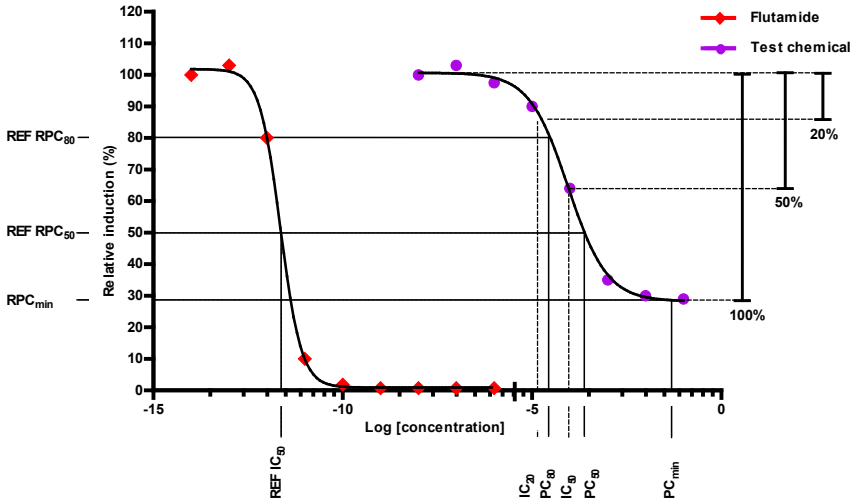
reported. In Figures D.4 (agonism) and D.5 (antagonism), a graphical representation of these parameters are given.

Figure D.4. Overview of parameters determined for a test chemical in the agonist assay



EC_{10} = concentration of a test chemical at which 10% of its maximum response is observed.
EC_{50} = concentration of a test chemical at which 50% of its maximum response is observed.
PC_{10} = concentration of a test chemical at which its response is equal to the EC_{10} of the reference chemical (REF RPC_{10}).
PC_{50} = concentration of a test chemical at which its response is equal to the EC_{50} of the reference chemical (REF RPC_{50}).
PC_{max} = concentration of a test chemical where the response is maximal (corresponding to RPC_{max})
REF EC_{50} = concentration of the reference chemical DHT at which 50% of its maximum response is observed

Figure D.5. Overview of parameters determined for a test chemical in the antagonist assay.



IC_{20} = concentration of a test chemical at which 80% of its maximum response is observed (20% inhibition).

IC_{50} = concentration of a test chemical at which 50% of its maximum response is observed (50% inhibition).

PC_{80} = concentration of a test chemical at which its response is equal to the IC_{20} of the reference chemical (REF RPC_{80}).

PC_{50} = concentration of a test chemical at which its response is equal to the IC_{50} of the reference chemical (REF RPC_{50})

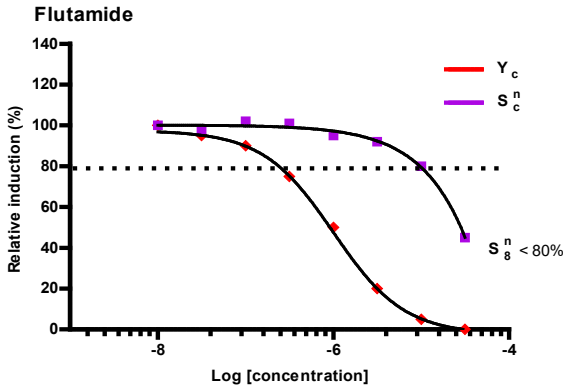
PC_{min} = concentration of a test chemical where the response is maximal (corresponding to RPC_{min})

REF IC_{50} = concentration of the reference chemical at 50% of its maximum response

68. For test chemicals, a full dose-response curve may not always be achieved due to e.g. cytotoxicity or solubility problems. In these cases, the EC_{50} and EC_{10} cannot be determined in the agonist testing, and, the IC_{50} and IC_{20} cannot be determined in the antagonist testing. Therefore it is then sufficient to determine the PC_{10}, PC_{50} and PC_{max} (agonist) and the PC_{80}, PC_{50} and PC_{min} (antagonist), if possible, which can be derived from e.g. linear interpolation between the two closest data points.

69. Specificity of an antagonist response (i.e. being a true competitive antagonist) is determined as indicated by the data interpretation criteria (Table D.7). When interpreting the results of the specificity control, the two dose response curves (Y_c and S_c^n) should be visually inspected and it should be verified whether the first positive criterion for antagonist testing can be applied (see Table D.7: S_c^n> 80% at all concentrations). Otherwise, calculate the square of the correlation coefficient (R^2) between the relative induction of the standard response (Y_c) and the relative induction of the normalized specificity response (S_c^n) of a test chemical. This second positive criterion of the antagonist testing should be verified (see Table D.7: R^2 is ≤ 0.9). Some caution should be applied as this criterion cannot be considered as 100% definitive (as shown in the AR-CALUX® validation study (5)). It may be influenced by the shape of the curves and by outliers. Expert judgment may need to be applied.

Figure D.6. Representation of the relative induction of the standard response (Y_c) and the relative induction of the normalized specificity response (S_c^n) of a true competitive antagonist ($R^2 \leq 0.9$)



Data Interpretation Criteria

70. For the interpretation of data and the decision whether a test chemical is considered positive or negative, the criteria in Table D.7 are to be used. One comprehensive run shall suffice for drawing a conclusion.

Table D.7. Decision criteria

AGONISM	
Positive	When the relative induction (Y_c) of the test chemicalis ≥ 10% (REF RPC_{10}) for two or more consecutive concentrations.
Negative	In all other cases
ANTAGONISM	
Positive	When the relative induction (Y_c) of the test chemical is ≤ 80% (REF RPC_{80}) for two or more consecutive concentrations and Either • the relative induction of the test chemical's normalised specificity control s_c^n > 80% at all concentrations or when the following two conditions are met: • the relative induction of the test chemical's normalised specificity control at the highest concentration $s_{c_8}^n$ is ≤ 80%, • the square of the correlation coefficient between the relative induction of the test chemical's normalized specificity control (s_c^n) and the relative induction (Y_c) (R^2) is ≤ 0.9
Negative	In all other cases

71. The given criteria in Table D.7 should be applied only to data that were generated in the absence of cytotoxicity.

72. In addition to the dichotomous categorisation (Table D.7), the potency measurements may also be used in integrated approaches.

Literature

1. Sonneveld, E., Jansen, H.J., Riteco, J.A., Brouwer, A, van der Burg B. (2004). Development of androgen- and estrogen-responsive bioassays, members of a panel of human cell line-based highly selective steroid-responsive bioassays. Toxicol. Sci. 83(1), 136-148.
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Annex E. (Method 3): Androgen Receptor Transactivation Assay for Detection of androgenic Agonist and Antagonist Activity of Chemicals using the stably transfected human 22Rv1/MMTV_GR-KO cell line

Initial Considerations and Limitations

1. The “General Introduction” should be read before using this test method (Main body page 6-9).

2. The 22Rv1/MMTV_GR-KO AR-mediated stably transfected transcriptional activation (TA) assay was established to screen chemicals for endocrine activity via interaction with the AR using a human prostate cancer cell line, 22Rv1, that endogenously expresses the AR (1, 2). This test method is specifically designed to detect human AR-mediated TA and inhibition by measuring luciferase activity as the endpoint. The information of chemical dependent interference with luminescence signals is limited in a GR-knockout 22Rv1/MMTV cell line.

3. Although the constitutively-acting truncated AR is expressed in 22Rv1/MMTV cells, the truncated AR does not significantly affect the activity. It is verified that the solvent control level (basal level) is not high, the induction fold is dose-dependently increasing by treatment with DHT, and the level of increase is very high compared to other reporter gene assay (2, 4). Furthermore, the full length AR is expressed to similar level with LNCaP cell (2).

4. The glucocorticoid receptor (GR) is expressed in 22Rv1 origin cells alongside AR, endogenously. The minimal GR-mediated response can interfere with the AR-mediated response because the GR is structurally similar to the AR and shares hormone response elements that exhibit cross-talk with the AR (1, 3, 5). To eliminate GR expression in cells, a GR-knockout 22Rv1/MMTV cell line was developed using the clustered regularly interspaced short palindromic repeats (CRISPR)/CRISPR-associated (Cas) 9 system (1, 2, 5).

5. The GR-knockout 22Rv1/MMTV cell line showed low metabolic activity in validation study (6). The validation was conducted using only monoconstituent chemicals. This test method can theoretically be applied to the testing of mixtures. Before applying this test method to mixtures, it should be considered whether the results will be scientific meaningful.

6. Definitions and abbreviations used in this test guideline are described in Annex A of this TG.

7. The 22Rv1/MMTV_GR-KO assay was validated by the National Institute for Food and Drug Safety Evaluation (NIFDS), the Korean Testing and Research Institute (KTR) and Dongguk University with support of a study management team comprised of members of the OECD VMG-NA expert group. The test method is used to detect AR agonists and antagonists of level 2 in “OECD Conceptual Framework for

the Testing and Assessment of Endocrine Disrupting Chemicals" (6, 7, 8). The validation study of the 22Rv1/MMTV_GR-KO method was conducted according to OECD Guidance Document (GD) 34. The relevance and reliability of the assay for its intended purpose was demonstrated (9, 10).

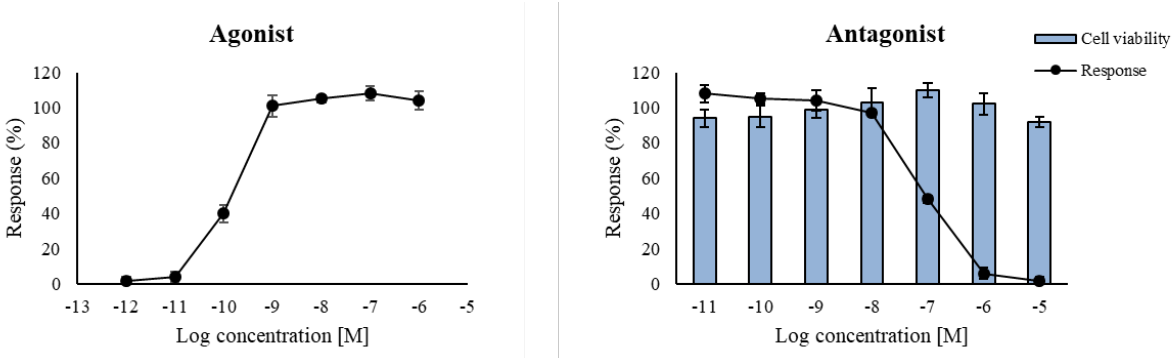
Principle of the test method

8. The test system provided in this method utilises the 22Rv1/MMTV_GR-KO cell line, which is derived from a 22Rv1 cell line. The 22Rv1 cells have been classified as a biosafety level 2 cell line from ATCC, because the 22Rv1 cell line produces the human retrovirus XMRV (xenotropic murine leukemia virus-related virus) (11). When conducting the experiment using the 22Rv1 cell line, the biological safety should be considered. The cell line developed consists of stably transformed 22Rv1 cells with one pGL4[*luc2P*/MMTV/Hygro] vector. The pGL4[*luc2P*/MMTV/Hygro] vector protocol is subject to a Promega limited use licence requiring i) the use of luminescent assay reagents purchased from Promega; or ii) to contact Promega to obtain a free license for commercial use.

9. AR agonist/antagonist assays using the 22Rv1/MMTV_GR-KO cell line should be conducted in a stepwise approach. After conduct of a pre-screen run, a comprehensive run and specificity control (only AR antagonist assay) are performed. The comprehensive run is only conducted if the pre-screen indicates positive activity in either the agonist or antagonist assay. A starting concentration of the test chemical for a comprehensive run is determined in the pre-screen run. To confirm whether a chemical is a true competitive AR binding antagonist, a specificity control test must be used (see paragraph 28).

10. 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-DHT, the AR agonist control (PC_{AGO}), the test chemical is considered a AR agonist. Data interpretation for an AR antagonist effect of a test chemical is decided by two steps. i) a cut-off of 30% inhibitory response of the test chemical in the presence of 800 pM DHT and ii) R^2 value less than 0.9 in the specificity control test (see paragraphs 40 and 43). If both criteria are met, then the chemical is considered a true AR antagonist. Data analyses is described in detail in paragraphs 37-41. Typical concentration-response curves of agonist and antagonist reference chemical (DHT and Bicalutamide) are shown in Figure E.1.

Figure E.1. Typical positive control responses from the pre-screen run in AR agonist and antagonist assay



Demonstration of laboratory proficiency

11. A proficiency test should be conducted by each laboratory to verify proficiency with the 22Rv1/MMTV GR-KO method. The proficiency chemicals for the agonist and antagonist assay are listed in Tables B.4a and B.4b of the Annex B in this TG. The proficiency test should be done at least twice, on different days, and the results should be consistent with the classifications and values for the proficiency chemicals listed in Tables B.4a and B.4b in Annex B.

Procedure

Cell line

12. The 22Rv1/MMTV_GR-KO cell line is an androgen-responsive stable transformed cell line derived from 22Rv1 human prostate cancer cells, which are adherent and AR-positive. The cell line can be obtained from the Korean Collection for Type Cultures ("KCTC")[2] , upon signing a Material Transfer Agreement (MTA).

13. Mycoplasma-free cells should be used in the method. The detection of mycoplasma infection should be conducted before starting any experiments using sensitive methods, such as PCR analysis (12).

Stability of the cell line

14. To maintain the stability and integrity of the response, the cells should be kept at less than -80℃ (e.g. in deep freezer or liquid nitrogen). Cells should be sub-cultured at least twice after thawing, and shall than be used to assess the (anti)androgenic activity of chemicals. Cells should not be sub-cultured for more than 30 passages The cell-doubling time is 48 hours.

Cell line maintenance and plating conditions

15. The following medium should be prepared (the details are described in the SOP of the validation report (10)):

- ▪ Culture medium: RPMI1640 supplemented with FBS (10% v/v), GlutaMAX™ (2 mM), Penicillin (100 units/mL), Streptomycin (100 µg/mL), and Amphotericin B (0.25 µg/mL).
- ▪ Test medium: phenol red-free RPMI1640 supplemented with Dextran-coated charcoal treated (DCC)-FBS (5% v/v), GlutaMAX™ (2 mM), Penicillin (100 units/mL), Streptomycin (100 µg/mL), and Amphotericin B (0.25 µg/mL)

16. The maintenance protocol for the 22Rv1/MMTV_GR-KO cell line is based on the ATCC 22Rv1 maintenance protocol (11). 22Rv1/MMTV_GR-KO cells are maintained in a culture medium that includes 200 µg/mL hygromycin as a luciferase gene selection marker to be used the first time after thawing cells. 0.1%Trypsin-EDTA is preferred over 0.05% Trypsin-EDTA for passage of 22Rv1/MMTV_GR-KO cell line, because the higher concentration improves cell dissociation from the cell culture plate. For the assay, cells should be suspended at 3.0 × 10^5 cells per 1 mL with test medium. 100 µL aliquots of suspended cells

[2] The 22Rv1/MMTV_GR-KO cell line can be obtained from the Korean Collection for Type Cultures("KCTC"), which is one of the largest biological resource center in Korea and performs acquisition, preservation, and distribution of biological resources. The cell line is available for sale at KCTC (https://kctc.kribb.re.kr/En/).

Cell line Name : 22Rv1/MMTV_GR-KO cell line (KCTC No. HC30009).

(corresponding to 3.0 × 10^4 cells /well) should be transferred into a 96-well white plate. Cells are pre-incubated for 48 hours at 37 ℃ in a 5%±0.5% CO_2 incubator prior to exposure.

17. DCC-FBS in test medium is used to minimize the interference of other serum ingredients.

Vehicle control, AR agonist control and AR antagonist control

18. For the AR agonist assay, the agonist control (PC_{AGO1}) wells (n = 4) treated with a 10 nM DHT and vehicle control (VC) wells (n =4) containing only 0.1% DMSO, and cytotoxicity control (PC_{CT}; 1 mM SDS) wells (n = 4) should be prepared on each plate. The 10 nM DHT concentration is selected in order to achieve 100% response in the AR agonist assay.

19. For the AR antagonist assay, VC wells (n = 3), agonist control (PC_{AGO2}; 800 pM DHT) wells (n = 3), AR antagonist control (PC_{ANTA}; 800 pM DHT and 1 µM of Bicalutamide) wells (n = 3), and cytotoxicity control (PC_{CT}; 800 pM DHT and 1 mM SDS) wells (n = 3) should be included for each plate.

Positive and negative references standards

20. Reference standards for each assay should be included in one plate of each run. For the AR agonist assay, three well-characterised reference standards; two positive reference standards (DHT and Mestanolone) and one negative reference standard (Diethylhexyl phthalate (DEHP)) should be included. Reference standards for the AR antagonist assay include two positive reference standards (Bicalutamide and Bisphenol A) and one negative reference standard (DEHP).

Quality criteria for AR agonist/antagonist assay

21. The mean luciferase activity of the PC (AR agonist assay: 10 nM DHT (PC_{AGO1}); AR antagonist assay: 800 pM DHT (PC_{AGO2})) should be at least 13-fold greater than the mean VC on each plate for the AR agonist assay, and at least 10-fold greater than the mean VC for the AR antagonist assay. With respect to the quality control of the assay, the induction fold of the PC_{10} must be greater than 1 + 2 Standard Deviations (SD) of the induction of the VC. Relative transcriptional activity (RTA) of PC_{ANTA} (800 pM DHT and 1 µM Bicalutamide), which is a single concentration without a dose response curve of Bicalutamide, should be less than 53.6% of the PC_{AGO2} in the AR antagonist assay.

Acceptability criteria

Table E.1. Acceptability criteria for AR agonist assay

Chemicals	Log PC_{10}	Log PC_{50}	Test Range
5α-Dihydrotestosterone (DHT)	−12.2 to −9.7	−10.6 to −9.0	1.0 x 10^{-6} to 1.0 x 10^{-12} M
Mestanolone	−12.3 to −9.8	−10.2 to −8.6	1.0 x 10^{-6} to 1.0 x 10^{-12} M
Diethylhexyl phthalate (DEHP)	–	–	1.0 x 10^{-5} to 1.0 x 10^{-11} M
Induction fold of PC_{AGO1}	≥ 13		

Induction fold of PC_{10}	Greater than 1+2SD (induction of VC)

Induction fold of PC_{10}: corresponding to the PC_{10} (10%) of AR agonist control (PC_{AGO1}:10 nM of DHT)
SD: Standard Deviation, VC: Vehicle Control

22. Induction fold of PC_{AGO1} is calculated using the following equation:

$$\text{Induction fold of } PC_{AGO1} = \frac{\text{Mean RLU of } PC_{AGO1} \text{ (10 nM DHT)}}{\text{Mean RLU of Vehicle control}}$$

▪ RLU: relative light units

Table E.2. Acceptability criteria for AR antagonist assay

Chemicals	Log IC_{30}	Log IC_{50}	Test Range
Bicalutamide	−7.5 to −6.2	−7.0 to −5.8	1.0×10^{-4} to 1.0×10^{-10} M
Bisphenol A	−6.6 to −5.4	−6.2 to −5.0	1.0×10^{-5} to 1.0×10^{-11} M
DEHP	-	-	1.0×10^{-5} to 1.0×10^{-11} M
Induction fold of PC_{AGO2}	≥ 10		
RTA of PC_{ANTA} (%)	≤53.6		

23. Induction fold of PC_{AGO2} is calculated using the following equation:

$$\text{Induction fold of } PC_{AGO2} = \frac{\text{Mean RLU of } PC_{AGO2} \text{ (800 pM DHT)}}{\text{Mean RLU of Vehicle control}}$$

$$\text{RTA of } PC_{ANTA} (\%) = \frac{\text{Mean RLU of } PC_{ANTA} - \text{Mean RLU of VC}}{\text{Mean RLU of } PC_{AGO2} - \text{Mean RLU of VC}} \times 100$$

▪ RTA: relative transcriptional activity

Solubility test

24. The solubility test is based on the OECD GIVIMP (13). Test chemical stocks are prepared at a maximum concentration of up to 1 M (stock solution; 0.1% of the stock solution in wells with cells, i.e. 1 mM) in DMSO or an appropriate solvent. If precipitation occurs, the stock solution should be re-prepared a new concentration solution at 10 times lower than the original stock solution until no precipitation is observed.

Test chemical exposure and assay plate organisation

Pre-screen run in AR agonist assay

25. The maximal stock concentration of each test chemical, determined by the solubility test (see above), should be serially diluted at a ratio of 1:10 in DMSO (or another appropriate solvent). Then the dilutions are added to aqueous medium to achieve a final DMSO concentration of 0.1%. The recommended final volume for each well is 100 µL (the test medium from the assay plate should be removed and replaced with the test chemicals in the test medium). Triplicate wells are used for each concentration. The reference standards for the AR agonist assay (DHT, Mestanolone and DEHP) should be tested in every assay. Wells treated with 10 nM DHT (PC_{AGO1}), wells treated with 0.1% DMSO alone (VC) and wells treated with 1 mM SDS (PC_{CT}) should be included in each plate for the AR agonist assay (Table E.3). An example of the plate design of test chemicals is provided in Table E.4. After adding the test chemicals, the assay plates should be placed at 37℃±1℃ in a 5%±0.5% CO_2 incubator for 20-24 hours.

Table E.3. Example of plate concentration assignment for the reference chemicals (in M).

	DHT			**Mestanolone**			**DEHP**			**Test Chemical**		
	1	2	3	4	5	6	7	8	9	10	11	12
A	$1.0x10^{-6}$	→	→	$1.0x10^{-6}$	→	→	$1.0x10^{-5}$	→	→	$1.0x10^{-3}$	→	→
B	$1.0x10^{-7}$	→	→	$1.0x10^{-7}$	→	→	$1.0x10^{-6}$	→	→	$1.0x10^{-4}$	→	→
C	$1.0x10^{-8}$	→	→	$1.0x10^{-8}$	→	→	$1.0x10^{-7}$	→	→	$1.0x10^{-5}$	→	→
D	$1.0x10^{-9}$	→	→	$1.0x10^{-9}$	→	→	$1.0x10^{-8}$	→	→	$1.0x10^{-6}$	→	→
E	$1.0x10^{-10}$	→	→	$1.0x10^{-10}$	→	→	$1.0x10^{-9}$	→	→	$1.0x10^{-7}$	→	→
F	$1.0x10^{-11}$	→	→	$1.0x10^{-11}$	→	→	$1.0x10^{-10}$	→	→	$1.0x10^{-8}$	→	→
G	$1.0x10^{-12}$	→	→	$1.0x10^{-12}$	→	→	$1.0x10^{-11}$	→	→	$1.0x10^{-9}$	→	→
H	**VC**	→	→	→	**PC_{AGO1}**	→	→	→	**PC_{CT}**	→	→	→

- VC: Vehicle control (0.1% DMSO)
- PC_{AGO1}: AR agonist control (10 nM DHT)
- PC_{CT}: Cytotoxic control (1 mM SDS)

Table E.4. Example of plate concentration assignment for the test chemicals (in M).

	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	$1.0x10^{-3}$	→	→	$1.0x10^{-3}$	→	→	$1.0x10^{-5}$	→	→	$1.0x10^{-6}$	→	→
B	$1.0x10^{-4}$	→	→	$1.0x10^{-4}$	→	→	$1.0x10^{-6}$	→	→	$1.0x10^{-7}$	→	→
C	$1.0x10^{-5}$	→	→	$1.0x10^{-5}$	→	→	$1.0x10^{-7}$	→	→	$1.0x10^{-8}$	→	→
D	$1.0x10^{-6}$	→	→	$1.0x10^{-6}$	→	→	$1.0x10^{-8}$	→	→	$1.0x10^{-9}$	→	→
E	$1.0x10^{-7}$	→	→	$1.0x10^{-7}$	→	→	$1.0x10^{-9}$	→	→	$1.0x10^{-10}$	→	→
F	$1.0x10^{-8}$	→	→	$1.0x10^{-8}$	→	→	$1.0x10^{-10}$	→	→	$1.0x10^{-11}$	→	→
G	$1.0x10^{-9}$	→	→	$1.0x10^{-9}$	→	→	$1.0x10^{-11}$	→	→	$1.0x10^{-12}$	→	→
H	**VC**	→	→	→	**PC_{AGO1}**	→	→	→	**PC_{CT}**	→	→	→

- VC: Vehicle control (0.1% DMSO)
- PC_{AGO1}: AR agonist control (10 nM DHT)
- PC_{CT}: Cytotoxic control (1 mM SDS)

Comprehensive run in AR agonist assay

26. The test chemicals, which are determined to be an AR agonist in the pre-screen run should be further tested with a comprehensive run. The maximal concentration of the test chemical, determined from the concentration response curve generated in the pre-screen run, should be serially diluted at a ratio of 1:3 or 1:5 in DMSO (see Appendix E.1). These dilutions are then added to aqueous medium to a final DMSO concentration of 0.1%, and all concentrations should be tested in triplicate. All tests should be conducted at concentrations where the concentration–response curve can be well characterised. To achieve these conditions, solutions found to contain insoluble solids or concentrations found to induce cytotoxic effects against cell lines should not be included in the final analysis. The recommended final volume for each well is 100 µL (test medium from assay plate should be removed and replaced with test chemicals in test medium). The plate layout for the reference standards and the test chemicals run in the comprehensive run is the same as for the pre-screen run. After adding the test chemicals, the assay plates should be placed at 37℃±1℃ in a 5%±0.5% CO_2 incubator for 20-24 hours.

Pre-screen run in AR antagonist assay

27. The maximal stock concentration of each test chemical, determined by the solubility test (see above), should be serially diluted at a ratio of 1:10 in DMSO. These dilutions are then added to aqueous medium to a final DMSO concentration of 0.1%. The recommended final volume for each well is 100 µL (test medium from the assay plate should be removed and replaced with the test chemicals in the test medium). The AR antagonist assay reference standards (Bicalutamide, Bisphenol A and DEHP) should be tested in every assay. An AR agonist control (PC_{AGO2}; 800 pM DHT), an AR antagonist control (PC_{ANTA}; 800 pM DHT and 1 µM Bicalutamide) and cytotoxic control (PC_{CT}; 800 pM DHT and 1 mM SDS) should be prepared for the AR antagonist assay (Table E.5). The plate design of the test chemicals is provided in Table E.6. Except for the VC, all other wells are spiked with a fixed concentration of the agonistic reference chemical (800 pM DHT) in order to measure attenuation of the agonistic response. After adding the test chemicals, the assay plates should be placed at 37℃±1℃ in a 5%±0.5% CO_2 incubator for 20-24 hours.

Table E.5. Example of plate concentration assignment for the reference standards (in M)

	Bicalutamide			Bisphenol A			DEHP			Test Chemical 1		
	1	2	3	4	5	6	7	8	9	10	11	12
A	$1.0x10^{-4}$	→	→	$1.0x10^{-5}$	→	→	$1.0x10^{-5}$	→	→	$1.0x10^{-3}$	→	→
B	$1.0x10^{-5}$	→	→	$1.0x10^{-6}$	→	→	$1.0x10^{-6}$	→	→	$1.0x10^{-4}$	→	→
C	$1.0x10^{-6}$	→	→	$1.0x10^{-7}$	→	→	$1.0x10^{-7}$	→	→	$1.0x10^{-5}$	→	→
D	$1.0x10^{-7}$	→	→	$1.0x10^{-8}$	→	→	$1.0x10^{-8}$	→	→	$1.0x10^{-6}$	→	→
E	$1.0x10^{-8}$	→	→	$1.0x10^{-9}$	→	→	$1.0x10^{-9}$	→	→	$1.0x10^{-7}$	→	→
F	$1.0x10^{-9}$	→	→	$1.0x10^{-10}$	→	→	$1.0x10^{-10}$	→	→	$1.0x10^{-8}$	→	→
G	$1.0x10^{-10}$	→	→	$1.0x10^{-11}$	→	→	$1.0x10^{-11}$	→	→	$1.0x10^{-9}$	→	→
H	**VC**			**PC_{AGO2}**			**PC_{ANTA}**			**PC_{CT}**		

- VC: Vehicle control (0.1% DMSO)
- PC_{AGO2}: AR Agonist control for AR antagonist assay (800 pM DHT)
- PC_{ANTA}: AR Antagonist control (1 µM Bicalutamide)
- PC_{CT}: Cytotoxic control (1 mM SDS)
- Grey wells include 800 pM DHT

Table E.6. Example of plate concentration assignment for test chemicals (in M)

	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	$1.0x10^{-3}$	→	→	$1.0x10^{-3}$	→	→	$1.0x10^{-5}$	→	→	$1.0x10^{-3}$	→	→
B	$1.0x10^{-4}$	→	→	$1.0x10^{-4}$	→	→	$1.0x10^{-6}$	→	→	$1.0x10^{-4}$	→	→
C	$1.0x10^{-5}$	→	→	$1.0x10^{-5}$	→	→	$1.0x10^{-7}$	→	→	$1.0x10^{-5}$	→	→
D	$1.0x10^{-6}$	→	→	$1.0x10^{-6}$	→	→	$1.0x10^{-8}$	→	→	$1.0x10^{-6}$	→	→
E	$1.0x10^{-7}$	→	→	$1.0x10^{-7}$	→	→	$1.0x10^{-9}$	→	→	$1.0x10^{-7}$	→	→
F	$1.0x10^{-8}$	→	→	$1.0x10^{-8}$	→	→	$1.0x10^{-10}$	→	→	$1.0x10^{-8}$	→	→
G	$1.0x10^{-9}$	→	→	$1.0x10^{-9}$	→	→	$1.0x10^{-11}$	→	→	$1.0x10^{-9}$	→	→
H	**VC**			**PC_{AGO2}**			**PC_{ANTA}**			**PC_{CT}**		

- VC: Vehicle control (0.1% DMSO)
- PC_{AGO2}: AR Agonist control for AR antagonist assay (800 pM DHT)
- PC_{ANTA}: AR Antagonist control (1 µM Bicalutamide)
- PC_{CT}: Cytotoxic control (1 mM SDS)
- Grey wells include 800 pM DHT

Comprehensive run and specificity control test in AR antagonist assay

28. To ensure the identification of AR antagonist that is determined to be positive in the pre-screen run, the comprehensive run and specificity control test should be conducted using both 800 pM DHT and 100 nM DHT. The inclusion of these two concentrations of DHT in the antagonist assay is expected to result in a shift between the concentration-response curves of "true" AR antagonists and distinguish these chemicals from potential false positives. The maximal concentration of the test chemical, determined from the concentration–response curves generated in the pre-screen run, should be serially diluted at a ratio of 1:3 or 1:5 in DMSO (see Appendix E.1). These dilutions are then added to aqueous medium to a final DMSO concentration of 0.1%, and all concentrations should be tested in triplicate. The recommended final volume for each well is 100 µL(the test medium from the assay plate should be removed and replaced with the test chemicals in test medium). The plate layout for the reference standards is the same as for the pre-screen run and the plate layout for the test chemicals is shown in Table E.7. An AR agonist control (PC_{AGO2}; 800 pM DHT), an AR antagonist control (PC_{ANTA}; 800 pM DHT and 1 µM Bicalutamide) and cytotoxic control (PC_{CT}; 800 pM DHT and1 mM SDS) should be prepared for the AR antagonist assay. The plate layout is given in Table E.7. After adding the test chemicals, the assay plates should be placed at 37℃±1℃ in a 5%±0.5% CO_2 incubator for 20-24 hours.

Table E.7. Example of plate concentration assignment of test chemicals (in log M)

	Test chemical 1						Test chemical 2					
	1	2	3	4	5	6	7	8	9	10	11	12
A	−5	→	→	−5	→	→	−4	→	→	−4	→	→
B	−5.7	→	→	−5.7	→	→	−4.7	→	→	−4.7	→	→
C	−6.4	→	→	−6.4	→	→	−5.4	→	→	−5.4	→	→
D	−7.1	→	→	−7.1	→	→	−6.1	→	→	−6.1	→	→
E	−7.8	→	→	−7.8	→	→	−6.8	→	→	−6.8	→	→
F	−8.5	→	→	−8.5	→	→	−7.5	→	→	−7.5	→	→
G	−9.2	→	→	−9.2	→	→	−8.2	→	→	−8.2	→	→
H	**VC**			**PC_{AGO2}**			**PC_{ANTA}**			**PC_{CT}**		

- VC: Vehicle control (DMSO);
- PC_{AGO2}: AR agonist control (800 pM of DHT);
- PC_{ANTA}: AR antagonist control (1 µM of Bicalutamide);
- PC_{CT}: Cytotoxicity control (1 mM of SDS);
- Grey wells are spiked with 800 pM DHT;
- Dark grey wells are spiked with 100 nM DHT

Endpoint measurements

29. Endpoint are measured using the Steady-Glo Luciferase assay system (e.g. Promega, E2510, or equivalents) for AR response, and the live-cell protease detection system (e.g. Cell Titer-Fluor™ Cell viability assay, Promega, G6080, or equivalents) for the cytotoxicity. The measurements of cell viability and luciferase activity are performed in the same plate.

30. For cell viability assay:

- Prepare the cell viability (CellTiter-Fluor™) reagent according to the manufacturer's instructions.
- Add directly 20 µL/well of cell viability assay reagent into the assay wells containing medium with test chemicals.
- Mix the assay plates briefly using an orbital shaker.
- Incubate the assay plates at 37℃±1℃ in a 5%±0.5% CO_2 incubator for 1–3 hour.
- Remove plates from incubator and measure the cytotoxicity using a fluorometer (380–400 nm Ex /505 nm Em).

31. For luciferase assay

- Prepare the luciferase assay (Steady-Glo) reagent according to the manufacturer's instructions.
- Add directly 50 µL/well of luciferase assay reagent into the assay wells after the cell viability assay.
- Cover the top of the assay plate with aluminium foil to block the light, and leave at room temperature for 5-10 min.
- Measure the luciferase activity using a luminescence reader.

Analysis of Data

Cytotoxicity

32. Cytotoxicity, as read by the fluorometer in RFU units, is recorded and is transformed as follows:

- The average for the AR agonist and AR antagonist control (AR agonist assay: 10 nM DHT, AR antagonist assay: 800 pM DHT) is set at 100%.
- The average for cytotoxicity control (AR agonist assay: 1 mM SDS, AR antagonist as say: 800 pM DHT and 1 mM SDS) is set at 0%

33. If the results of the cell viability test indicate that the concentration of the test chemical has reduced cell viability by 20% or more, this concentration is regarded as cytotoxic. All concentrations considered cytotoxic should be excluded from the evaluation

34. For the cell viability assay, the data transformation from RFU units is as follows:

$$\text{Cell viability (\%)} = \frac{\text{Mean RFU of test chemical - Mean RFU of } PC_{CT}}{\text{Mean RFU of PC} - \text{Mean RFU of } PC_{CT}} \times 100$$

▪ RFU: relative fluorescence units

Luciferase activity

35. The luminescence signal data, as read by the luminometer in RLU units, is recorded and is transformed as follow:

- The average for the AR agonist and AR antagonist control (AR agonist assay: 10 nM DHT, AR antagonist assay: 800 pM DHT) is set at 100%.
- The average for vehicle control (0.1% DMSO) is set at 0%

36. For the agonist, and antagonist assay, the data transformation from RLU units is as follows:

$$\text{RTA (\%)} = \frac{\text{Mean RLU of test chemical - Mean RLU of VC}}{\text{Mean RLU of PC} - \text{Mean RLU of VC}} \times 100$$

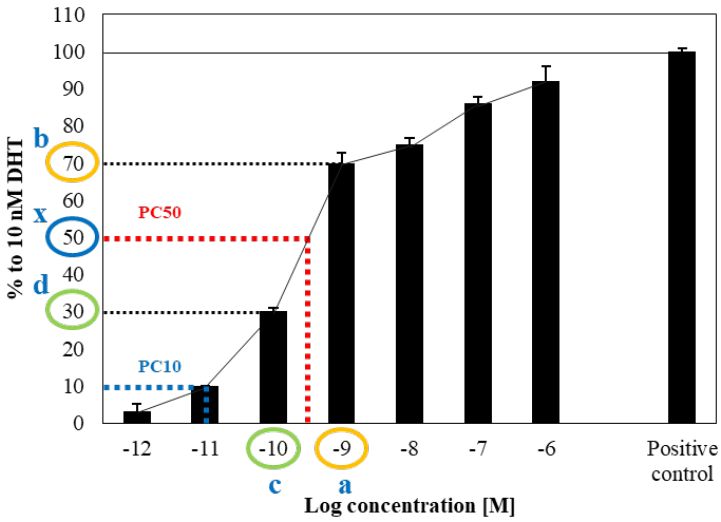
▪ RLU: relative light units▪ RTA: relative transcriptional activity

Calculation of parameters

37. In the AR agonist assay, the following information should be provided for a positive test chemical: the concentrations that induce an effect corresponding to that of a 10% effect for the positive control (log PC_{10}) and, if appropriate, the 50% effect for the positive control (log PC_{50}). Descriptions of log PC_x values, where "x" is a selected response, e.g. 10% or 50% induction, compared to PC_{AGO1}, are provided in Figure E.2. 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 by PC_{AGO1} (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 equation below and Figure E. 2 shows the method for the calculation of log[PC_{50}]:

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

Figure E.2. Schematic illustration of the calculation of log PC_x values

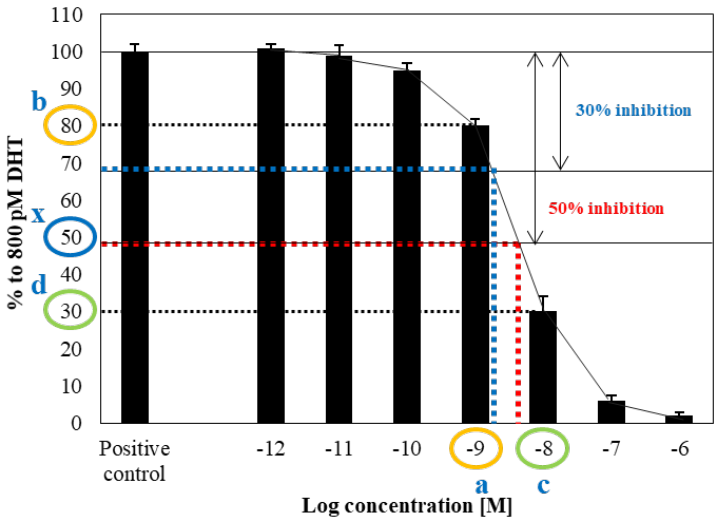


- The PC_{AGO1} (10 nM of DHT) is included on each assay plate in AR agonist assay.

38. For the AR antagonist assay, the following information should be provided for a positive test chemical: the concentrations for 30% inhibition of transcriptional activity induced by 800 pM DHT (log IC_{30}) and, if appropriate, for 50% inhibition of activity by 800 pM DHT (log IC_{50}). Descriptions of log IC_x values, where "x" is a selected response, e.g. 30% or 50% inhibition, compared to PC_{AGO2}, are provided in Figure E.3. 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 800 pM DHT. 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 equation below and Figure E.3 shows an illustration of the calculation of log[IC_{50}]:

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

Figure E.3. Schematic illustration of the calculation of log IC_x values



▪ The PC_{AGO2} (800 pM DHT) is included on each assay plate in AR antagonist assay.

39. In case of the specificity control test, to distinguish the responses by the two concentrations of DHT, the Y_C represents the relative induction at concentration c when the 800 pM DHT is used, and the symbol S_C represents the relative induction at concentration c when the 100 nM DHT is used. The data transformation from RLU of Y_C or S_C is as follows:

$$Y_C \text{ or } S_C\ (\%) = \frac{\text{Mean RLU of test chemical} - \text{Mean RLU of VC}}{\text{Mean RLU of } PC_{AGO2} - \text{Mean RLU of VC}} \times 100$$

40. For test chemicals to be a true AR antagonist (competitive), the square of the coefficient of determination, R^2, was calculated between the relative induction of the standard response Y_C and the relative induction of the specificity response S_C. If R^2 is less than 0.9, this test chemical was determined to be a true AR antagonist. The formula of R^2 for identifying the true AR antagonist can be found in the validation report (6). Some caution should be applied as this criterion cannot be considered as 100% definitive (as shown in the AR-CALUX® validation study report). It may be influenced by the shape of the curves and by outliers. Expert judgment may need to be applied.

41. The presence of increasing levels of cytotoxicity can significantly alter or eliminate the typical sigmoidal response and should be considered when interpreting the data in the agonist and antagonist assay. Accordingly, AR-mediated transcriptional activity and cytotoxicity should be evaluated simultaneously in the same assay plate. Should the results of the cytotoxicity test show that the concentration of the test chemical has reduced cell viability 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.

Data Interpretation Criteria

42. The interpretation of data and the decision, whether a test chemical in the absence of cytotoxicity is considered positive or negative, are shown in Table E.8.

43. To classify a chemical as an AR agonist, a positive pre-screen run in which a log PC_{10} can be determined should be followed by concordant results in two comprehensive runs or if not concordant, a third comprehensive run. In the case of a negative pre-screen run, the result should be confirmed in a (second) follow-up pre-screen run. If the second pre-screen run is positive after a first negative pre-screen run, a third pre-screen run should be additionally conducted. In the case of AR antagonist, the log IC_{30} is calculated in a pre-screen run and is confirmed in at least two (of up to three) comprehensive runs (in the absence of cytotoxicity) alongside a specificity control test. If the R^2 of test chemical in the specificity control is less than 0.9, the test chemical can be considered a true AR antagonist, however this may require additional expert judgement (see paragraph 40). Chemicals that are not AR antagonists are classified based on negative results (in the absences of positive results) in at least two pre-screen runs (Table E.8).

Table E.8. Positive and negative decision criteria

AR agonist assay	**Positive**	If obtained RPC_{max} is equal to or exceeds 10% of the response of the positive control.
	Negative	In all other cases.
AR antagonist assay	**Positive**	If the test chemical satisfies the following: i) the log IC_{30} of test chemical is calculated in the absence of cytotoxicity and ii) the R^2 is less than 0.9 in specificity control test.
	Negative	In all other cases.

▪ All results are in the absence of cytotoxicity.

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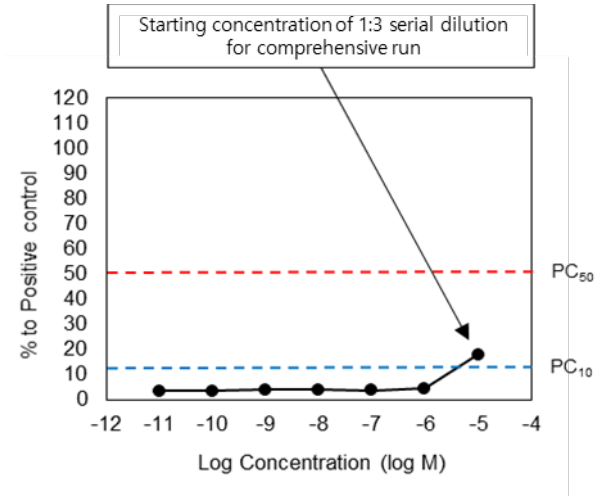
Appendix E.1

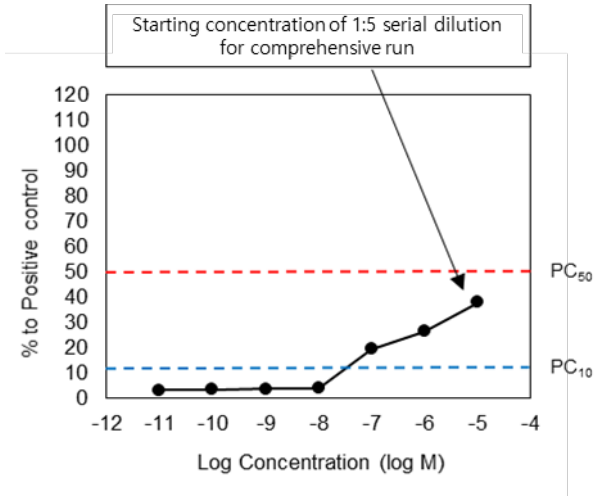
1. The method to determine the max concentration for the comprehensive run

2. If test chemicals are determined to be positive in the pre-screen run, comprehensive run should be conducted to accurately determine the potency of test chemicals. All test chemicals classified as positive for AR agonistic activity should have a concentration–response curve consisting of a baseline, and a positive slope; all test chemicals classified as positive for AR antagonistic activity should have a concentration response curve consisting of a baseline, and a negative slope. If possible, PC_{10}, PC_{50}, IC_{30} and IC_{50} value should be calculated for each positive decision. The comprehensive AR agonist/antagonist assay consists of a seven-point serial dilution (1:3 or 1:5 serial dilution) with each concentration tested in triplicate wells of the 96-well plate. To determine the starting concentrations for comprehensive run, use the following criteria:

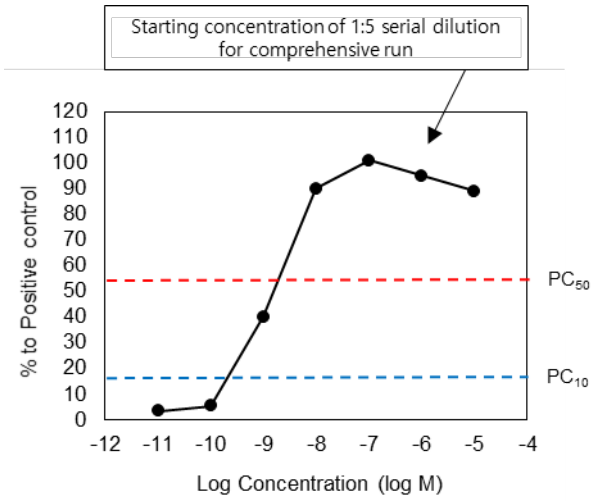
- If results in the pre-screen run suggest that the test chemical is positive with only PC_{10} value for AR agonist assay (if there is only one point on the test chemicals concentration curve that is greater than the positive decision criteria without cytotoxicity), the comprehensive run should be conducted using the 7-point 1:3 serial dilution starting at the maximum exposure concentration (see example 1).
- If results in the pre-screen run suggest that the test chemical is positive with only PC_{10} value for AR agonist assay (if there are several points on the test chemical concentration curve that are greater than the positive decision criteria without cytotoxicity), the comprehensive run should be conducted using the 7-point 1:5 serial dilution starting at the maximum exposure concentration (see example 2).
- If results in the pre-screen run suggest that the test chemical is positive with PC_{10} and PC_{50} values for AR agonist assay (i.e., if there are points on the test chemical concentration curve that are greater than the positive decision criteria without cytotoxicity), the starting concentration to be used for the 7-point dilution scheme in the comprehensive run should be 10 times greater than the concentration associated with the highest level of response in the pre-screen run (see example 3).
- If results in the pre-screen run suggest that the test chemical is positive with only PC_{50} value (or a PC_{50} value cannot be calculated but maximum activity is more than 10%) for AR agonist assay (i.e., if all testing points on the test chemical concentration curve are greater than the positive decision criteria without cytotoxicity), the starting concentration to be used for the 7-point dilution scheme in the comprehensive run should be 10 times greater than concentration associated with the highest level of response in the pre-screen run (see example 4).



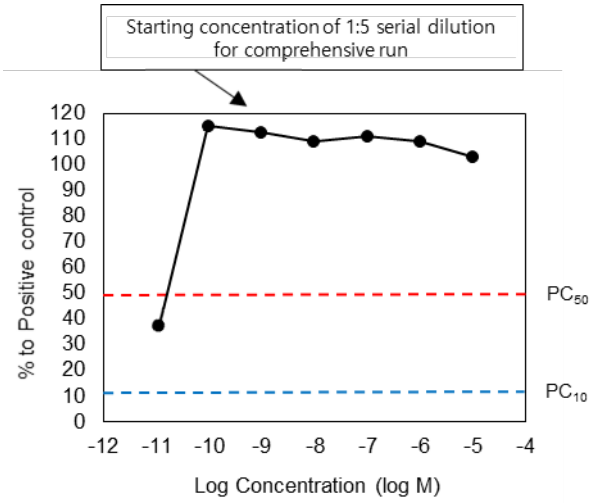
Example 1



Example 2



Example 3



Example 4

- If results in the pre-screen run suggest that the test chemical is positive with only IC_{30} value for AR antagonist assay (if there is only one point on the test chemical concentration curve for which the response is greater than the positive decision criteria without cytotoxicity), the comprehensive testing will be conducted using the 7-point 1:3 serial dilution starting at the maximum exposure concentration (see example 5).
- If results in the pre-screen run suggest that the test chemical is positive with only IC_{30} value for AR antagonist assay (if there are points on the test chemical concentration curve for which the response is greater than the positive decision criteria without cytotoxicity), the comprehensive testing should be conducted using the 7-point 1:5 serial dilution starting at the maximum exposure concentration (see example 6).
- If results in the pre-screen run suggest that the test chemical is positive with IC_{30} and IC_{50} values for AR antagonist assay (i.e., if there are points on the test chemical concentration curve that have a response greater than the positive decision criteria without cytotoxicity), the starting concentration to be used for the 7-point dilution scheme in the comprehensive testing should be the concentration giving the highest level of response in the pre-screen run (see

example 7).

- If results in the pre-screen run suggest that the test chemical is positive with only IC_{50} value (or not calculate IC_{50} value) for AR antagonist assay (i.e., if all testing points on the test chemical concentration curve are greater than the positive decision criteria without cytotoxicity), the starting concentration to be used for the 7-point dilution scheme in the comprehensive testing should be the concentration giving the highest level of response in the pre-screen run (see example 8).

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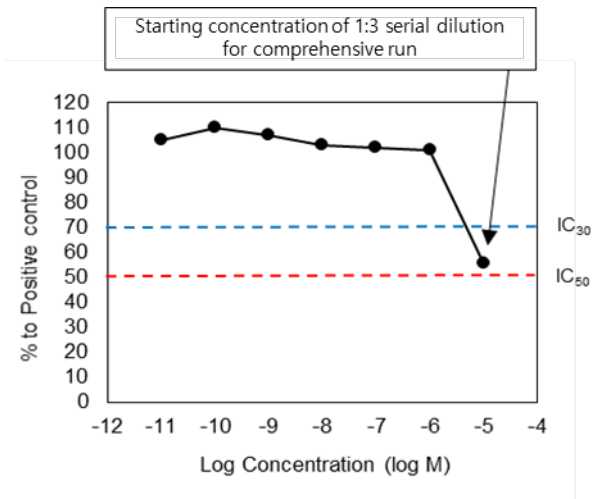
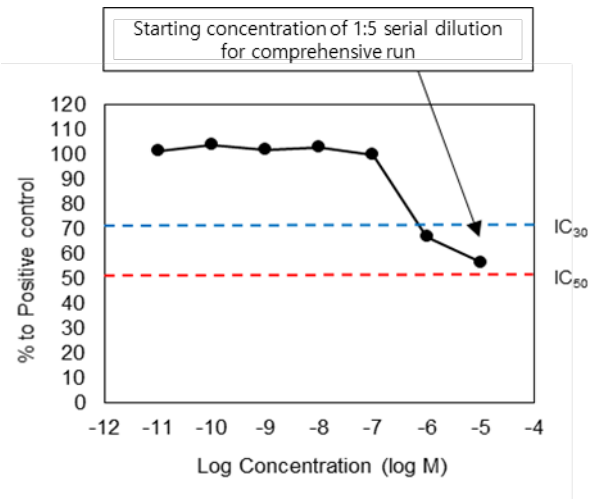
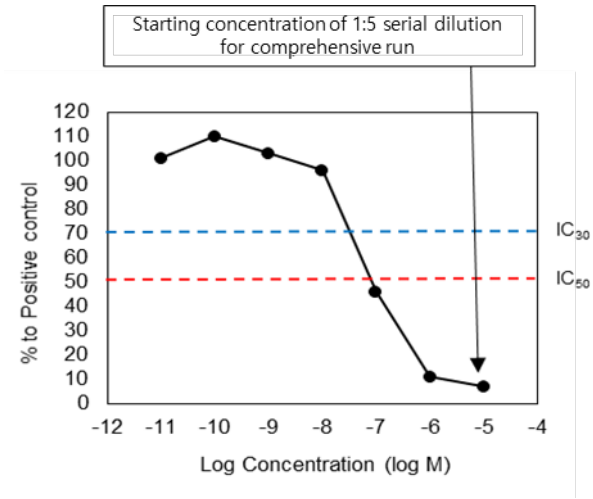


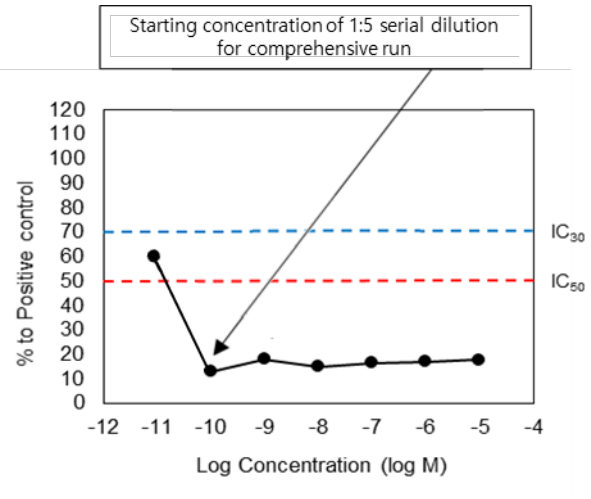
Example 5



Example 6



Example 7



Example 8